

**A Dissertation Presented**

**By**

**Nunciada Salma**

**Submitted to the Faculty of the University of Massachusetts Graduate School of  
Biomedical Sciences, Worcester in partial fulfillment of the requirements for the  
degree of**

**DOCTOR OF PHILOSOPHY**

**March 2, 2006**

**Cell Biology**

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Parts of this dissertation have appeared in the following publications:

Salma, N., Xiao, H., Mueller, E., and Imbalzano, A. N. (2004). Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. *Mol Cell Biol* 24, 4651-4663.

Salma, N., Xiao, H. and Imbalzano, AN. (2006). Temporal recruitment of C/EBP proteins to early and late adipogenic promoters *in vivo*. *J. Molec. Endocrinol.* 36: 139-151.

TRANSCRIPTIONAL REGULATION DURING ADIPOCYTE DIFFERENTIATION:  
A ROLE FOR SWI/SNF CHROMATIN REMODELING ENZYMES

A Dissertation Presented

By

Nunciada Salma

Approved as to style and content by:

---

Janet Stein, Ph.D., Chair of Committee

---

Craig L. Peterson, Ph.D., Member of Committee

---

Jacqueline M. Stephens, Ph.D., Member of Committee

---

Jeffrey Nickerson, Ph.D., Member of the Committee

---

Michael Czech, Ph.D., Member of Committee

---

Anthony N. Imbalzano, Ph.D., Thesis Advisor

---

Anthony Carruthers, Ph.D., Dean of the Graduate  
School of Biomedical Sciences

March 2, 2006

## ACKNOWLEDGMENTS

I would like to first thank my dissertation mentor Anthony Imbalzano. I owe him the opportunity he gave me of working in such a great project and for convincing me that University of Massachusetts was my best choice within other options. I immensely thank him for his advices, support and help during all of these years. I also thank him for openly sharing his knowledge and strategies as well as making each of our project discussions graceful and pleasant moments. I truly enjoyed, from the beginning to the end, my stay in his laboratory.

I would like to thank all of the Imbalzano's laboratory members for their support and magnificent advices. Special thanks for David Hill for always being willing to help every time it was necessary, including the development of *in vitro* experiments and grammar corrections on my written thesis. I also would like to acknowledge Xiao Hengyi for her contributions. To the old members of the laboratory I make especial mention about Kanaklata Roy and Cindy Guidi for being such a great help during my first steps in the laboratory. I also would like to thank Tim Veal for his generosity and willingness to help any time I needed it. I want to express profound gratitude to Ivana de la Serna for her support during these years and her invaluable contribution with the grammar corrections of my thesis. Ivana represents to me the great example of what scientific efficiency and quality is all about.

From Dr. Steve Jone's laboratory, I would like to thank Liang Hulliing and Heather Steinman for their unconditional friendship during all these years. I want to make especial mention about Rajini Mudhasani for her immense and invaluable help and for giving me her friendship. Words cannot express my deepest appreciation for her



time and unconditional support. I enormously value the scientific discussions with Rajini, which have given me the understanding that there is always a positive side even in those craziest experiments. I also would like to thank Rajini's husband, Prasat Kota, who has made me understand that even though we cannot avoid moments of tension in our lives there is always the possibility of erasing the darkest clouds with a simple smile.

I enormously thank the member of Drs. Stein's laboratory for the great help when it was needed.

Special thanks to the members of my committee for dedicating part of their precious time reading my Thesis and providing me with remarkable suggestions.

I would like to express profound gratitude to all of my friends in the USA for their devotion, love, spiritual support and advice during all of these years. Very special thanks go to Amalfi Viera, Maria Elena Zoghbi, Jose Francisco Perez, and Judith Campos. Thank all of you for being such extraordinary friends.

My debt is extended, of course, to my Venezuelan friends whom during so many years have been sharing a part of them with me. They have always been present even through the distance, and have filled me with positive, spiritual and refreshing energy. My especial thank to Vestalia Rodriguez, Odalis Herrera, Valentina Salas, José Fernández, Claudia Bisits, Lourdes Azurmendi, Cecilia Jiménez, Freida Monterola, Julia de la Peña, Juana Jiménez, Patrizia Mastromatteo, Zhaida Duran, Alvaro Acosta... I am highly blessed to have all of you...

I thank with all of my heart, my family who I love so much, my mother Lucia de Salma, my sister Maria Salma, my aunt Catena Salma and my brother-in-law Jose Albani for the love they constantly transmit to me and their constant and invaluable support. Even those that are no longer with us; I thank my father Attilio Salma and my

Godfather Sebastiano Rigano because I know that wherever they are, they are filling me with love and strength which I need so much. This Thesis is dedicated to all of you!

...and thank you God for giving me this spiritual and unforgettable experience and send me His Angels to take me to the right place at the right moment...

## ABSTRACT

Chromatin has a compact organization in which most DNA sequences are structurally inaccessible and functionally inactive. Reconfiguration of the chromatin is required to activate transcription. This reconfiguration is achieved by the action of enzymes that covalently modify nucleosomal core histones, and by enzymes that disrupt histone-DNA interactions via ATP hydrolysis.

The SWI/SNF family of ATP-dependent chromatin remodeling enzymes has been implicated not only in gene activation but also in numerous cellular processes including differentiation, gene repression, cell cycle control, recombination and DNA repair. PPAR $\gamma$ , C/EBP $\alpha$  and C/EBP $\beta$  are transcription factors with well established roles in adipogenesis. Ectopical expression of each of these factors in non-adipogenic cells is sufficient to convert them to adipocyte-like cells

To determine the requirements of SWI/SNF enzymes in adipocyte differentiation, we introduced PPAR $\gamma$ , C/EBP $\alpha$  or C/EBP $\beta$  into fibroblasts that inducibly express dominant-negative versions of the Brahma-Related Gene 1 (BRG1) or human Brahma (BRM), which are the ATPase subunits of the SWI/SNF enzymes. We found that adipogenesis and expression of adipocyte genes were inhibited in the presence of mutant SWI/SNF enzymes. Additionally, in cells expressing C/EBP $\alpha$  or C/EBP $\beta$ , PPAR $\gamma$  expression was SWI/SNF dependent. These data indicate the importance of these remodeling enzymes in both early and late gene activation events.

Subsequently, we examined by chromatin immunoprecipitation (ChIP) assay the functional role of SWI/SNF enzymes in the activation of PPAR $\gamma$ 2, the master regulator of adipogenesis. Temporal analysis of factors binding to the PPAR $\gamma$ 2 promoter showed

that SWI/SNF enzymes are required to promote preinitiation complex assembly and function.

Additionally, our studies concentrated on the role of C/EBP family members in the activation of early and late genes during adipocyte differentiation. During adipogenesis, C/EBP $\beta$  and  $\delta$  are rapidly and transiently expressed and are involved in the expression of PPAR $\gamma$  and C/EBP $\alpha$ , which together activate the majority of the adipocyte genes. Our studies determined the temporal recruitment of the C/EBP family at the promoters of early and late genes by ChIP assay during adipocyte differentiation. We found that all of the C/EBP members evaluated are present at the promoters of early and late genes, and the binding correlated with the kinetics of the C/EBPs expression. Binding of C/EBP $\beta$  and  $\delta$  is transient, subsequently being replaced by C/EBP $\alpha$ . These studies demonstrated that C/EBP $\beta$  and  $\delta$  are not only involved in the regulation of PPAR $\gamma$  and C/EBP $\alpha$ , but also in the activation of late expressed adipocyte genes.

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## ABBREVIATIONS

**SREBP1/ADD1:** Sterol Response Element Protein 1/Adipocyte Determination and Differentiation Factor 1

**aP2:** Lipid binding protein

**ARID:** A/T-Rich Interaction Domain

**BAP:** Brahma Associated Factors

**BAF:** BRG1 Associated Factors

**bp:** base pair

**B22:** Cell line that inducibly expresses dominant negative BRG1

**36B4:** Acidic ribosomal phosphoprotein PO

**BRG1:** Brahma Related Gene 1

**BRM:** Brahma

**C/EBP:** CAATT/Enhancer Binding Protein

**C<sub>2</sub>H<sub>2</sub> zinc finger:** DNA-binding module

**CHD:** Chromo Helicase DNA binding protein

**CTD:** C-terminal domain

**DNA:** Deoxyribonucleic acid

**DNase I:** Deoxyribonuclease I

**EMSA:** Electrophoretic Mobility Shift Assay

**FLAG:** Polypeptide protein tag which sequence is: N-DYKDDDDK-C

**H17:** Cell line that inducibly expresses dominant negative hBRM

**HAT:** Histone Acetyl Transferase

**HDAC1:** Histone deacetylase 1

**HMG:** High Mobility Group B box

**HMT:** Histone Methyl Transferases

**IP:** Immunoprecipitation

**ISWI:** Imitation SWItch

**KLF2:** Kruppel-Like Factor 2

**MCE:** Mitotic Clonal Expansion

**MSC:** Mesenchymal stem cells

**PHD:** Plant HomeoDomain

**PPAR $\gamma$ :** Peroxisome Proliferator Activated Receptor  $\gamma$

**SANT:** Swi3, ADA, N-CoR and TFIIIB domain (Putative histone tail-binding module)

**s:** Seconds

**STAT:** Signal Transducers and Activator of Transcription

**SWI/SNF:** SWItching defective/Sucrose Non Fermenting

**SWP:** SWI/SNF-associated Protein

**TBP:** TATA Binding Protein

**Tet-VP16:** Cell line that express Tet-VP16 regulator protein



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# CHAPTER I

## CHAPTER I

### INTRODUCTION

#### 1) Chromatin structure and transcriptional control

Many biological processes that take place in eukaryotic organisms such as cell replication, development and cellular differentiation are highly regulated. During these nuclear processes, precise temporal and spatial expression of specific genes is required. However, in the nucleus of eukaryotic cells, DNA is inaccessible and compacted into chromatin, which is formed by association of the DNA with histones and non histone proteins. Since DNA needs to be compacted in order to fit into the nucleus, cells face a repressive environment, a highly packaged chromatin structure, which is responsible for controlling the dynamics of DNA dependent processes.

The fundamental repeat unit of chromatin is the nucleosome which consists of 147 base pairs of DNA wrapped around a histone octamer core, which is composed of two copies of each of the histone proteins H3 and H4, located at the center of the core, flanked by two H2A/H2B heterodimers (Fig. I.1) (Kornberg and Thomas, 1974; Luger and Hansen, 2005; Luger et al., 1997). Each of the core histones has a central domain and a flexible tail domain located at the amino terminal (N-terminal) region; an additional carboxy terminal (C-terminal) tail is also found exclusively on histone H2A. The central domain contributes to histone-histone interactions inside the octamer and the tail domains allow histones to interact with DNA and other proteins that control nucleosome folding and function. In addition, these tails are also subjected to a considerable number of post-translational modifications which are critical for the regulation of the structural and functional activities of the nucleosome. These enzymatic activities, which include acetylation, phosphorylation, methylation, ubiquitination, sumoylation, ADP ribosylation,



**Figure I.1.** Nucleosome core particle: the 146 base pair DNA backbones is shown in light green and light brown, histone protein H3 is shown in blue, H4 in dark green, H2A in yellow and H2B in pink (Luger et al., 1997).

glycosylation, biotinylation and carbonylation or some combination of thereof, are important for mediating structural alterations between repressive and active chromatin states [Reviewed in (Luger and Hansen, 2005; Margueron et al., 2005) and references therein]. Additional covalent modifications that occur in the central domain of the histones have recently been identified. A number of them are located at the lateral surface of the nucleosome where histone-DNA interactions occur. These modifications may affect histone-DNA interactions and nucleosome positioning (Cosgrove et al., 2004).

The regulation of chromatin structure, as well as the regulatory aspects of chromatin, is not only influenced by covalent modification of the histones but also by the presence of specific histone variant proteins (for instance H3.3 and H2AZ, which are H3 and H2A variants, respectively). Even though many aspects of the variant deposition are still debated, it seems that histone variants contribute to the generation of functional domains in the chromatin with different properties. For instance, histone H3 variant CENP-A which is located to centromeres, is essential for the formation of a functional kinetochore in all eukaryotes [Reviewed in (Cairns, 2005; Jin et al., 2005; Korber and Horz, 2004; Peterson and Laniel, 2004; Sarma and Reinberg, 2005)].

DNA wrapped around a series of nucleosome core particles that are separated by 10-60 base pairs (bp) generates the 10 nm fiber, also known as "beads on a string". This constitutes the first level of DNA organization inside the nucleus. At the next level, the nucleosomal array is subsequently condensed into a secondary structure known as the 30 nm fiber, which is stabilized by linker histones (e.g. H1, H5) (Carruthers et al., 1998). How are the nucleosomes organized within the 30 nm fiber? Although the *in vivo* 30 nm organization has not been elucidated, a series of studies performed *in vitro* using



nucleosomal arrays have shown how packaging of the array depends on interactions between the N-terminal tails of histone H4 from one nucleosome with the H2A/H2B dimer of the adjacent one. These interactions can direct the connection of nucleosomes into a zigzag arrangement resulting in a "two-start helix" pattern under physiological conditions (Dorigo et al., 2003; Dorigo et al., 2004; Hansen, 2002). Although these studies are convincing, the possibility exists that other three-dimensional structures coexist *in vivo*. Chromosomal architecture beyond the 30 nm fiber is still unclear; however it is known that chromatin fiber can form loops that allow the connection of two distal regions of a particular gene. In addition, a number of nucleosome-binding proteins such as MENT (myeloid and erythroid nuclear termination stage-specific protein) and MeCP2 (methyl CpG binding protein 2), are also involved in the formation of secondary and tertiary chromatin structures that contribute to the further packaging and stabilization of the DNA [Reviewed in (Luger and Hansen, 2005) and references therein].

In a scenario where the highly condensed DNA is inaccessible to gene regulatory proteins, cells have developed mechanisms that respond to specific signals that facilitate the remodeling of the chromatin structure, thus, alleviating the impediment imposed by chromatin and assuring gene function. In contrast, when repression of a gene is required, alteration of chromatin occurs in a way that results in a condensed and inaccessible chromatin structure. Two major classes of chromatin modifying enzymes have been identified so far, including the histone modifying enzymes that covalently modify histones, and the ATP-dependent chromatin remodeling enzymes that disrupt histone DNA contacts in the nucleosomal core using the energy derived from ATP hydrolysis.

Remodeling that involves covalent modifications, particularly at the N and C-termini of histone tails is mediated by a set of enzymes, including histone acetyltransferases (HATs), histone methyl transferases (HMTs), kinases, ubiquitinylases, sumoylases, and ADP-ribosylases. The covalent modifications of the histone tails not only alter the chromatin structure, making it more accessible or inaccessible (depending of the modification(s), but also allow chromatin to recruit regulatory proteins responsible for the final outcome: gene activation or silencing. The enzymes involved in reversing histone modifications such as acetylation and methylation have been identified. They include the well known histone deacetylases (HDACs) and the recently discovered lysine-specific demethylase (LSD1) [Reviewed in (Margueron et al., 2005; Peterson and Laniel, 2004; Schreiber and Bernstein, 2002; Shi et al., 2004)].

Combinations of these covalent modifications are complex and it is believed that they might influence the structure of the chromatin or generate binding sites for regulatory factors that contain specific binding domains. For instance, transcription factors and chromatin remodeling enzymes containing bromodomains that recognize acetylated lysines and those containing some chromodomains recognize methylated lysines (Berger, 2002; Brehm et al., 2004; Cosgrove et al., 2004; Zeng and Zhou, 2002).

The second class of chromatin modifiers alters contacts between DNA and histones using the energy from ATP hydrolysis. The first ATP-dependent chromatin remodeling enzyme identified was the yeast SWI/SNF complex (Winston and Carlson, 1992). The complex was discovered in *S. cerevisiae* while studying the regulation of the gene HO, which is involved in mating type switching and SUC2, which regulates the growth on sucrose. The mutations that affected the expression of HO (SWI; for switching defective mutants) and SUC2 (SNF: sucrose non fermenting mutants) resulted

in a similar phenotype, suggesting that these proteins might work in a complex. The link between SWI/SNF and chromatin was established when the analysis of mutations that suppressed SWI and SNF phenotypes resulted in the identification of genes that encoded histones and chromatin associated proteins. [Reviewed in (Imbalzano, 1998; Mohrmann and Verrijzer, 2005; Sif, 2004; Smith and Peterson, 2005) and references therein]. The sequence analysis of the Swi2/Snf2 gene, the motor of the complex, showed that the central domain of the protein is homologous to the later identified, SF2 superfamily of helicases (Laurent et al., 1992). The Swi2/Snf2 protein and its family members do not display DNA unwinding activity, however, they all possess DNA-stimulated ATPase activity that is required for altering DNA-histone core contacts, resulting, at least in *in vitro* assays, in relocation of nucleosomes, displacement of histones or alteration of the nucleosome structure (Becker and Horz, 2002; Cote et al., 1994; Flaus and Owen-Hughes, 2004; Laurent et al., 1993; Narlikar et al., 2002; Peterson, 2002).

## **2) The SWI/SNF superfamily of ATP-dependent chromatin remodeling enzymes.**

The yeast SWI/SNF complex consists of eleven subunits (Table I), and its activity is required for the regulation of the expression of constitutive and inducible genes. Analysis of mutations of SWI/SNF have revealed that this complex is not only involved in gene expression but also in gene repression [Review in (Wang, 2003) and references therein]. Biochemical analyses demonstrated that Swi1, Swi2 (Snf2), Swi3, Snf5 and Snf6 and the subsequently identified SWPs (SWI/SNF-associated proteins) were indeed components of the 1.14 MDa SWI/SNF complex which alters the structure of the nucleosome in an ATP dependent manner (Cairns et al., 1994; Peterson et al., 1994).

YEAST		DROSOPHILA		HUMAN	
ySWI/SNF	RSC	BAF	PBAF	hSWI/SNF-A (BAF)	hSWI/SNF-B (PBAF)
Swi2/Snf2	Sth1	Brahma (dBrm)	Brahma (dBrm)	hBRG1	hBRG1
Swi1		Osa		BAF250/p270	
Swi3	Rsc8/Swh3	BAP155 (Maira)	BAP155 (Maira)	BAF170 and BAF155	BAF170 and BAF155
Snf5	Sfh1	Snr1	Snr1	Ini1/hSnf5	Ini1/Snf5
Snf6					
Snf11					
Swp29/Tfg3/Anc1/Taf30					
Swp73	Rsc6	BAP60	BAP60	BAF60a	BAF60 a or BAF60b
Swp82					
Arp9/Swp59	Rsc12/Arp9	BAP55	BAP55	BAF53	BAF53
Arp7/Swp61	Rsc11/Arp7	BAP47/ACT1/2	BAP47/ACT1/2	$\beta$ -actin	$\beta$ -actin
	Rsc1,Rsc2,Rsc3		Polybromo		Polybromo/BAF180
			BAP170		
		BAP111	BAP111	BAF57	BAF57

**Table I.** SWI/SNF class chromatin remodeling enzymes from yeast, *Drosophila* and Human.

After the discovery of the SWI/SNF complex, a second member of the family was identified in yeast. The RSC (remodels the structure of chromatin) complex includes fifteen polypeptides some of which are highly homologous to the SWI/SNF subunits, and moreover, two of the subunits, Arp7 and Arp9, are shared in both complexes (Table I) [Reviewed in (Mohrmann and Verrijzer, 2005) and references therein]. Even though the remodeling activities of both complexes are similar *in vitro*, several subunits of RSC are essential for viability, including the ATPase subunit (Sth1). Whole genome analysis has shown that RSC activates or represses the transcription of genes that are not regulated by SWI/SNF, thus yeast has two SWI2/SNF2 remodeling complexes, the yeast SWI/SNF (ySWI/SNF) and RSC [Review in (Mohrmann and Verrijzer, 2005; Wang, 2003)].

Homologues of Swi2/Snf2 have been identified in *Drosophila*, mouse, frog, and human (Cairns et al., 1994; Wade et al., 1998). All of them share the same basic structure, a catalytic subunit that contains an ATPase domain (related to the yeast Swi2/Snf2), and each is associated with additional subunits that regulate the structure, function, and efficiency of the complex (Table I) (Cote et al., 1994; Laurent et al., 1993).

To date, the Swi2/Snf2 superfamily has been divided into four families including SWI/SNF, ISWI, CHD and Ino80 that were classified based on sequence homology within the ATPase subunit and also by the presence of different domains outside the ATPase region, which include the bromodomains, SANT domain, chromodomains, PHD fingers, and TELY/GTIE motifs. These structural and functional domains are not limited to ATPase subunits; they are also present in a number of the associated subunits in the remodeling complexes and in a number of chromatin binding proteins.

The additional domains within the catalytic subunit might function by mediating protein-protein interactions, targeting the remodeling complex to regulatory regions on

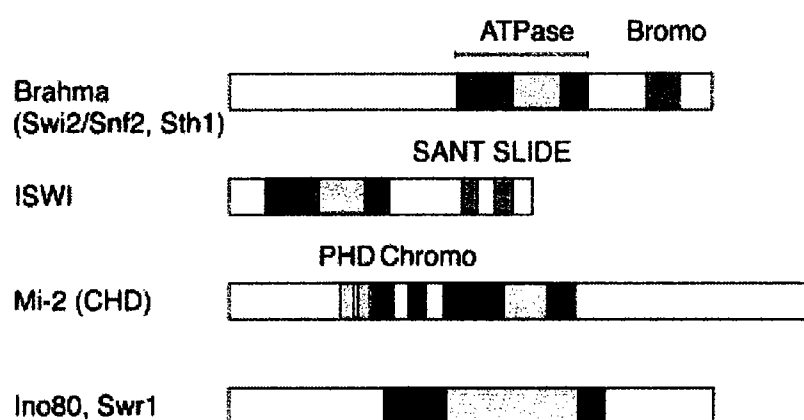
the DNA and/or stabilizing the interactions with chromatin, and mediating complex formation (Fig.1.2) (Boyer et al., 2000; Eisen et al., 1995; Fan et al., 2005; Sif, 2004; Smith and Peterson, 2005).

The bromodomains, which are about 110 amino acids in size, are present in the ATPase subunit of the SWI/SNF family members. This domain permits interaction with specific acetylated lysines in histone tails, facilitating the targeting of the ATPase to chromatin or perhaps allowing the interactions with other proteins that stabilize the binding to chromatin. The SANT domain present in the ATPase subunit of the ISWI family members is a small motif of approximately fifty amino acids. There is a high degree of homology between this domain and the DNA-binding domain of the c-Myb proteins. This domain is implicated in the targeting of the catalytic subunit to the regulatory sequences via interactions with the unmodified histone tails, stabilizing the chromatin-enzyme interactions. Two chromodomains are present in the catalytic subunit of the CHD family, and these promote interactions with nucleosomal DNA in a histone tail independent manner [Reviewed in (de la Cruz et al., 2005; Mohrmann and Verrijzer, 2005; Smith and Peterson, 2005)].

In the next sections of this thesis the characterization of the SWI/SNF complex in *Drosophila* and human will be presented. Additionally, it will mention the mechanisms of remodeling, and finally it will be discuss the role of the complex in development and differentiation.

## **2.1) *Drosophila* SWI/SNF complexes and subunits**

After the identification of the SWI/SNF complex in *Saccharomyces cerevisiae*, SWI/SNF related complexes were characterized in *Drosophila melanogaster* and then in

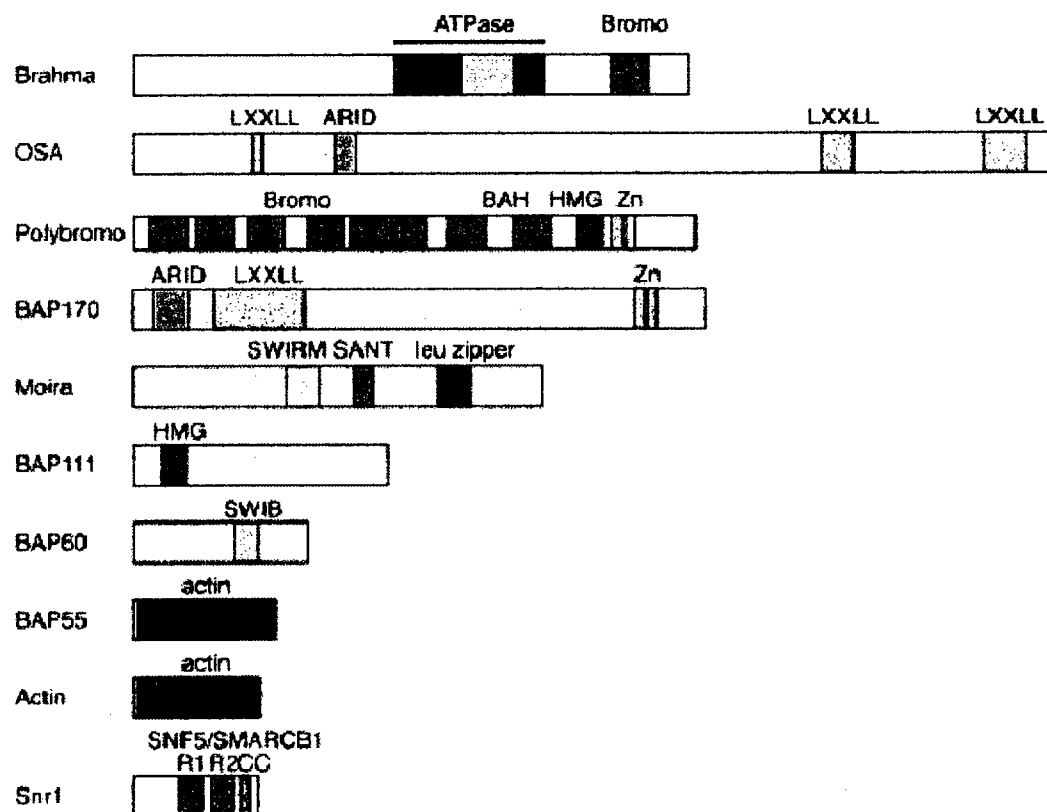


**Figure I.2.** ATPases of the four main families of ATP-dependent SWI/SNF-related chromatin remodeling enzymes: SWI/SNF, ISWI, CHD and Ino80. The ATPase domain and other characteristic domains are shown (Mohrmann and Verrijzer, 2005).

mammals. The Swi2/Snf2 protein in *Drosophila* is known as Brahma or Brm and was identified in genetic screens looking for genes essential for embryonic development (Dingwall et al., 1995; Tamkun et al., 1992). The first purification of the *Drosophila* Brm complex (dBrm) revealed that it consists of eight subunits. The Brm associated proteins, also referred to as BAPs, were named according to their molecular weight as BAP45, BAP47, BAP55, BAP60, BAP74, BAP111, and BAP155. At least four of the subunits Brm, BAP155 (also known as moira), BAP60 (Swp73/Rs6 homolog), and BAP45 (also called Snr1) are conserved in yeast and human; see below (Table I). In contrast, BAP111 is unique to higher eukaryotes and belongs to the HMG domain family of proteins (Fig. 1.3). A number of HMG domains contact DNA in a sequence-dependent manner, although the HMG motifs present in the BAP111 subunit bind DNA independently of the DNA sequence (Mohrmann and Verrijzer, 2005; Papoulas et al., 2001). Later studies demonstrated that BAP111 interacts with Brm *in vivo* suggesting a possible role of this subunit in the functional activity of the Brm and mammalian complexes (Papoulas et al., 2001). BAP74 is a HSP70 equivalent to HSC4 chaperone; BAP55 is an actin-related protein; BAP47 matched conserved regions of the actin-related proteins ACT1 and ACT2 (Fig. 1.3) (Papoulas et al., 1998).

Later, another subunit known as Osa (closest homolog to the yeast Swi1) was identified as a component of the dBrm complex. Osa encodes a large protein of approximately 300 kDa that contains two domains, a putative DNA binding domain (ARID: AT-rich interaction domain) which is also present in the yeast Swi1 subunit, and a C-terminal domain with highly conserved regions, HRI and HRII. In addition, Osa contains variant LXLL motifs (L: leucine; X: any amino acid), which are characteristic of protein domains involved in ligand-dependent binding of hormone receptors to their co-





**Figure I.3.** Domain architecture of *Drosophila* BAP and PBAP subunits. Proteins and their structural modules are shown approximately to scale (Mohrmann and Verrijzer, 2005).

activators (Fig. I.3) (Collins et al., 1999; Inoue et al., 2002; Mohrmann and Verrijzer, 2005).

Recently, two novel subunits of the *Drosophila* Brm complex were purified and characterized by mass spectrometry: Polybromo and BAP170. The Polybromo subunit contains 6 bromodomains, 2 BAH (bromo-adjacent homology) domains, a HMG box, and 2 putative  $C_2H_2$  zinc fingers (Fig. I.3). The presence of numerous bromo domains possibly controls targeting of the complex to regions of hyperacetylated chromatin.

Nevertheless, it is not possible to generalize since there are SWI/SNF complexes that do not contain polybromo subunits and these still localize to the same regions (Mohrmann et al., 2004; Mohrmann and Verrijzer, 2005). BAH domains have also been identified in Rsc1 and Rsc2, in DNA methyltransferases, and in other proteins implicated in gene regulation. The function of both domains in the polybromo subunits has not been determined. The  $C_2H_2$  zinc fingers are known to mediate sequence-dependent DNA interactions as well as protein-protein interactions (Mohrmann and Verrijzer, 2005).

The BAP170 subunit is present in many species. Structurally the protein contains an N-terminus with an ARID domain, an LXXLL (L: leucine; X: any amino acid) motifs which contributes to protein-protein interactions, a region rich in the amino acids proline and glutamine, a region rich in serine, and a C-terminus including two  $C_2H_2$  zinc finger motifs (Fig. I.3). As in the Polybromo subunit, BAP170 contains multiple conserved motifs that might be important function in targeting. Biochemical analysis of the dBrm associated subunits revealed that in *Drosophila* there are two complexes one including the Osa subunit, known as **BAP** (Brahma Associated Proteins) and the other one containing a polybromo or **PBAP** (Polybromo Brahma-Associated Proteins) which is also characterized by the presence of BAP170 subunit (Table 1) (Mohrmann et al., 2004;

Mohrmann and Verrijzer, 2005; Smith and Peterson, 2005). Furthermore, the composition of the dBrm complexes might vary in different stages during development.

Using indirect immunofluorescence the distribution of BAP and PBAP complexes was determined in polytene chromosomes. The complexes exhibited an overlapping but a different distribution pattern which suggests specific gene regulation by each complex (Mohrmann et al., 2004). It has not been reported yet how both complexes regulate genes *in vivo*.

## 2.2) Human SWI/SNF complexes and subunits

After the discovery of yeast Swi2/Snf2 and the *Drosophila* Brahma ATPase subunits, two human homologues, BRG1 and hBRM, were identified. BRG1 (Brahma related gene 1) was isolated by screening a human HeLa cell cDNA library with the Brahma cDNA probe (Khavari et al., 1993). Likewise, hBRM (human Brahma) was isolated by screening a human liver cDNA library with a *Drosophila* Brm probe. Simultaneously with the hBRM isolation, the mouse counterpart (mBrm) was obtained from a lung cDNA library probed with a fragment of the ATPase domain from hBRM (Muchardt and Yaniv, 1993).

Initially the human SWI/SNF complexes were partially purified as two multi-protein complexes (A and B), each containing 7 peptides, and ATP-dependent nucleosome disruption activity that facilitated the binding of transcription factors and the TBP (TATA-binding protein) to nucleosomal DNA (Imbalzano et al., 1994; Kwon et al., 1994). Subsequent purifications demonstrated that the human complexes contained either BRG1 or hBRM and consisted of 9 to 12 subunits depending on the cell type used in the purification (Wang et al., 1996b). Like dBrm complexes, BRG1 is found as a

component of two complexes, in human referred to as complex **BAF** (BRG1-Associated Factors) or hSWI/SNF-A and in complex **PBAF** (Polybromo-BRG1-Associated Factors) or hSWI/SNF-B; BRM is found as a component in one complex, the BAF (Table I).

The human subunits BAF170 and BAF155, which are highly homologous, were purified, sequenced and cloned. The alignment of both genes with the yeast Swi3 revealed that all of them have 4 conserved regions in the proteins including a hydrophobic region, a tryptophan-repeat domain or SANT, a leucine-zipper or coiled-coil region (identified as dimerization domain) and a SWIRM (Swi3, Rsc8, and Moira) domain. The SWIRM domain is a conserved domain of about 85 residues present in Swi3, Rsc8 and Moira as well as other chromosomal proteins involved in chromatin remodeling (Aravind and Iyer, 2002; Qian et al., 2005). BAF170 and BAF155 form hetero or homodimers.

The mouse BAF60 (mBAFa) was purified and sequenced before the human counterpart, and confirmed to be the homolog of the yeast Swp73 subunit. This subunit contains the SWIB domain which is similar to the p53-binding domain of MDM2 and a region present in some topoisomerases (Mohrmann and Verrijzer, 2005). The human BAF60 includes 3 members, BAF60a, b and c, differing at the N-terminal regions which are expressed differentially in tissues. The human BAF60a, which is ubiquitously expressed, is highly homologous to the mouse (mBAF60a), and it has been shown to copurify with BRG1. BAF60b is expressed in many tissues, but more so in pancreas, whereas BAF60c is expressed particularly in muscle (Wang et al., 1996b). Recently, a new isoform of BAF60c, BAF60c2, was identified. Both isoforms are expressed in a number of tissues, and it has been found that they bind to several nuclear receptors and transcription factors and enhance their activities (Debril et al., 2004).

Another subunit, BAF47, obtained from rat liver, is the counterpart from the previously identified human INI1 (integrator interactor 1), the homolog of the yeast SNF5 gene (Kalpana et al., 1994; Wang et al., 1996a; Wang et al., 1996b). BAF47 contains a highly conserved domain with 2 direct repeats and a coiled-coil region. The human BAF47 interacts directly with BRG1 and hBRM and these interactions mediate the ATPase motor activity (Kalpana et al., 1994; Muchardt et al., 1995).

BAF45 and BAF53 were identified as  $\beta$ -actin and actin-related proteins respectively. It has been proposed that BAF45 and BAF53 are required for the association of the SWI/SNF complex with chromatin/nuclear matrix. However, further studies will be necessary to establish this directly (Zhao et al., 1998).

The cloning of the human and mouse BAF57 subunit (BAP111 in *Drosophila*) revealed that it contains 4 domains. At the N-terminus are present a proline-rich region and a high-mobility-group (HMG) domain. At the C-terminus is found a kinesin-like coiled-coil domain and an acidic region. The BAF57 subunit does not have a yeast homologue but it is a component of all mammalian SWI/SNF complexes (Papoulas et al., 1998; Wang et al., 1998).

The human BAF180 subunit, which is the homologue of the *Drosophila* polybromo, was purified and cloned. BAF180 together with BAF200 (see below) are specific subunits of the PBAF complex. BAF180 contains the highly conserved structural domains, already described in *Drosophila*. Because the yeast Rsc1, 2 and 4 from the yeast Rsc complex contain two bromodomains and a BAH (Bromo Adjacent Homology) region homologous to BAF180, it was proposed that Rsc1, 2 and 4 are the closest relatives of the human BAF180 (Xue et al., 2000). Xue and colleagues proposed that the presence of BAF180 and the absence of BAF250 (see below) in the PBAF

complex gives a structural and a functional specificity to the complex (Xue et al., 2000). Therefore, BAF180 and BAF200 in PBAF and BAF250 in BAF might be critical components that functionally discriminate BAF from PBAF. It would be interesting to determine if BAF180 and BAF200 are responsible for targeting PBAF to specific genes *in vivo*.

Recently, BAF200 subunit was isolated by improved immunopurifications procedures using antibodies against BRG1, BAF180 and hSNF5. BAF200 is the homologue of the *Drosophila* BAP170 and it is an integral component of PBAF complex (Wang et al., 1996a; Xue et al., 2000). BAF200 contains in addition, a putative sequence-specific DNA binding region that is not present in BAP170. Similar to BAF180, BAF200 contains various domains involved in DNA and protein-protein interactions, which might permit the PBAF complex to be targeted to specific regions (Yan et al., 2005).

In reference to the Osa subunit, the cloning of two human Osa, hOsa1 and hOsa2 was reported. hOsa1 and hOsa2 are the largest subunits present in the human SWI/SNF-A (PBAF) complex. hOsa1 and hOsa2 are approximately 60% identical and contain the AT-rich DNA binding domain and a C-terminal region known as Osa homology domain that confers the ability to interact with nuclear hormone receptors (Inoue et al., 2002; Kozmik et al., 2001). Likewise, the C-terminal domain is involved in the interaction of hOsa1 and hOsa2 with BRG1 and hBRM. Immunoprecipitations using HeLa nuclear extracts showed that hOsa1 and hOsa2 antibodies were able to precipitate BRG1, hBRM and other subunits, indicating that both hOsa1 and hOsa2 interact with BRG1 and hBRM (Inoue et al., 2002).

In summary, SWI/SNF complexes not only consist of many subunits but they also exhibit significant variation in composition. We have seen that almost all of the subunits have been structurally characterized in different organisms. The presence of conserved structural domains in these subunits establishes the functional properties of the complex they form part of (Mohrmann and Verrijzer, 2005). Diverse roles for the SWI/SNF subunits have been proposed; one potential function may be to regulate the motor activity of the ATPase subunit. In fact, at least four subunits are needed to stimulate the BRG1 ATPase activity *in vitro* to levels comparable to the intact complex. These subunits, Ini1, BAF155 and BAF170 form, together with BRG1, a functional catalytic core (Phelan et al., 1999). It is interesting that these minimal subunits are highly conserved from yeast through humans (see Table I) (Marenda et al., 2004; Phelan et al., 1999). It has been proposed that the subunits of the catalytic core might be assisting the ATPase subunit either by increasing the affinity of the ATPase for nucleosomes, stabilizing a particular conformation that is more active, and/or assisting remodeling (Phelan et al., 1999). Do these minimal subunits also constitute a catalytic core *in vivo*? This question does not have a complete answer yet. Knockout mice for Brg1, Ini1 and BAF155 were generated, showing that all are critical for development (See section 2.4). Furthermore, some subunits of the complex have independent functions and it is possible that they are involved in functions other than remodeling, such as specific targeting through regulators or repressors, complex assembling, and/or controlling complex functions (Lusser and Kadonaga, 2003; Marenda et al., 2004). Moreover, different mammalian tissues contain SWI/SNF complexes that are heterogeneous in subunit composition, indicative of specialized function [Reviewed in (Chi, 2004;

Sudarsanam and Winston, 2000)]. Thus, further investigations are required in order to precisely establish the role of each subunit within different complexes.

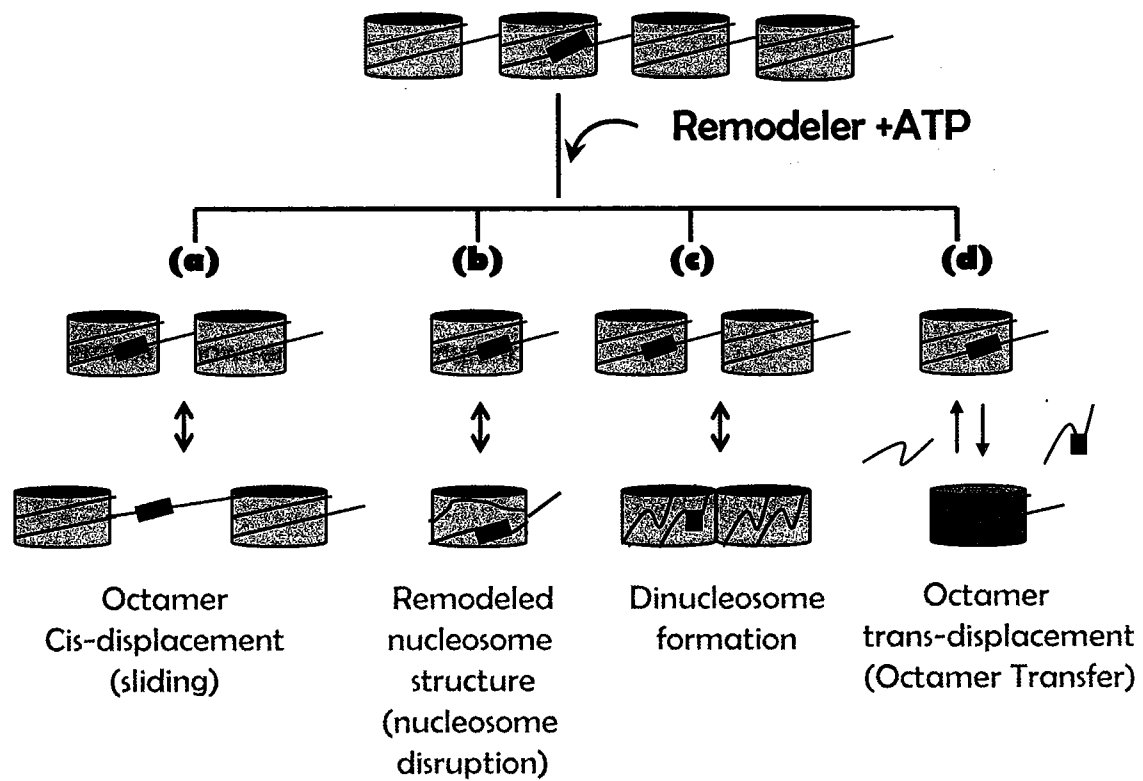
### **2.3) Mechanism of ATP-dependent chromatin remodeling**

Even though there is clear involvement of ATP-dependent chromatin remodeling enzymes in a variety of nuclear functions, the DNA-histone remodeling process has not been completely revealed (Imbalzano and Xiao, 2004; Johnson et al., 2005; Smith and Peterson, 2005). The studies of chromatin remodeling have been concentrated to *in vitro* systems since *in vivo* studies are technically restricted.

The mechanism of remodeling is demonstrated by changes in the structure and/or position of the nucleosomal DNA on the nucleosome that could be brought about by disruption, sliding, dinucleosome formation or transfer of individual nucleosomes (Fig. 1.4) [Reviewed in (Imbalzano and Xiao, 2004; Johnson et al., 2005; Smith and Peterson, 2005) and references therein]. In addition, remodeling can cause removal or replacement of histone dimers between nucleosomes [Review in (Flaus and Owen-Hughes, 2004)]. How are these structural changes generated? A number of potential mechanisms have been proposed to explain chromatin remodeling, however two major models have been preferred, the twisting and the bulging or spooling models (Flaus and Owen-Hughes, 2004; Smith and Peterson, 2005).

In the twisting model a small local twist disrupts histone-DNA contacts and propagates through the length of the nucleosomal DNA, generating a change in the DNA position in respect to the core histone. This model is consistent with the capacity of the ATP-chromatin remodeling enzymes to create superhelical torsion. The bulge or looping





**Figure I.4.** Models for changes in chromatin structure mediated by ATP-dependent chromatin remodeling enzymes (Imbalzano and Xiao, 2004).

model suggests that a region of the DNA is released as a loop from the nucleosome and it is propagated along the surface of the nucleosome (Imbalzano and Xiao, 2004; Smith and Peterson, 2005). Recently, Fan and colleagues proposed an alternative model where the remodeler slides the histone octamer to a different position, via intermediates that contain DNA loops on the histone surface. This occur, even though the remodeled nucleosome is constrained by adjacent ones, indicating that a particular nucleosome can be remodeled without affecting the structure of the surrounding ones (Fan et al., 2003).

Although experimental data support these models, there is some discrepancy. Further investigations are necessary to clarify the mechanism of remodeling, specially determining how remodeling occurs *in vivo*.

#### **2.4) The mammalian SWI/SNF subunits in development and differentiation.**

In order to determine the role *in vivo* of the SWI/SNF complexes in development, targeted disruption of specific subunits has been generated in mice. In the next subsections these results are presented.

##### **BRG1 and BRM**

Initial studies were focused on determining the pattern of expression of mBrg1 and mBrm during early mouse development at preimplantation stages. In mature oocytes both genes are expressed, indicative of maternal contribution. Levels of mBrg1 mRNA continued to be high during the preimplantation period. Levels of mBrm instead showed a decline from the oocyte to the 4-cell stage and an increase from the 8 cells to the blastocyst stage and mBrm protein was limited to the inner cell mass at the blastocyst stage. From this study it was evident that mBrg1 and mBrm likely have

different roles regulating gene expression during early development of mammalian cells (LeGouy et al., 1998). To determine the levels of expression at later stages in mouse development, embryos and extraembryonic tissues (yolk sack and allantoid) were evaluated from 7.5 to 18 days. mBrg1 protein was present at high concentrations in both embryonic and extraembryonic tissues, however the levels of mBrm were 20 to 30 fold lower than the levels of mBrg1. After birth levels of hBrm protein exceeded those of mBrg1 in a number of organs (Reyes et al., 1998)

Later, the mBrm gene was inactivated by homologous recombination with the purpose of determining the role of the mBrm-containing SWI/SNF complexes *in vivo* and investigating the functional differences between mBrg1 and mBrm. Surprisingly, mBrm null mice were viable and developed and reproduced normally. However, these mice expressed high levels of mBrg1 protein in different organs compared to wild type mice, indicating that possibly a compensatory mechanism operated. In fact, it was found that a post-transcriptional mechanism of regulation was involved since the mRNA levels of mBrg1 in homozygotes, heterozygotes and wild-type mice were similar. These results indicated that mBrm and mBrg1 functions are in part redundant (Reyes et al., 1998).

Although null mBrm mice seemed normal, they showed some different characteristics than the wild type mice. mBrm (-/-) mice were heavier than wild-type, and proliferation of cells in liver was higher as well. Cultures of mouse embryonic fibroblasts (MEFs) obtained from null mice did not arrest after confluence. Certain observations have suggested a role of mBRM in G0 arrested cells. These include the high expression in adult mice in post-mitotic cell types, cell cycle arrested cells show more Brm than exponentially growing cells, and downregulation of Brm expression has been observed in transformed cell lines. These observations may point to a role for Brm

in the control of cell proliferation and terminal differentiation that cannot be compensated by mBrg1 (Reyes et al., 1998).

In contrast with mBrm, a mBrg1 null mutation in mice is lethal and embryos die at the peri-implantation stage. The *in vitro* culture of recovered null blastocysts at day 3.5 (E3.5) showed that they failed to hatch from their zona pellucidae or to stay alive in culture. The absence of mBrg1 causes a more general effect to the embryo than the failure to hatch. In fact, the death of the embryos at early stages and the failure of the inner cell mass (ICM) and the trophectoderm (TE) to survive was suggestive of the requirement of mBrg1 beyond the peri-implantation stage. This result indicates that mBrg1 is essential for early embryogenesis, but, it is not a general cell viability factor, since mBrg1 deficient fibroblasts proliferate as well as control cells (Bultman et al., 2000).

Although mBrg1 (+/-) mice are viable and seen normal, 15% of them show exencephaly and they are susceptible to developing tumors at low frequency. The tumors were epithelial in origin, contained glandular structures, and occurred in subcutaneous tissues. Tumor formation in mBrg1 (+/-) mice has been ascribed to haplo insufficiency since loss of heterozygosity has not been revealed in tumors (Bultman et al., 2000; Roberts and Orkin, 2004). In contrast, Brm (-/-) mice did not develop tumors (Reyes et al., 1998).

Both mBrm and mBrg1 null mutations surprisingly generated different phenotypes. What would be expected, considering the main function of these ATPases, is the inactivation of the whole complex and the generation of the same phenotype (Roberts and Orkin, 2004). One possible explanation is that complexes containing Brm or Brg1 might be recruited to different downstream target genes (Bultman et al., 2005).

In order to answer to this question a new mBrg1 mutation was obtained. This mutation avoided the early embryonic lethality produced in the Brg1null mice (Bultman et al., 2005). Mice containing a Brg1 hypomorph allele in the ATPase domain were isolated from an N-ethyl-N-nitrosourea (ENU) mutagenesis screen. The allele contained a point mutation where amino acid glutamine was substituted to glycine at the putative helicase motifs IV and V of the catalytic ATPase domain. This region is conserved from yeast to human and it is involved in coupling the energy from ATP hydrolysis to chromatin remodeling (Bultman et al., 2005). The mutant mBrg1 protein was stable, assembled into the SWI/SNF complex and exhibited normal ATPase activity, but the nucleosome remodeling properties was reduced. mBrg1<sup>null/ENU</sup> mice developed normally until E11.5 to E14.5 stages, dying because of anemia caused by a blockage in the development of the erythroid lineage. The normal development of the mBrg1<sup>null/ENU</sup> mice raises the possibility that mBrm could functionally compensate for mBrg1 activities in many cell types where it is expressed; however, it could not compensate for mBrg1 activity in the erythroid lineage, where it is absent.

These results indicate that the ATPase activity of Brg1 needs to be coupled with chromatin remodeling activity in order for this energy to be connected to a change in conformation and position of the nucleosomes (Bultman et al., 2005).

### **Ini1/Snf5**

Investigations in malignant rhabdoid tumors and atypical teratoid tumors (also refer as atypical teratoid/rhabdoid tumors), which are aggressive and highly lethal cancers of early childhood, have revealed that these tumors carry specific, biallelic, inactivating mutations in the SNF5/Ini1 gene [Review in (Gibbons, 2005; Mohrmann and

Verrijzer, 2005; Roberts and Orkin, 2004) and references therein]. In order to investigate the mechanism by which *Ini1* is involved in tumorigenesis, and its possible role as a tumor suppressor, three independent groups simultaneously generated, by different targeting strategies, *Ini1* knockout mice. Disruption of *Ini1* expression is embryonic lethal and occurs during the periimplantation stage, between 3.5 and 5.5 days (Guidi et al., 2001; Klochender-Yeivin et al., 2000; Roberts et al., 2000). Analysis of *Ini1* expression during embryogenesis confirmed the presence of the protein in fertilized oocytes and two cell embryos, due to maternal stores. *Ini1* expression starts at the 4 cell stage and is maintained during all subsequent developmental periods. *Ini1* protein is present in embryos ubiquitously, but predominantly concentrated in the headfolds, neural folds, first branchial arch and hindlimb bud (Guidi et al., 2001; Klochender-Yeivin et al., 2000; Roberts et al., 2000).

The *in vitro* culture of recovered null *Ini1* blastocysts at day 3.5 (E3.5) showed two different results. Klochender-Yeivin and colleagues found that one third of the *Ini1*-null embryos failed to hatch from the zona pellucida, while the rest of them emerged, but the trophectoderm did not spread, suggestive of a trophectoderm defect (Klochender-Yeivin et al., 2000). However, Guidi and colleagues showed that 100% of the null blastocysts in culture did not hatch and expand in culture, which is indicative of hatching defect. Even more, manual disruption of the zona pellucida from null blastocysts showed a failure of the inner cell mass and the trophectoderm to expand. As was the case of *mBrg1* knockout mice, this result indicates that *Ini1* was required beyond the peri-implantation stage (Guidi et al., 2001).

*Ini1* heterozygous mice developed normally but are highly susceptible to tumor formation in the head or neck regions of the mice, predominantly in the soft tissue of the

face. The analysis of the expression of Ini1 protein from tumors showed the absence of Ini1, indicative of loss of heterozygosity (LOH) at the Ini1 locus. LOH is the main reason for the tumor development in the heterozygote mice, a characteristic of classical tumor suppressors (Guidi et al., 2001; Knochendler-Yeivin et al., 2000; Roberts et al., 2000).

In order to understand the molecular mechanisms by which INI1 suppresses tumor formation, several groups have investigated the effect of reintroduction of the INI1 gene in INI1-deficient cancer cell lines. The results have shown that cells undergo cell cycle arrest in G0/G1, which indicates that INI1 function limits cell division. However, the mechanisms differ in different tumor cell lines (Roberts and Orkin, 2004).

Is the tumor suppressor activity of INI1 mediated via effect on the integrity of the SWI/SNF complex? The answer should be yes if we extrapolate from results obtained from the yeast SWI/SNF complex. Deletions and/or mutation of any subunit causes disassembly of the complex (Peterson et al., 1994). However, it was shown that the assembly of the SWI/SNF complex in human cells can occur independently of INI1 subunit. Moreover, some genes that require BRG1 do not require INI1 (Doan et al., 2004). Therefore, it is possible that the mechanism of tumor suppression by INI1 is not related to the integrity of a functional SWI/SNF complex, but instead to an independent activity (Doan et al., 2004).

### **BAF155 /Srg3**

Srg3 is the mouse homolog of the yeast Swi3, the *Drosophila* Moira/BAP155, and the human BAF155. As it was mentioned previously, this subunit is a component of the catalytic core of SWI/SNF complexes. To examine the *in vivo* function of Srg3, Kim and colleagues generated knockout mice for Srg3 (Kim et al., 2001). As was

demonstrated for mBrg1 and mIni1, absence of Srg3 in mice resulted also in early embryonic lethality. The expression pattern of this subunit during mouse development, from E7.5 to E10.5 embryos, showed that Srg3 protein was present constitutively in all of the stages. Following stages from E12.5 to E18.5 were evaluated with *in situ* hybridization. Srg3 expression pattern coincided with that of mBrg1, but with some differences. Embryos at E12.5 and E14.5 expressed high levels of Srg3 in almost all organs, excluding heart and liver; mBrg1 was found in spinal cord, brain and thymus. At E16.5 Srg3 was evidenced in lung, intestine, thymus and central nervous system. As the embryos developed further, expression of Srg3 changed, becoming restricted to the thymus and central nervous system (Kim et al., 2001). Since Srg3 is expressed in additional regions of the embryos, the authors suggested that Srg3 may have alternative and independent function exclusive of its role in SWI/SNF remodeling activity. However, pattern of expression of mBRM was not determined in these embryos; therefore it may be possible that Srg3 is present in mBRM complex.

Blastocysts from null embryos at E3.5 cultured *in vitro* undergo hatching, adherence, and formation of trophoblast giant cells, although the inner mass degenerate. Levels of Brg1 were stable during these experiments (Kim et al., 2001).

Although heterozygous mice looked normal, around 20% of them developed exencephaly a condition that includes defects in brain growth and organization (Kim et al., 2001). Exencephaly was also seen in heterozygous Brg1 mice (Bultman et al., 2000). It has not been confirmed if Srg3 heterozygotes are susceptible to spontaneous neoplasia, as was demonstrated for mBrg1 and Ini1 (Kim et al., 2001).



### **Polybromo/BAF180**

As mentioned in a previous section, BAF180 represents a specific subunit of the SWI/SNF (PBAF) complex (Table I). In order to determine its role in the PBAF complex, BAF180 knockout mice were generated (Wang et al., 2004). The expression of BAF180 is ubiquitous during mouse embryogenesis and also in extraembryonic tissues, such as the yolk sac and placenta. Disruption of BAF180 is embryonic lethal and mice embryos die between E12.5 and E15.5 of gestation. Examination of null embryos showed that the majority of the organs were present, but liver and lung were smaller and hearts exhibited incomplete development of the cardiac ventricular free walls and ventricular septa. Analysis of the nature of the heart defects showed that it was not a consequence of apoptosis but instead a failure in cell growth and differentiation (Wang et al., 2004). Because BAF180 is expressed also in placenta, examination for defects was performed. Placentas were abnormal at the labyrinthine layer, where groups of trophoblasts replaced maternal and fetal blood space, putting at risk the maternal-fetal exchange. In order to determine that the defect in heart development was due to a direct effect of BAF180 disruption, Wang and colleagues performed tetraploid aggregation experiments to rescue the placenta and complementary fusion experiments. The results confirmed a direct role for BAF180 in the heart development and not an indirect effect caused by the placenta (Wang et al., 2004).

Gene expression profiles of the wild type and mutant hearts were obtained by microarray analysis. Potential target genes involved in heart development were identified and the presence of BAF180 was found on the promoters of some of these genes. Therefore, these studies demonstrated a contribution of BAF180 in the maturation of the cardiac chamber by mediating the expression of genes involved in cell

growth and proliferation. This function is specific to this subunit and differs from the roles of Brg1, BAF155, and Ini1 subunits in early embryogenesis.

### **BAF60c**

Another subunit of the mammalian SWI/SNF BAF complexes examined during mouse development was BAF60c. Levels of BAF60c mRNA and protein are detected starting at day 7.5 during development, being concentrated in the lateral mesoderm (LPM) that will contribute to the heart. As development progresses, BAF60c is found in the heart at the looping heart tube, in somites and in the midline of the neural tube (Debril et al., 2004; Lickert et al., 2004). The role of BAF60c in developing mice was determined by Lickert and colleagues using an embryonic stem cell-mediated transgenic RNA interference strategy. Knockdown of BAF60c caused cardiac malformations with lethality at E10.0 to E11.0. The heart contained a single ventricle connected to an abnormal outflow tract and the myocardial wall looked rougher. Additionally, the silencing of BAF60c caused failure of forebrain closure and disordered somites. The analysis of markers of primary and anterior/secondary heart fields were delayed in the knockdown mice, but the expression of primary and secondary heart fields was normal (Lickert et al., 2004).

The transcriptional activity of BAF60c was demonstrated *in vitro* by transient reporter assays. BAF60c overexpressed in cell culture mediated interactions between specific heart transcription factors and SWI/SNF through Brg1, potentiating the expression of the target genes. The tissue-specific expression of BAF60c is a good example of how the interactions of transcription factors and remodeling enzymes are established specifically to regulate particular genes during tissue morphogenesis.

Disruption of the core subunits in the SWI/SNF complex, including Brg1, Ini1 and BAF155, produced early lethality at the peri-implantation stage. Disruption of other subunits, like BAF180, which distinguishes BAF from PBAF complexes, and BAF60c which is expressed in a tissue specific manner, resulted in lethality, but at later stages where they affect a particular organ/tissue. Individual subunits have differential functions within a cell type. There are isoforms of BAF subunits that are thought to mediate the functions of the SWI/SNF complexes in a tissue specific manner, such as the neuron-specific BAF53b (Olave et al., 2002). Detailed structural and functional analysis of the unique and distinguishing subunits in the SWI/SNF complexes will ultimately help us better understand how these complex chromatin remodeling cofactors work individually or in concert to guide expression, cell growth, and organ development (Wang et al., 2004).

We have seen the involvement of SWI/SNF in early and middle development and its role in tumor suppression. The following will summarize the differentiation events in which SWI/SNF remodeling enzymes participate. These studies not only have demonstrated the contribution of SWI/SNF in cellular differentiation, but also have defined the mechanisms by which SWI/SNF activity promotes gene expression. To perform these studies the use of tissue culture models of differentiation has been pivotal.

### **SWI/SNF and cellular differentiation**

#### **Myeloid differentiation**

The first endogenous mammalian genes shown to be controlled by SWI/SNF complexes were the myeloid specific genes (Kowenz-Leutz and Leutz, 1999). During

myeloid differentiation two major transcription factors, C/EBP $\beta$  and Myb, collaborate to activate specific myeloid genes. In fact, overexpression of both activators in heterologous cell lines, such as fibroblasts, induces the expression of originally silent myeloid-specific genes. It was demonstrated that transcriptional activation mediated by C/EBP $\beta$ -Myb involves the recruitment of SWI/SNF complex, via direct interaction of Brm with the N-terminal region of the C/EBP $\beta$  activator. If this C/EBP $\beta$  domain is transferred to Myb, the resulting chimera is able to activate myeloid genes in the absence of C/EBP $\beta$  (Kowenz-Leutz and Leutz, 1999). Thus, interaction of C/EBP $\beta$  and SWI/SNF is crucial for activating a subgroup of genes involved in myeloid differentiation.

### **Enterocyte differentiation**

The human epithelial cell line CaCo-2 spontaneously differentiates from a cryptlike to a villous like enterocyte and expresses several markers, including the well characterized  $\alpha$ 1-antitrypsin gene. The  $\alpha$ 1-antitrypsin gene is regulated by the hepatocyte nuclear factors HNF-1 $\alpha$  and HNF-4 $\alpha$  that function synergistically (Hu and Perlmutter, 1999). In an analysis of the temporal recruitment of factors to the human  $\alpha$ 1-antitrypsin promoter during enterocyte differentiation, transient binding of hBRM occurred. The major events during differentiation included the stable association of HNF-4 $\alpha$  and hBRM at the promoter, the remodeling of one of the two nucleosomes (the one containing the TATA box and the transcription start site) and transcription initiation (Soutoglou and Talianidis, 2002). Therefore, chromatin remodeling by the SWI/SNF complex is crucial for the activation of the  $\alpha$ 1-antitrypsin gene expression.

### **Muscle differentiation**

Skeletal muscle differentiation involves the activation of specific genes by the action of two families of transcription factors: the myogenic basic helix loop helix (bHLH) family, which includes MyoD, Myf5, myogenin, and MRF4 and the myocyte enhancer factor 2 (MEF2) family (Berkes and Tapscott, 2005). Each of the members of the myogenic family of transcription factors is able to initiate the muscle differentiation program when they are ectopically expressed in different cell lines. Activation of muscle specific genes in fibroblasts overexpressing any of the members of the myogenic family, is abrogated by the presence of dominant negative versions of hBRG1 or hBRM (see below) (de la Serna et al., 2001b; Roy et al., 2002). Induction of the muscle-specific genes required SWI/SNF activity to facilitate chromatin remodeling at the endogenous specific promoter (de la Serna et al., 2001b). Consequently, these studies demonstrated the crucial role of SWI/SNF enzymes in the initiation of the muscle differentiation program. Furthermore, a recent study using microarray analysis of cells differentiated by MyoD in the presence or absence of dominant-negative BRG1, showed that one-third of the MyoD-induced genes were dependent on SWI/SNF enzyme activity (de la Serna et al., 2005). Chromatin immunoprecipitation (ChIP) at the myogenin promoter confirmed the presence of BRG1 during differentiation. In the presence of dominant-negative BRG1, the recruitment of MyoD transcription factor was prevented and transcription was prevented (de la Serna et al., 2005).

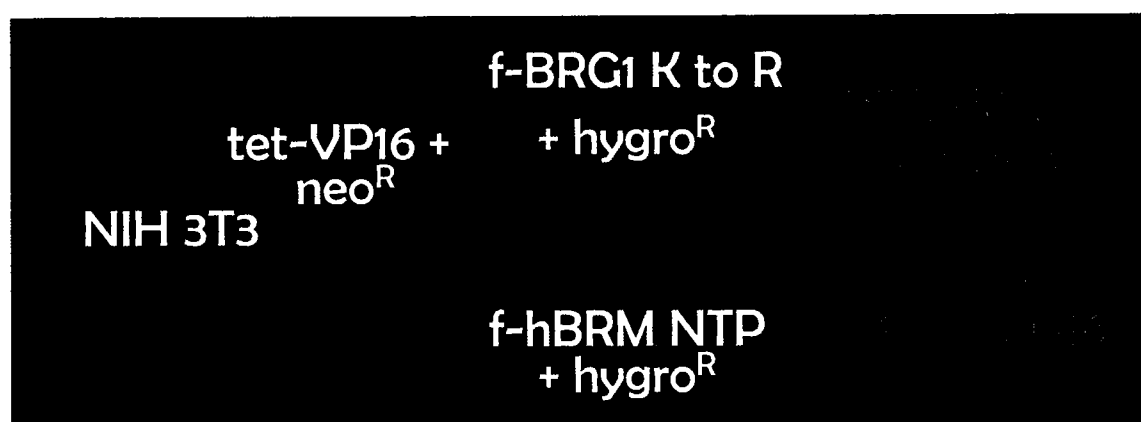
### **Adipocyte differentiation**

The main goal of this thesis was to determine the role of SWI/SNF chromatin remodeling enzymes in adipocyte differentiation. Prior to this work there were not

publications on the role of chromatin remodeling enzymes during adipocyte differentiation.

After the isolation and characterization of the human SWI/SNF complexes as ATP dependent chromatin remodeling machines that alter nucleosome structure (Imbalzano et al., 1994; Kwon et al., 1994), Anthony Imbalzano's laboratory major aim was the identification of endogenous cellular loci whose activation and expression is dependent on chromatin remodeling by the mammalian SWI/SNF complexes. In order to determine the role of the mammalian SWI/SNF complexes, Imbalzano and colleagues generated cell lines that inducibly express either hBRG1 or hBRM subunits that are mutated in the ATP binding site and FLAG tag. The purpose was to create non-functional hSWI/SNF complexes via sequestration of BAF subunits into complexes with the mutated hBRG1 or BRM. Thus, genes which activation requires SWI/SNF activity would be impaired in cells expressing dominant negative version of the complexes (de La Serna et al., 2000).

Conditional expression was performed using the Tet repressor system. Briefly, NIH3T3 cells were stably transfected with the vector that encodes a Tet-VP16 regulatory protein (Fig. 1.5), which is a fusion of the DNA binding domain of the Tet repressor with the activation domain of the herpes simplex virus activator VP16, and a vector encoding drug resistance. The fusion protein is active in the absence of tetracycline and can promote expression from promoters containing a binding site for Tet repressor. Subsequently, the cells were transfected with the Tet responsive vector containing an epitope (FLAG) tagged dominant versions of the hBRG1 or BRM (Fig. 1.5). The cell lines generated that inducibly express FLAG tagged dominant negative hBRM were named



**Figure I.5.** Generation of dominant negative BRG1 and hBRM cell lines.

"H" lines and the cell lines that inducibly express FLAG tagged dominant negative BRG1 were named "B" lines. The parental cell line Tet-VP16 which contains the Tet regulator was used as control. Initially, these cell lines were used to demonstrate that dominant negative SWI/SNF enzymes inhibit the activation of the cellular stress response when activated by environmental stresses (de La Serna et al., 2000). Subsequently, de la Serna and colleagues demonstrated that hSWI/SNF enzymes are required for muscle differentiation (see above) (de la Serna et al., 2001a). Because components of the mammalian SWI/SNF complexes were required for the initiation of skeletal muscle differentiation, we ask if these remodeling enzymes were also involved in adipocyte differentiation.

Why are adipocyte differentiation studies important? Adipogenesis is the process by which mature fat cells are formed from preadipocytes. Adipose tissue is considered as a global regulator of energy metabolism. Excessive development of adipocyte tissue causes pathologies, including obesity. Adipocyte related diseases including diabetes type II and atherosclerosis have been established as serious health problems. Consequently, all aspects of adipocyte biology, including adipogenesis, and the sequence of transcriptional events during adipogenesis have become targets of intense scientific investigation.

A brief summary of this thesis research is presented here. In chapter II this work will be discussed thoroughly.

The main transcription factors involved in adipocyte differentiation are members of the basic leucine zipper (bZIP) family of CCAAT/enhancer binding proteins (C/EBPs), specifically C/EBP $\beta$ ,  $\delta$  and  $\alpha$  and the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR $\gamma$ ). Any of these activators, except for C/EBP $\delta$ , when



ectopically expressed in non-adipocyte cell lines is sufficient to drive the complete program of adipogenesis (Tontonoz et al., 1994b; Wu et al., 1995a; Yeh et al., 1995).

We found that adipogenesis and induction of PPAR $\gamma$  forced by overexpression of the adipogenic activators was blocked upon expression of dominant negative hBRG1 or hBRM. The recruitment of BRG1, BRM, and Ini1 to adipocyte specific promoters indicated that SWI/SNF enzymes were involved in the activation of these genes during adipocyte differentiation (Salma et al., 2004).

During this thesis work, using a different approach, Pedersen and colleagues showed that the capacity of C/EBP $\alpha$  to mediate adipocyte differentiation depends on a domain present in C/EBP $\alpha$  which is responsible for establishing the interaction with SWI/SNF components Brm and BAF155. Therefore, SWI/SNF recruitment is critical for C/EBP $\alpha$ -mediated adipogenesis (Pedersen et al., 2001).

In the following sections of this thesis, general aspects of adipogenesis, including adipocyte tissue and development, sequence of events during adipocyte differentiation, and transcription factors involved in adipogenesis will be covered.

### 3) Adipogenesis

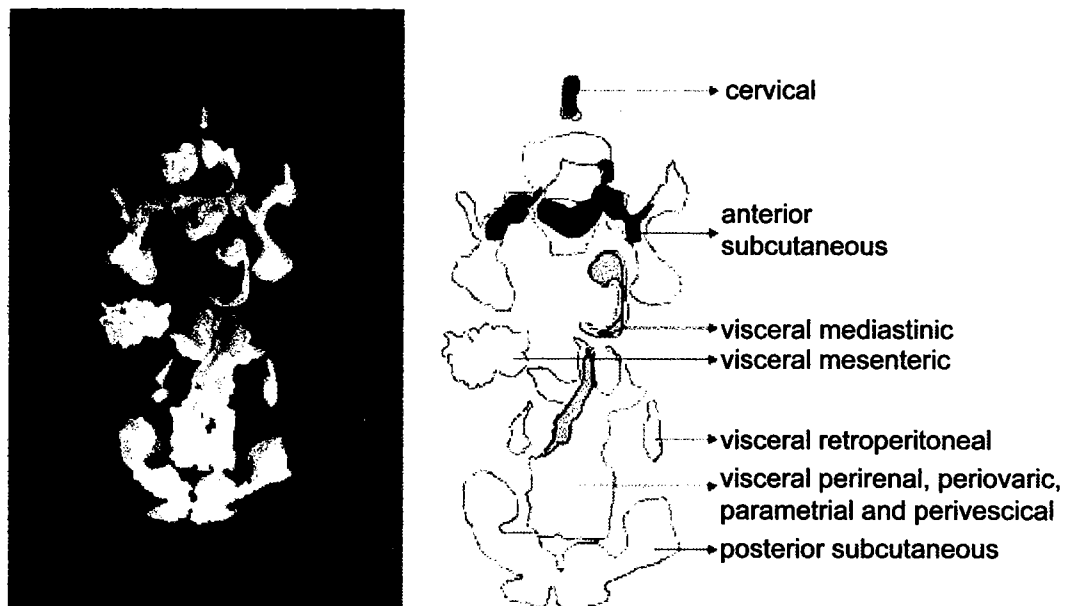
To study the molecular events and the involvement of SWI/SNF function during adipocyte differentiation, a well established cell model was chosen. Adipocyte differentiation can be examined when immortalized preadipocytes undergo differentiation to adipocytes after hormonal stimulation. These adipocytes exhibit a typical morphological and biochemical characteristic of fat cells found *in situ* (Green and Kehinde, 1975; Green and Meuth, 1974; Hwang et al., 1997). Adipogenesis can also be assessed using non-adipogenic cell lines (such as dominant negative BRG1, BRM and

Tet-VP16 cell lines) by forcing them to differentiate by ectopic expression of specific adipocyte transcription factors.

### **3.1) Adipose tissue**

Adipose tissue is a specialized connective tissue that functions as the main storage place for fat in the form of triglycerides during times when caloric input exceeds expenditure. In contrast, when caloric expenditure exceeds intake, the reserves are mobilized to supply energy in the form of fatty acids or heat. There are two kinds of adipose tissue found in mammals, the white adipose tissue (WAT) and the brown adipose tissue (BAT). Adipose tissue is found distributed at sites rich in loose connective tissues such as subcutaneous layers. It is also found in fat depots throughout the body and is divided in subcutaneous and visceral depots (Fig.1.6). In small mammals BAT is primarily found in the intercapsular, subcapsular, axillary, intercostal, perineal and periaortic regions. In humans and in larger mammals, BAT is found during development but after birth the BAT become more like WAT. In small mammals, both WAT and BAT are found in the same depots (Cinti, 2005; Sell et al., 2004).

Brown adipose tissue differs in structure and function from the white adipose. Structurally, brown adipose cells contain small multilocular lipid droplets whereas the white adipose cell is composed of larger unilocular lipid droplets. In addition, brown adipose cells are rich in large mitochondria that express the unique uncoupling protein-1 (UCP-1). The UCP-1 gives the cell's mitochondria the ability to uncouple oxidative phosphorylation and use the substrates to generate heat. Exposure to cold stimulates the sympathetic nervous system, releasing norepinephrine, which in turn promotes the



**Figure I.6.** The adipose organ of an adult mouse. The organ is made up of two subcutaneous and several visceral depots. The most representative visceral depots are shown. White areas made up of WAT and brown areas of BAT are indicated (Cinti, 2005).

oxidation of fatty acids in the mitochondria and the consequent release of heat. On the other hand, sympathetic stimulation on white fat promotes the hydrolysis of triglycerides, and the release of fatty acid and glycerol (Cinti, 2005; Klaus, 1997; Kuroshima, 1993).

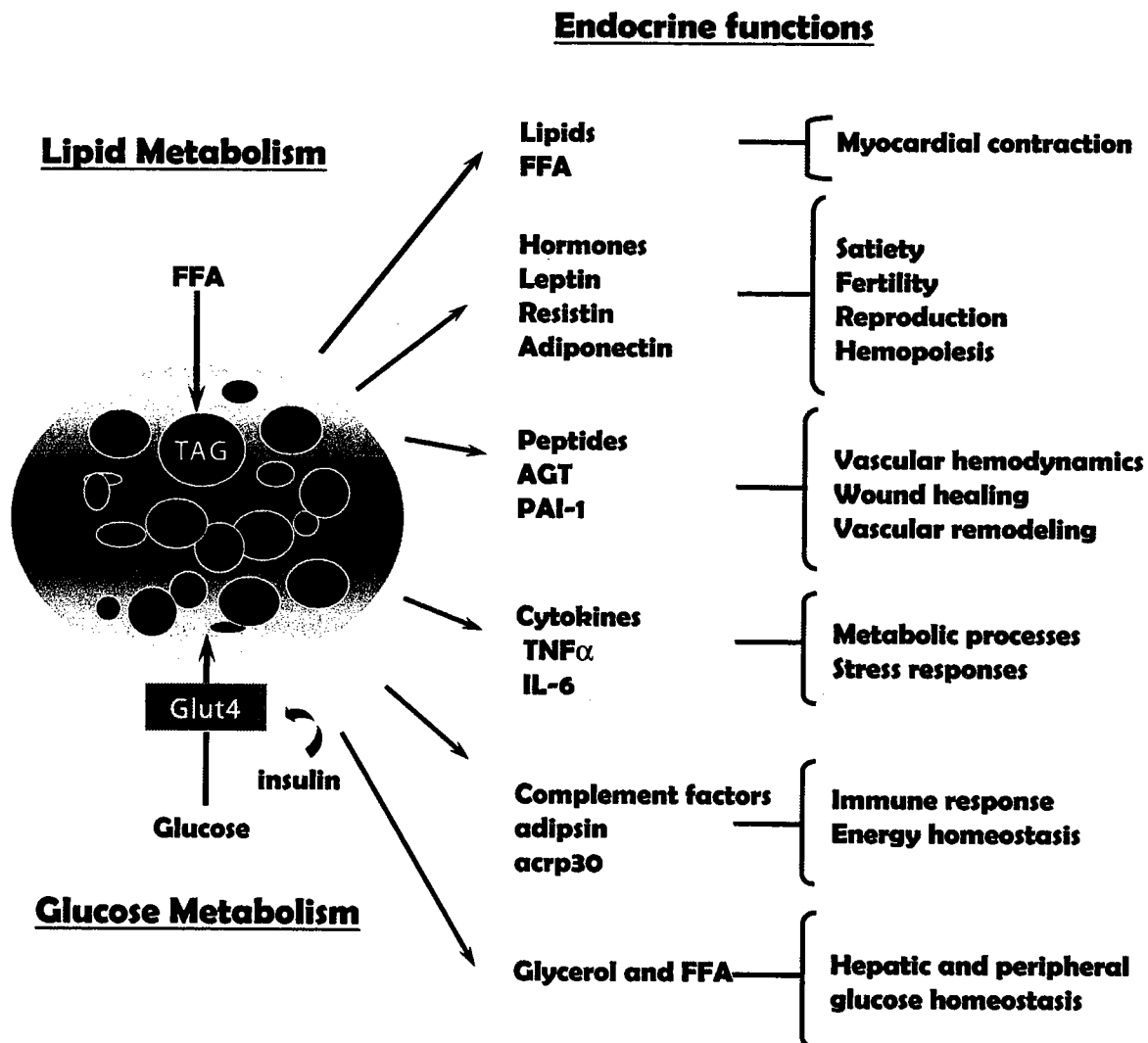
Adipose tissue is no longer considered a passive reservoir of energy. Numerous studies indicate that adipocytes play an active role in the control of the whole body metabolism and homeostasis through the secretion of paracrine and endocrine factors in addition to other regulatory factors (Fig. 1.7) (Kershaw and Flier, 2004; MacDougald and Mandrup, 2002; Morrison and Farmer, 2000; Otto and Lane, 2005). Understanding the development and mechanisms that regulate adipogenesis, adipocyte growth and metabolism as well as the identification of adipose-secreted factors has been the focus of many investigations since adipose tissue is involved in a number of pathologies associated with obesity and its related disorders (Morrison and Farmer, 2000).

Lastly, it is important to mention that different adipose depots exhibit significant heterogeneity. It has been proposed that the observed difference is possibly the result of variation of their endocrine function. Adipose depots show particular adipokine (bioactive peptides expressed by adipose tissue) expression and an exclusive receptor expression, which gives them the capacity to respond appropriately. All of these differences between adipose depots indicate that this tissue not only function as an endocrine organ, but also as group of "distinctive organs" (Kershaw and Flier, 2004).

### **3.2) Adipose development**

#### ***In vivo***

Mammalian adipose cells originate from multipotent mesenchymal stem cells (MSC) in the mesoderm during mid to late gestation. In the emergent fat pad, MSC



**Figure I.7.** Pleiotropic functions of the adipocyte. Adipocytes play roles in lipid metabolism in the storage of free fatty acid and in glucose metabolism through expression of the insulin-dependent glucose transporter, Glut 4. Endocrine functions include secretion of tumor necrosis factor  $\alpha$ , interleukin-6, adipsin and adiponectin (Morrison and Farmer, 2000).

become committed to the adipocyte lineage under the control of factors still unidentified *in vivo*. The process that converts MSC into preadipocytes is known as determination. The time of adipose development varies between species and the adipose depot. After birth, adipocytes expand as a result of an increase in adipocyte size and in the number of preadipocytes. New adipocytes are continually generated and expand even in the adult stage (Fig. 1.8) [Reviewed in (Gregoire et al., 1998; Novakofski, 2004; Rosen, 2002) and references therein].

### ***In vitro***

Since the development of immortal preadipocyte cell lines by Green and colleagues in the 1970s, the study of adipocyte development and physiology has been possible. Even though there are some disadvantages of the *in vitro* system, fat differentiation *in vitro* is reliable and valid, since it recapitulates almost all of the characteristics during the adipogenesis process *in vivo*, including morphological changes and expression of adipocyte genes such as those involved in lipid metabolisms, glucose metabolism, as well as those involved in insulin sensitivity (Rosen, 2002). All *in vitro* systems have limitations; with increasing culture passages, the proliferative capacity and the differentiation potential decreases (Feve, 2005).

### **Cell culture models of differentiation**

Various cell lines have been used to study the molecular and cellular events that take place during the adipocyte differentiation process. These include the multipotent stem cells 10T1/2 that can undergo differentiation into adipocytes, myocytes or chondrocyte and the well characterized preadipocyte cell line 3T3-L1 that are

## **Adipose Tissue Development**

### **Early Gestation**

**Fetal Mesenchyme**

**Endothelial/Mesenchymal Cords**

**Primitive Fat Organs**  
**(dense capillaries)**

**Presumptive Fat Lobules**  
**(identifiable adipocytes,**  
**nerves, blood, lymph vessels)**  
**40-60 days gestation of pig**  
**18-20 weeks in humans**

### **Mid Gestation**

**Adipocytes contain small lipid droplets**  
**Number of lobules increasing**

### **Late Gestation**

**Number of preadipocytes increasing**  
**Number of small adipocytes increasing**  
**Lipids droplets still small**

### **Nursing**

**Most rapid adipose tissue growth**  
**Adipocytes fill rapidly**  
**Preadipocytes proliferate and differentiate**

**Figure I.8.** Adipose tissue development.

(<http://classes.aces.uiuc.edu/AnSci312/Adipose/Adiplect.htm>)

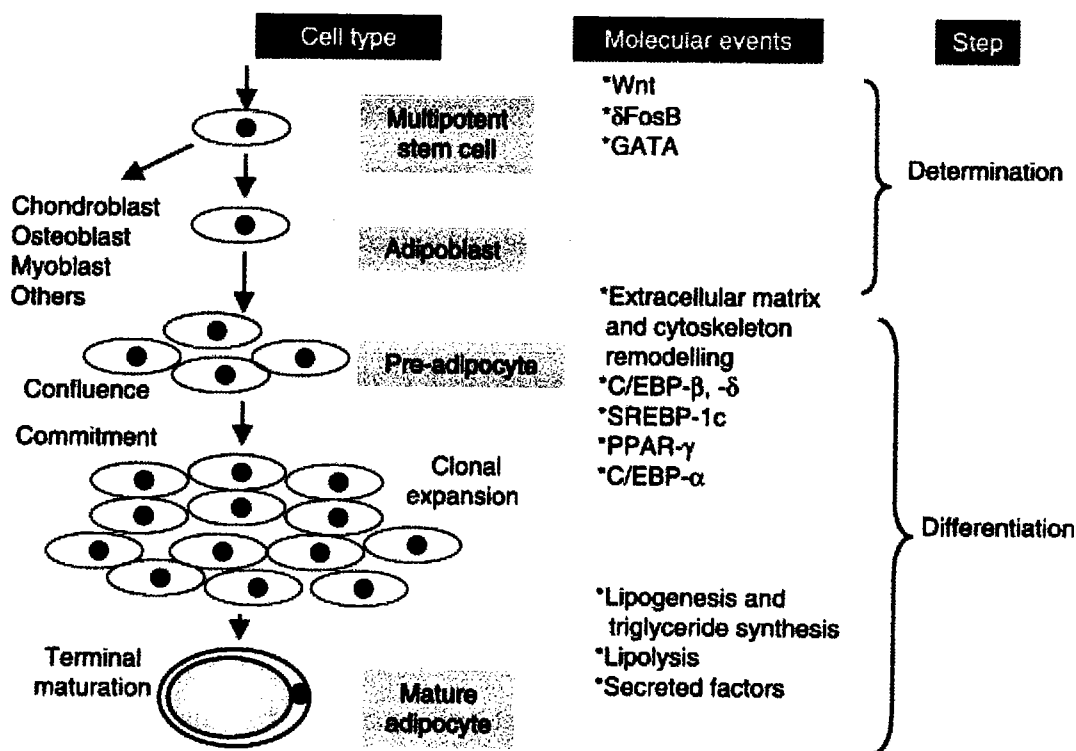
commitment to differentiate into adipocytes. 10T1/2 cells represent an early stage in the adipose development and 3T3-L1 a late stage (Fig. 1.9) (Cornelius et al., 1994; Feve, 2005). Preadipocyte cell lines such as 3T3-L1 and 3T3-F442A were isolated from disaggregated mouse embryo cells and selected for their ability to accumulate fat (Green and Kehinde, 1975; Green and Meuth, 1974). When preadipocyte cell lines are treated with external inducers, they differentiate into cells that are morphologically, biochemically, and functionally identical to mature adipocytes. Evidence indicate that these cell systems recapitulate the whole differentiation process *in vivo*, in fact, transplantation of these preadipocyte cell lines into mice results in development of normal fat pads indistinguishable from the adipose pad [Reviewed in (Cornelius et al., 1994)].

Non-adipogenic fibroblasts can be forced to differentiate into adipocytes when master regulators of adipocyte differentiation, such as C/EBP $\alpha$  or PPAR $\gamma$  are ectopically overexpressed in these cells. After overexpression of regulators, the cells are treated with the same differentiation cocktail used to differentiate preadypocytes.

Protocols that allow a highly efficient differentiation of preadipocytes in culture have been developed. For 3T3-L1 cell lines, the well established differentiation cocktail consists of dexamethasone, a synthetic glucocorticoid agonist, insulin, a hormone that acts through the insulin-like factor 1(IGF-1) receptor in preadipocytes, methylisobutylxanthine (MIX), a cAMP phosphodiesterase inhibitor, and fetal calf serum (Cornelius et al., 1994; Student et al., 1980).

How do these differentiation inducers activate adipocyte differentiation? The insulin/insulin-like growth factor (IGF-1) signaling pathway contributes to adipocyte differentiation through complex signaling networks that play a significant role during





**Figure I.9.** Adipogenesis is a multi-step process. Mesenchymal cells proliferate (clonal expansion). At some point, some of these cells differentiate into preadipocytes. The signal for this critical differentiation, commitment step is unknown *in vivo*; *in vitro* factors have been identified. Preadipocytes proliferate at the site of adipogenesis (clonal expansion). Preadipocytes undergo a second differentiation step and begin to fill with lipids (Feve, 2005).

adipogenesis. Insulin exerts its effect on differentiation through the insulin-like growth factor (IGF-1R) receptor at high concentrations and not through the insulin receptor (IR), whose concentration is low in preadipocytes. During adipogenesis, IR levels increase. The participation of IGF-1R in adipocyte differentiation implies that a tyrosine kinase-mediated signaling pathway is involved. Data support the idea that the tyrosine kinase involved may be the IGF-1R tyrosine kinase. Insulin effects include the activation of the extracellular signal-regulated kinase (ERK) pathway, which is important at the proliferative phase. ERK activity is also necessary for the expression of the adipogenic activators C/EBP $\alpha$ ,  $\beta$ , and  $\delta$  and PPAR $\gamma$  [Reviewed in (Bost et al., 2005)].

Glucocorticoids are another inducer required for preadipocytes to progress through clonal expansion and terminal differentiation. The role of glucocorticoid receptor during adipogenesis is still not clear at molecular level; evidence suggest that adipogenesis stimulated by glucocorticoids may be mediated by its capacity to reduce the levels of preadipocyte factor-1, an antiadipogenic epidermal growth factor that is expressed in preadipocytes (Rangwala and Lazar, 2000). It is known that glucocorticoids activate phospholipase A2, which triggers the release of arachidonic acid for prostaglandin (PGI2 and PGF2 $\alpha$ ) synthesis. The increase of PGI2 causes an increase in cAMP and Ca<sup>2+</sup> levels, which then regulate differentiation (MacDougald and Lane, 1995b). Glucocorticoid activity through its receptor is also involved in the activation of the expression of the adipogenic regulators, C/EBP $\delta$  and PPAR $\gamma$  (Cao et al., 1991; Wu et al., 1996). Methylisobutylxanthine (MIX) is other component of the differentiation cocktail. It is a synthetic organic chemical that inhibits phosphodiesterases, causing an increase in the intracellular level of cAMP (Wu et al., 1996; Wu et al., 1995b). The cellular target of cAMP is the protein kinase A, which catalyzes the phosphorylation of

the cAMP response element binding protein (CREB) at serine 133, and its ability to activate the transcription of C/EBP $\beta$ . Additionally, CREB can be phosphorylated by insulin action. The transcription factor Sp1 negatively regulates C/EBP $\alpha$  through a Sp1 site on the C/EBP $\alpha$  promoter. The levels of Sp1 decrease by the action of (MIX) (Rangwala and Lazar, 2000; Zhang et al., 2004a). Fatty acids and peroxisome proliferators are also activators of adipogenesis, acting synergistically with other inducers (MacDougald and Lane, 1995a).

The use of preadipocyte cell lines has allowed the characterization of the sequence of events during adipogenesis as well as the identification of the master regulatory proteins that control the process (Hwang et al., 1997).

### **3.3) Sequence of events during differentiation *in vitro***

The developmental program *in vitro* can be defined by four stages, preconfluent proliferation, confluence/growth arrest, hormonal induction/mitotic clonal expansion (MCE) and terminal differentiation. Each stage is characterized by the coordinated expression of specific genes. Preconfluent preadipocytes express the preadipocyte factor 1 (Pref-1) which is responsible for maintaining the undifferentiated state. Once confluence is reached, cells arrest at the G0/G1 phase of the cell cycle. At this stage occurs early expression of genes such as lipoprotein lipase (LPL),  $\alpha$ 2 chain type VI collagen, and fatty acid activated receptor (FAAR). The induction of these genes is regulated by autocrine/paracrine mechanism(s) and mediated by cell-cell contacts. [Review in (Amri et al., 1986; Gregoire et al., 1998; MacDougald and Lane, 1995b)]. When cells are exposed to adipogenic inducers, they synchronously enter S phase of the cell cycle and undergo one or two rounds of mitosis. Early changes that occur

during clonal expansion include the early expression of two regulators, the CCAAT enhancer binding proteins C/EBP $\beta$  and C/EBP $\delta$ , which are induced by the effects of IBMX and dexamethasone, respectively (Rangwala and Lazar, 2000; Yeh et al., 1995). After clonal expansion, expression of both regulators decreases and is replaced by C/EBP $\alpha$  (see below) (Cao et al., 1991; Yeh et al., 1995).

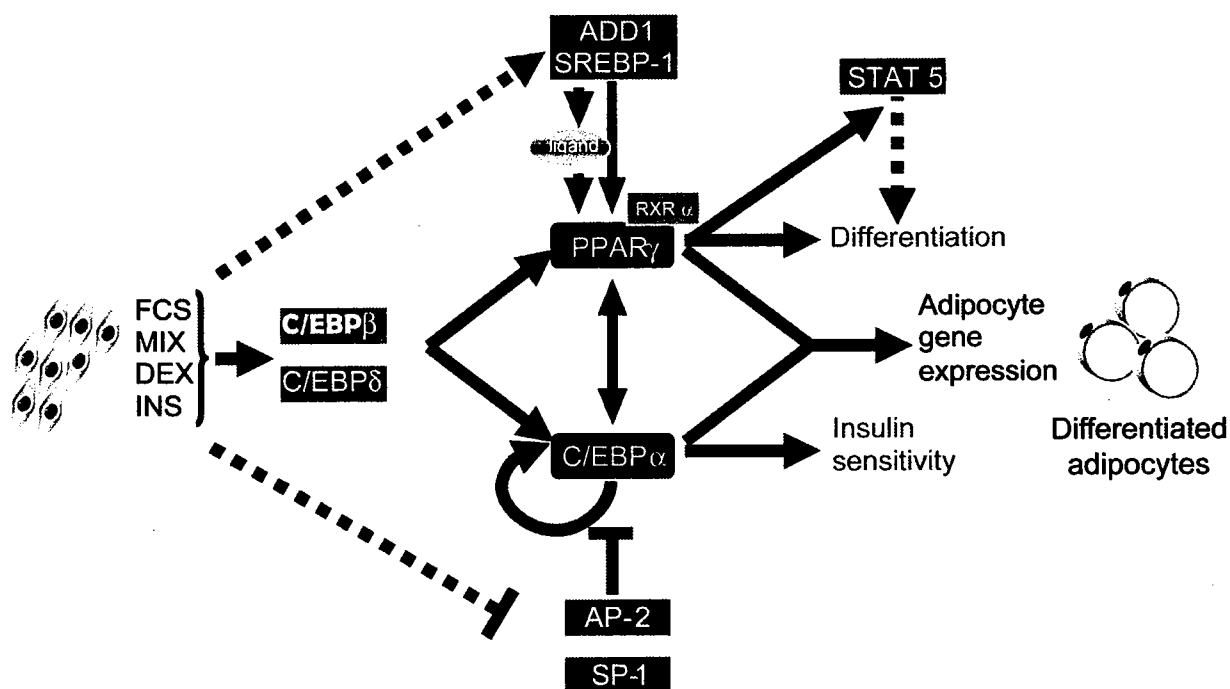
It is important to emphasize that MCE is required for the progression of the differentiation program, and that C/EBP $\beta$  participation is essential (Tang et al., 2003a; Tang et al., 2003b; Zhang et al., 2004b). To demonstrate the role of C/EBP $\beta$  in MCE, Tang and colleagues examined the effect of C/EBP $\beta$  deficiency in mouse embryo fibroblasts (MEFs). They found that C/EBP $\beta$  (-/-) MEFs formed neither mitotic foci nor adipocyte cells. Reintroduction of C/EBP $\beta$  LAP or LIP isoforms (described later) in the deficient MEFs showed that the C/EBP $\beta$  LAP (active form) re-established both MCE and adipogenesis. In contrast C/EBP $\beta$  LIP (dominant negative) was not able to restore these functions. These results indicate that C/EBP $\beta$  has a dual role in adipogenesis, as a transcriptional activator involved in the expression of PPAR $\gamma$  and C/EBP $\alpha$  (both gene promoters contain C/EBP sites) and playing a role during MCE (Tang et al., 2003a). Experiments that support C/EBP $\beta$  function in MCE were performed by Zhang and colleagues using a dominant negative A-zip C/EBP, which contains the leucine zipper but lacks functional DNA-binding and transactivation domains. This protein heterodimerizes with the bZip region of C/EBP $\beta$  and generate a stable coiled-coil complex unable to bind DNA. Forced expression of the A-zip C/EBP prevented C/EBP $\beta$  translocation to the nucleus, resulting in a block to MCE and adipogenesis (Zhang et al., 2004b).

During the terminal phase of differentiation, cells exit the cell cycle and express the master regulators of adipogenesis, peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and C/EBP $\alpha$ , which then activate a large number of adipocyte genes. Expression of both genes is maintained in mature adipocytes (Rangwala and Lazar, 2000). In addition to C/EBPs and PPAR $\gamma$  expression, other regulators are involved (see below).

C/EBP $\alpha$  is responsible for the maintenance of the terminal differentiated state in adipocytes, since it functions by blocking mitosis (Christy et al., 1989; Umek et al., 1991). PPAR $\gamma$  is also involved in growth arrest, and acts cooperatively in this activity with C/EBP $\alpha$  (Altioek et al., 1997). C/EBP $\alpha$  has the capacity to autoregulate its own expression due to the presence of a C/EBP binding in the proximal promoter (Christy et al., 1991; Lin and Lane, 1992). Also, C/EBP $\alpha$  and PPAR $\gamma$  regulate each others expression (Hamm et al., 1999; Shao and Lazar, 1997).

### **3.4) Transcription factors involved in adipogenesis**

Many molecular aspects of adipogenesis can be illustrated as a cascade of gene expression regulated by a set of transcription factors. At least four transcription factors play a significant regulatory role during adipocyte differentiation. These include the already mentioned master regulators C/EBPs and PPAR $\gamma$ , and also the SREBP1/ADD1 and the STAT proteins (Fig. 1.10) (Kim and Spiegelman, 1996; Kim et al., 1995; Stephens et al., 1996). Because this thesis concentrated in the role of C/EBPs and PPAR $\gamma$  proteins, in the following sections the role of these factors during adipogenesis will be discussed.

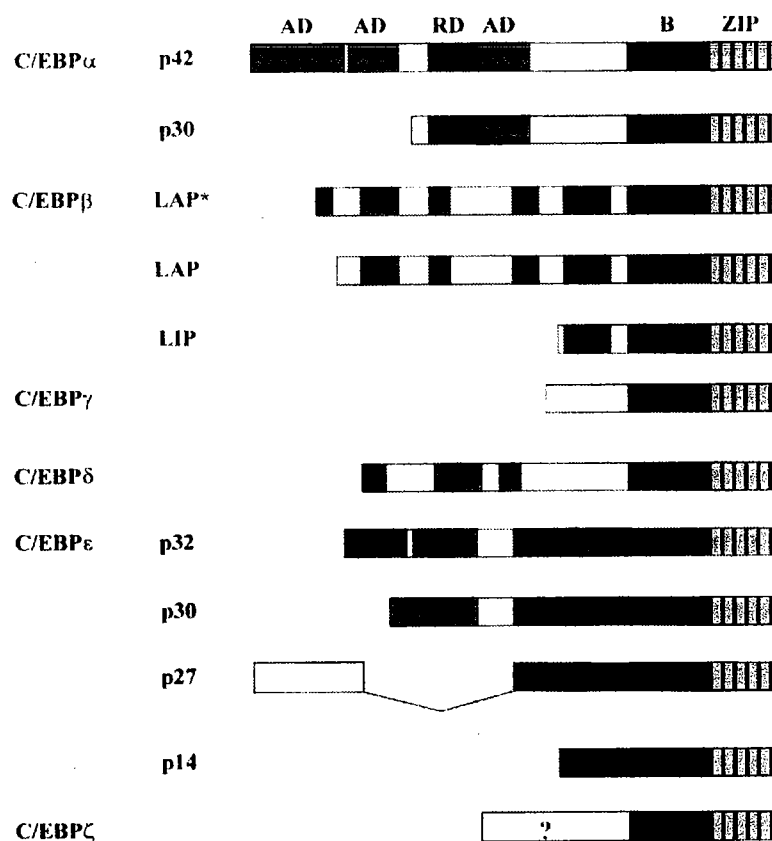


**Figure I.10.** Molecular pathway of transcriptional events mediating adipocyte differentiation. Direct or indirect transcriptional events are indicated in solid lines. Broken lines represent interactions that are less understood. Abbreviations: ADD1, adipocyte determination and differentiation dependent factor 1; C/EBP, CCAAT enhancer binding protein; PPAR $\gamma$ , peroxisome proliferator activated receptor  $\gamma$ ; RXR, In the retinoic X receptor; SREBP, sterol regulatory element binding proteins; STAT, signal transducers and activators of transcription (Morrison and Farmer, 2000).

### 3.4.1) C/EBPs

CCAAT/enhancer-binding protein (C/EBP) was discovered as a heat stable protein in rat liver nuclei able to bind to a CCAAT box motif present in a number of cellular gene promoters and in the "core homology" sequence of viral enhancers (Landschulz et al., 1988). The cloning of the C/EBP gene (later designated C/EBP $\alpha$ ) led to the discovery of the basic-leucine zipper (bZIP) class of DNA-binding and dimerization domain present in a number of transcription factors [Review in (McKnight, 2001; Ramji and Foka, 2002)]. Later on, other members of the family were cloned and sequenced, and they were called C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , C/EBP $\gamma$ , C/EBP $\epsilon$ , and C/EBP $\zeta$  (also known as CHOP-10) (Otto and Lane, 2005; Schrem et al., 2004). These transcription factors are involved in a number of cellular processes like cell cycle control, liver regeneration and liver specific gene regulation, apoptosis, immune and inflammatory processes, circadian gene regulation and in the control of a number of differentiation processes (Ramji and Foka, 2002; Schrem et al., 2004). The isoforms involved in adipocyte differentiation are: C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , and CHOP10.

The protein structure of all of the members of the family consist of three structural components including a C-terminal leucine-zipper (consisting of a heptad repeat of four or five leucine residues), a basic DNA-binding region, and an N-terminal transactivating region (Fig. 1.11) [Reviewed in (Otto and Lane, 2005; Ramji and Foka, 2002; Schrem et al., 2004)]. Electrostatic interactions between amino acids along the dimerization domain (leucine-zipper) establish the specificity of dimer formation [Reviewed in (Ramji and Foka, 2002)]. Dimerization of the C-terminal region is highly conserved and confers the ability to bind DNA and to form homo or heterodimers with other members of the family. C/EBP dimerization is a requirement for DNA binding (Landschulz et al., 1988).



**Figure I.11.** Schematic representation of the C/EBP family members. The leucine zipper is shown in yellow, with black vertical lines indicating the leucine residues, and the basic region is colored red. The position of the activation domains (AD) and negative regulatory domains (RD) are shown in green and blue respectively (Ramji and Foka, 2002).



In the case of CHOP-10, the DNA-binding domain is not functional; as a consequence heterodimers formed with this isoform cannot bind to the C/EBP site in the promoter of target genes, thus acting as a dominant negative of C/EBP function. However, CHOP-10/CEBP heterodimers can recognize a distinct DNA sequence when cells are exposed to stress (Otto and Lane, 2005; Ramji and Foka, 2002). The basic DNA-binding region of the C/EBP proteins determines the DNA binding specificity [Reviewed in (Lekstrom-Himes and Xanthopoulos, 1998)].

The N-termini of the C/EBPs are more divergent, although there are three separated subregions, also termed transactivation elements that are conserved in most of the members. These subregions are important to establish direct or indirect interaction with components of the basal transcription machinery (Ramji and Foka, 2002). In fact, the transactivation elements in C/EBP $\alpha$  activate transcription and cooperatively mediate *in vitro* binding of C/EBP $\alpha$  to TBP and TFIIB (Nerlov and Ziff, 1994; Nerlov and Ziff, 1995). Functional interaction of transactivation elements with components of the SWI/SNF complex has been demonstrated (Pedersen et al., 2001). Furthermore, it was shown *in vitro* that C/EBP $\alpha$ , C/EBP $\beta$  and C/EBP $\delta$  interact with p300 and CREB-binding protein (CBP) coactivator. These interactions potentiate C/EBP transactivation (Erickson et al., 2001; Kovacs et al., 2003; Schwartz et al., 2003). The N-terminus also contain one or two regulatory domains, involved in DNA binding inhibition in a cell type specific manner (Lekstrom-Himes and Xanthopoulos, 1998).

Some of the C/EBP members produce N-terminal truncated proteins, which are products of alternative usage of translation initiation codons in the same mRNA due to leaky ribosomal scanning or by regulated proteolysis. C/EBP $\alpha$  produces two isoforms of 42 kDa and 30kDa. The smaller isoform is a less potent transcriptional activator.

C/EBP $\beta$  mRNA produces three isoforms: the 38 kDa and 35 kDa species termed liver activating protein (LAP), and the 20 kDa liver inhibitory protein (LIP). LIP does not contain the N-terminal domain, and it functions as a dominant-negative regulator [Reviewed in (Otto and Lane, 2005; Ramji and Foka, 2002) and references therein].

The expression of the C/EBP family members differs in different tissues.

C/EBP $\alpha$  is highly expressed in adipose tissue, liver, intestine, lung, adrenal gland, blood mononuclear cells and placenta. In liver and adipose tissue, significant levels of

C/EBP $\alpha$  are detected in differentiated tissue (Lekstrom-Himes and Xanthopoulos, 1998).

C/EBP $\beta$  is present in liver, intestine, lung, adipocyte tissue, spleen, kidney, and

myelomonocytic cells. C/EBP $\delta$  is expressed in adipose tissue, lung and intestine.

C/EBP $\gamma$  and CHOP-10 are present in all tissues, and C/EBP $\epsilon$  is only present in myeloid and lymphoid cells [Reviewed in (Schrem et al., 2004)].

### **C/EBP deficient animal models**

#### **Disruption of C/EBP $\alpha$**

Mice homozygous for the targeted deletion of the C/EBP $\alpha$  gene appear normal to wild mice, but they die 8 hours after birth. The levels of blood glucose were significantly low, a sign of hypoglycemia. Analysis of embryo (E18) and newborn livers showed the absence of glycogen, which is indicative of interference of hepatic glycogen synthesis. The analysis of subcutaneous inguinal WAT and BAT confirmed their absence in the null animals. In contrast, the intercapsular regions presented the same amount of immature BAT as the control mice at seven hours after birth; however, by 32 hours postpartum the volume of BAT did not increase. Analysis of the brown adipocytes indicated that null

mice failed to accumulate fat in BAT. Molecular analysis of the adipocyte markers showed a reduction in the expression of UCP1, but no effects on the expression of Glut 4 and aP2 (Wang et al., 1995). In conclusion, C/EBP $\alpha$  null mice show significant reduction in lipid accumulation in both WAT and BAT depots and failure of gluconeogenesis in the liver. Mice die very early after birth as a result of a severe metabolic disorder that causes hypoglycemia. C/EBP $\beta$  and  $\delta$  were not able to compensate for the lack of C/EBP $\alpha$ , since the levels of both proteins are normal in those mice. However, normal levels of Glut 4 and aP2 could be explained by C/EBP $\beta$  and/or  $\delta$  transactivation activities (Rangwala and Lazar, 2000).

#### **Disruption of C/EBP $\beta$ and C/EBP $\delta$**

To investigate the role of C/EBP $\beta$  and C/EBP $\delta$  during adipocyte differentiation *in vivo*, Tanaka and colleagues generated mice lacking the C/EBP $\beta$  and/or C/EBP $\delta$  by gene targeting. C/EBP $\delta$  (-/-) mice did not die, BAT was only slightly impaired, intracytoplasmic lipid content is almost the same or to some extent reduced compared with wild-type mice and C/EBP $\alpha$  and PPAR $\gamma$  mRNA levels in the interscapular BAT were similar with that in wild-type mice (Tanaka et al., 1997). On the other hand, 35% of the C/EBP $\beta$  (-/-) knockout mice die at early neonatal stage, BAT was considerably reduced compared with that of wild-type mice, the distribution of the intracytoplasmic lipid droplets was sparsely distributed and small in size and as in the C/EBP $\delta$  (-/-) mice, the levels of C/EBP $\alpha$  and PPAR $\gamma$  mRNA were normal. UCP1 expression in the C/EBP $\delta$  (-/-) or C/EBP $\beta$  (-/-) mice was reduced compared with the wild type mice (Tanaka et al., 1997).

In contrast, disruption of both C/EBP $\beta$  (-/-) and  $\delta$  (-/-) produced a more severe phenotype: 85% of the double knockout mice died at perinatal or early postnatal stage of unknown reasons; the remaining mice (15%) showed a significant reduction of lipid accumulation at the interscapular BAT and minor decrease in WAT (due to a decreased in the cell number). The analysis of BAT demonstrated the absence of fat droplets (Tanaka et al., 1997). Expression of adipocyte genes in the double knockout mice showed that most of the genes evaluated were not altered in WAT and BAT, but UCP1 levels were significantly reduced. Levels of PPAR $\gamma$  and C/EBP $\alpha$  in these mice were not affected in BAT or WAT even though contradictory *in vitro* results were obtained from embryonic fibroblasts. Fibroblasts isolated from C/EBP $\beta$  (-/-) and  $\delta$  (-/-) double knockout mice did not express PPAR $\gamma$  and C/EBP $\alpha$  and they did not differentiate. *In vivo*, some kind of mechanism may be involved regulating the expression of PPAR $\gamma$  and C/EBP $\alpha$  in the absence of upstream regulators (Rangwala and Lazar, 2000; Tanaka et al., 1997).

Thus, these results demonstrated C/EBP $\beta$  and C/EBP $\delta$  work together in terminal fat differentiation. Even though PPAR $\gamma$  and C/EBP $\alpha$  are normally expressed in the double knockout mice, adipogenesis was impaired. Additionally, expression of PPAR $\gamma$  and C/EBP $\alpha$  at least *in vivo* does not require C/EBP $\beta$  and C/EBP $\delta$ , but induction of the master regulators PPAR $\gamma$  and C/EBP $\alpha$  does not completely compensate the absence of C/EBP $\beta$  and C/EBP $\delta$ .

### Role of C/EBP in adipogenesis

The essential role of the C/EBP proteins in adipocyte differentiation has been well established using cell culture models. Ectopic expression of C/EBP $\beta$  or C/EBP $\alpha$  is able to force non-adipogenic cell lines to differentiate into adipocytes (Freytag et al., 1994; Wu et al., 1995b; Yeh et al., 1995). Expression of antisense C/EBP $\alpha$  RNA in preadipocyte cell lines prevents the differentiation program (Lin and Lane, 1992). The analysis of promoter regions of adipogenic genes as well as studies of knockout mice have demonstrated the involvement of this family of transcription factors in regulating adipogenesis and other important physiological processes [reviewed in (Cornelius et al., 1994; Darlington et al., 1998; Gregoire et al., 1998; Lane et al., 1999; MacDougald and Lane, 1995b; Ramji and Foka, 2002; Rangwala and Lazar, 2000; Tanaka et al., 1997)]. A number of adipocyte genes contain C/EBP binding sites in their proximal promoter and they are transactivated by C/EBP factors.

C/EBP $\beta$  and  $\delta$  are the first transcription factors induced after exposure to the inducers of differentiation; this implies that both factors direct the first steps of the differentiation process. *In vitro* results have shown that lack of C/EBP $\beta$  and C/EBP $\delta$  cause a block in differentiation and inhibition of PPAR $\gamma$  and C/EBP $\alpha$  expression, which then affects adipocyte gene expression. Reintroduction of C/EBP $\beta$  rescues the differentiation process but not the overexpression of C/EBP $\delta$ . C/EBP $\delta$  is not sufficient by itself, but does accelerate the differentiation process together with C/EBP $\beta$  (Yeh et al., 1995). This is indicative that C/EBP $\delta$  activity has a minor role, and C/EBP $\beta$  is a key in the cascade promoting adipogenesis. The ability of C/EBP $\beta$  to replace C/EBP $\alpha$  activity suggests a dual role for C/EBP $\beta$  as stimulator of cell determination as well as a

promoter of differentiation (Darlington et al., 1998; Rosen, 2002; Wu et al., 1995b). The same role is also applicable to C/EBP $\alpha$  (Freytag et al., 1994; Lin and Lane, 1994).

To establish the role of C/EBP $\alpha$  and PPAR $\gamma$  in adipocyte differentiation, Wu and colleagues studied the patterns of adipocyte gene expression after ectopic expression of these regulators using fibroblasts derived from C/EBP $\alpha$  knockout mice. These cells go through differentiation when PPAR $\gamma$  is overexpressed, however the levels of adipocyte genes expression involved in lipid storage were lower compared to control. This reduction could explain the smaller lipid droplets within the cytoplasm of these cells. Also, these cells overexpressing PPAR $\gamma$  did not show insulin dependent glucose intake compared to C/EBP $\alpha$ . These results defined an additional role for C/EBP $\alpha$  in adipogenesis. Overexpression of both activators in the C/EBP (-/-) cells clearly demonstrated full activation of the differentiation program. This study clearly showed that PPAR $\gamma$  activates expression of C/EBP $\alpha$ , and C/EBP $\alpha$  is necessary to activate PPAR $\gamma$ . Cross-regulation of C/EBP $\alpha$  and PPAR $\gamma$  expression is essential to maintain the differentiation state (Wu et al., 1999).

We have already seen a number of studies that have established the key role for C/EBP transcription factors in adipogenesis. Nevertheless some aspects have remained unresolved and require further investigation. One issue came from the studies of the C/EBP $\beta$  and C/EBP $\delta$  double knockout mice. It is clear that both activators are involved in BAT and in WAT differentiation. In general, expression of most adipocyte markers evaluated looked normal, except that aP2 showed reduced levels, and the BAT marker UCP1 was almost absent. Since PPAR $\gamma$  and C/EBP $\alpha$  are expressed at high levels in those mice, it is not surprising to observe expression of some of the late adipocyte genes. However, some genes were not fully induced in the double knockout even

though the presence of the master regulators was indicative of a possible role of C/EBP $\beta$  and C/EBP $\delta$  in activating late adipocyte genes. Another aspect that has remained ambiguous is the binding of these transcription factors at the promoter of genes early during differentiation. In order to determine the role of C/EBP $\beta$  and  $\delta$  in the regulation of late gene markers we examined the kinetic of C/EBP $\alpha$ ,  $\beta$  and  $\delta$  binding to early and late adipocyte genes in differentiating 3T3-L1 by chromatin immunoprecipitations. The results of these studies are presented in chapter III of this Thesis.

### 3.4.2) PPAR $\gamma$

Peroxisome proliferators are a structurally varied group of substances, including some clinically tested hypolipidemic agents, herbicides, etc., that activate hepatic peroxisomal proliferation and the expression of enzymes of the fatty acid  $\beta$ -oxidation pathway (Tontonoz et al., 1995). They control gene expression through the activation of a member of the steroid hormone receptor family. The activity of nuclear receptors is regulated by interaction with small lipophilic ligands. The receptor was isolated for the first time and cloned from a mouse liver cDNA library and named peroxisome proliferator activated receptor (PPAR) (Issemann and Green, 1990). Subsequently, the human PPAR gene was cloned (Sher et al., 1993). Then, three related PPAR genes were isolated in *Xenopus*, named  $\alpha$ ,  $\beta$  and  $\gamma$ , and in mouse they were referred as PPAR $\alpha$ ,  $\gamma$ , and  $\delta$  or NUC-1 isoforms. Many cell types express more than one PPAR isoform (Tontonoz et al., 1995).

The PPARs bind to DNA sequences called PPAR response elements (PPREs). The analysis of these target sequences showed they consist of direct repeats of AGGNCA-like motifs spaced by one nucleotide (DR-1). In addition, it was found that

PPARs form heterodimers with the a second member of the nuclear receptor family, the retinoid X receptors (RXR) $\alpha$  (Brun et al., 1996; Tontonoz et al., 1995).

The pattern of expression of different PPARs in tissues differs. PPAR $\alpha$  is found in liver, heart, kidney, skeletal muscle, small intestine, and BAT. PPAR $\delta$  is expressed in many tissues, but markedly in the brain, WAT, and muscle, and PPAR $\gamma$  is highly expressed in adipose tissue (both BAT and WAT), placenta, large intestine, and macrophages (Grimaldi, 2001; Tontonoz et al., 1995).

The first study that demonstrated the involvement of PPAR $\gamma$  in adipocyte differentiation was performed with the preadipocyte cell line 3T3-L1. Treatment of 3T3-L1 with different peroxisome proliferators caused adipocyte differentiation and the induction of PPAR $\gamma$  (Chawla et al., 1994). These results and the presence of PPAR $\gamma$  in adipose tissue were indicative of a role for this activator in adipocyte differentiation (Tontonoz et al., 1995). Later, it was showed that ectopic expression of PPAR $\gamma$  in different fibroblast cells lines enabled them to differentiate into adipocytes (Tontonoz et al., 1994c).

The role of PPAR $\gamma$  as a master key regulator was later verified by studies using immortalized fibroblasts lacking PPAR $\gamma$ . Overexpression of C/EBP $\alpha$  in those cells did not stimulate adipogenesis, however, reintroduction of PPAR $\gamma$  reestablished the differentiation process (Rosen et al., 2002).

PPAR $\gamma$  is activated by polyunsaturated fatty acids, and in response to these lipids, PPAR $\gamma$  regulates the induction of adipocyte genes. Earlier findings demonstrated that the antidiabetic thiazolidinediones (TZDs) were capable of initiating adipogenesis and were later linked to PPAR $\gamma$  by Lehmann and colleagues. These investigators

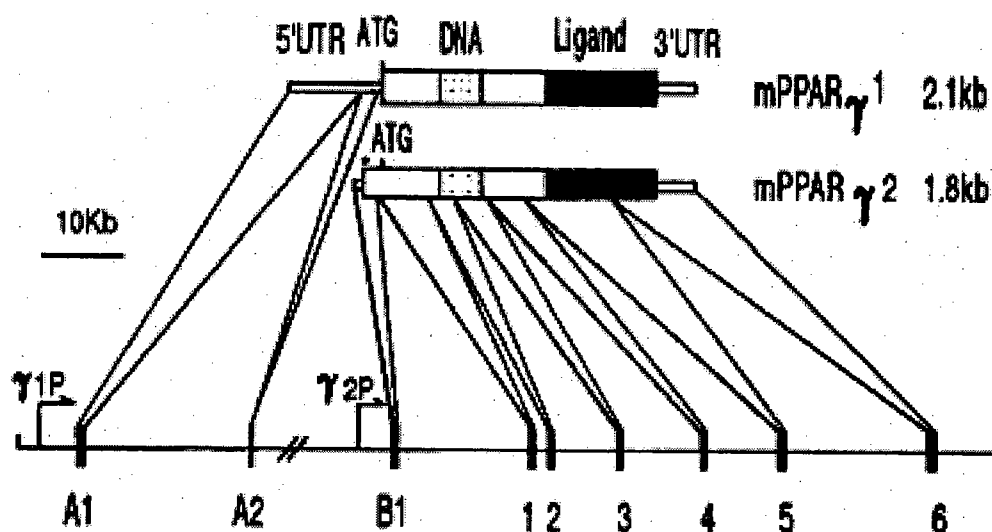


reported that TZDs selectively and efficiently activated PPAR $\gamma$  transcription factor (Lehmann et al., 1995). After their discovery, TZDs have been used extensively in *in vitro* differentiation protocols.

### **PPAR $\gamma$ protein structure**

The isolation and cloning of the mouse PPAR $\gamma$  (later named PPAR $\gamma$ 1) was reported by Zhu and colleagues (Zhu et al., 1993). Simultaneously, another isoform of the receptor, PPAR $\gamma$ 2, which showed expression largely confined to adipocytes, was isolated and cloned (Tontonoz et al., 1994a). PPAR $\gamma$ 1 and PPAR $\gamma$ 2 proteins are produced from differential promoter usage and alternative splicing at the 5' end. The transcription of PPAR $\gamma$ 1 is controlled by the promoter P1, which generates messenger RNA (mRNA) containing two untranslated exons, A1 and A2, which are specific to PPAR $\gamma$ 1 (Fig. I.12). The 5' untranslated sequence and the extra thirty N-terminal amino acids of PPAR $\gamma$ 2 are encoded by exon B, which is located between the second and the third exons that encode PPAR $\gamma$ 1. Exons one to exon 6 encode the same sequences in both isoforms (Fig. I.12). The cDNA of PPAR $\gamma$ 2 encodes an extra thirty amino acids N-terminal to the first ATG codon of the PPAR $\gamma$ 1 and shows a distinct 5'-untranslated sequence (Fajas et al., 1997; Zhu et al., 1995). PPAR $\gamma$ 1 is expressed in adipose tissue but also in colon, macrophages and cell of the vasculature. PPAR $\gamma$ 2 is specifically expressed in adipose tissue.

PPAR $\gamma$  conforms to the established domain structure that is present in the superfamily of nuclear hormone receptors. The N-terminal region contains the



**Figure I.12.** Organization of mPPAR $\gamma$  gene. The eight exons (A1, A2, and 1-6) encoding the mPPAR $\gamma$ 1 (solid boxes) as well as the seven exons (B1 and 1-6) encoding mPPAR $\gamma$ 2 are shown in the genomic DNA.  $\gamma$ 1P and  $\gamma$ 2P represent the promoter of mPPAR $\gamma$ 1 and mPPAR $\gamma$ 2, respectively. The corresponding positions of the exons with respect to mPPAR $\gamma$ 1 and mPPAR $\gamma$ 2 cDNAs are shown above. Exons numbered 1-6 code for identical sequences in both isoforms starting at the ATG. The mPPAR $\gamma$ 2 cDNA encodes an additional 30 amino acids N-terminal to the first ATG of mPPAR $\gamma$ 1 from an upstream ATG (indicated by \*) from exon B1 shown on the genomic DNA. At the top, the location of 5' and 3' untranslated regions (UTR) and the DNA and ligand-binding domains are shown (Zhu et al., 1995).

transcriptional activation function 1 (AF-1) that functions independently of ligand. This region contains a mitogen-activated protein kinase (MAPK) phosphorylation site that when phosphorylated decreases the transcriptional activity of PPAR. The central domain contains the DNA binding motif, which includes two zinc fingers. The C-terminal domain includes the dimerization and the ligand binding domain (AF-2) (Rosen and Spiegelman, 2001; Willson et al., 2001).

### **Functional roles of PPAR $\gamma$ 1 and PPAR $\gamma$ 2**

The functional role of the extra thirty amino acids present on PPAR $\gamma$ 2 is unclear. Experimental data have suggested that the extra amino acids in PPAR $\gamma$ 2 contribute to transcriptional activation function and insulin increases that effect (Werman et al., 1997). To access the specific functions of PPAR $\gamma$ 1 and PPAR $\gamma$ 2, these factors were independently overexpressed in PPAR $\gamma$  null fibroblasts. The results showed that both proteins have the capacity to stimulate adipogenesis. However, at low ligand concentrations, PPAR $\gamma$ 2 was more adipogenic than PPAR $\gamma$ 1. Analysis of coactivator binding and transcriptional analysis showed that PPAR $\gamma$ 2 has an increased capacity to interact with cofactors essential in adipocyte differentiation (Mueller et al., 2002). Contradictory results were obtained when endogenous expression of PPAR $\gamma$ 1 and PPAR $\gamma$ 2 was abrogated by targeting a zinc finger repressor protein to specific regions on the PPAR $\gamma$  gene promoter. Adipogenesis was rescued only by overexpression of PPAR $\gamma$ 2 and not by PPAR $\gamma$ 1 (Ren et al., 2002).

Additionally, it is important to point out that PPAR $\gamma$ 2 seems to control the expression of the PPAR $\gamma$ 1 in WAT. In a time course of differentiation, PPAR $\gamma$ 1 induction

followed PPAR $\gamma$ 2 expression. This was demonstrated previously by Ren and colleagues and Saladin and colleagues (Ren et al., 2002; Saladin et al., 1999), and confirmed later *in vivo* by Koutnikova and colleagues (Koutnikova et al., 2003)

### PPAR $\gamma$ deficient animals

The use of genetically modified mice has confirmed the role of PPAR $\gamma$  in adipogenesis and also has been a tool to examine the role of PPAR $\gamma$  in metabolic responses (Gray et al., 2005).

The complete absence of PPAR $\gamma$  is embryonic lethal affecting trophoblast differentiation and placental vascularization. The defects in placental vascularization cause myocardial thinning and consequently death at E10 stage. The placental defects were rescued by generation of tetraploid embryos, allowing the recovery of one mouse born PPAR $\gamma$  (-/-). All the tetraploid chimeras between E10.0 and term survived, demonstrating that PPAR $\gamma$  is not vital during embryogenesis. Analysis of the null pup demonstrated total absence of WAT and BAT, confirming the essential role of PPAR $\gamma$  in adipogenesis *in vivo* (Barak et al., 1999). Likewise, through use of a different approach, Rosen et al demonstrated the pivotal role of PPAR $\gamma$  in adipocyte differentiation both *in vivo* and *in vitro*. The authors designed a chimeric mouse using wild type and PPAR $\gamma$  (-/-) embryonic stem (ES) cells. Because PPAR $\gamma$  (-/-) ES cells contributed weakly to the to adipose depots formation and not at all to the sebaceous glands of the skin, they concluded that PPAR $\gamma$  is involved in the development and maturation of fat cells (Rosen et al., 1999).

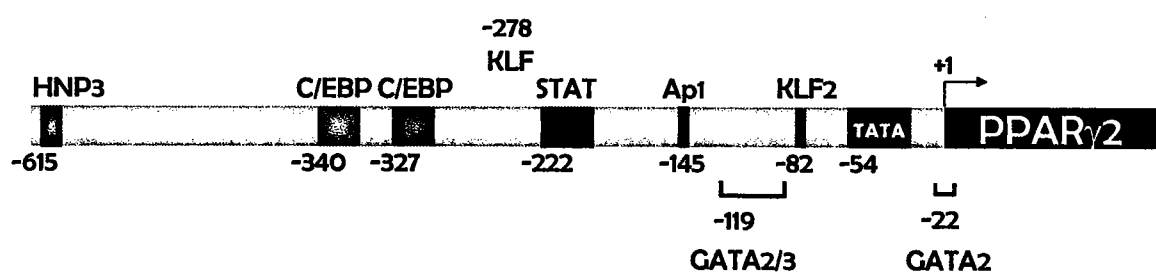
### Regulation of PPAR $\gamma$ expression

The sequence analysis of the mouse PPAR $\gamma$ 2 promoter identified the TATA-like element (TATTA) located 49 base pair upstream of the transcription start site and consensus binding sites for HNF3, C/EBP, and Ap1 transcription factors (Fig. 1.13) (Zhu et al., 1995). The role of the C/EBP sites in PPAR $\gamma$ 2 promoter was established in co-transfection studies and demonstrated that the C/EBP proteins are involved in the activation of PPAR $\gamma$ 2 expression (Clarke et al., 1997; Elberg et al., 2000; Zhu et al., 1995).

Another transcription factor involved in adipogenesis through regulation of the PPAR $\gamma$ 2 expression is GATA. GATA family members share highly conserved zinc-finger DNA binding domains and bind specifically to a consensus DNA sequence (A/T) GATA(A/G) (Tong et al., 2000). The proximal PPAR $\gamma$ 2 promoter region contains two GATA sites. GATA2 and GATA 3, which are expressed in preadipocytes, bind directly to both GATA sites on the PPAR $\gamma$ 2 promoter to negatively regulate its basal transcriptional activity (Tong et al., 2000).

In addition to GATA, STAT proteins also negatively regulate PPAR $\gamma$ 2 expression. Interferon- $\gamma$  (INF $\gamma$ ) is a potent activator of STAT1 and treatment of 3T3-L1 with INF $\gamma$  has been shown to inhibit adipogenesis. The mechanism by which INF $\gamma$  performs its effect is through regulation of the PPAR $\gamma$ 2 expression. PPAR $\gamma$ 2 promoter contains a STAT site at -222 from the start site. This site specifically binds STAT1 and negatively regulates PPAR $\gamma$ 2 expression (Hogan and Stephens, 2001).

Krüppel-like factors (KLF) are also involved in the regulation of the PPAR $\gamma$ 2 expression. KLF are zinc finger proteins that constitute an important class



**Figure I.13.** The mouse PPAR $\gamma$ 2 promoter.

of transcription regulators. They are characterized to contain multiple zinc fingers at the C-terminus of the protein and at the N-terminus, which is implicated in transcriptional activation and/or repression a protein-protein interactions domain. This family of transcription factor is involved in the regulation of cellular differentiation (Bieker, 2001; Segre et al., 1999). KLF5 is highly expressed in adipose tissue and KLF2 is highly expressed in preadipocytes. It has been demonstrated that KLF2 negatively regulates PPAR $\gamma$ 2 expression. The KLF2 inhibitory effect on PPAR $\gamma$ 2 expression is mediated by direct binding to the promoter as was demonstrated in transient transfection studies (Banerjee et al., 2003). In contrast, the other member of the family, KLF5, positively regulates adipocyte differentiation (Oishi et al., 2005). It has been demonstrated using reporter assays that KLF controls PPAR $\gamma$ 2 expression in concert with C/EBP $\beta$  and  $\delta$ . The proximity of one of the KLF sites at -278 to the C/EBP sites on the PPAR $\gamma$ 2 promoter suggested a possible interaction between KLF factor and C/EBP $\beta$  and  $\delta$ . This interaction was later confirmed to occur *in vivo*. ChIP analysis demonstrated the presence of KLF5 at early time points during differentiation. Mechanisms by which KLF proteins modulate PPAR $\gamma$ 2 expression are still unknown.

While studying the role of SWI/SNF in adipogenesis, we observed that PPAR $\gamma$ 2 expression was affected by the presence of dominant negative BRG1. To study the mechanisms by which SWI/SNF modulate PPAR $\gamma$ 2 expression, a series of chromatin immunoprecipitations were performed to temporally characterize pivotal protein interactions at the PPAR $\gamma$ 2 promoter. The results and analysis of these experiments are presented in the following Chapter of this thesis.

# CHAPTER II

The work presented in this chapter was published in *Molecular and Cellular Biology* (2004) volume 24: 4651-4663. Data presented in figure II.7 was generated by Hengyi Xiao, Ph.D.



## CHAPTER II

# TEMPORAL RECRUITMENT OF TRANSCRIPTION FACTORS AND SWI/SNF CHROMATIN REMODELING ENZYMES DURING ADIPOGENIC INDUCTION OF THE PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR $\gamma$ NUCLEAR HORMONE RECEPTOR

### Abstract

The peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) regulates adipogenesis, lipid metabolism, and glucose homeostasis, and roles have emerged for this receptor in the pathogenesis and treatment of diabetes, atherosclerosis, and cancer. We report here that induction of the PPAR $\gamma$  activator and adipogenesis forced by overexpression of adipogenic regulatory proteins is blocked upon expression of dominant negative BRG1 or hBRM, the ATPase subunits of distinct SWI/SNF chromatin remodeling enzymes. We demonstrate that histone hyperacetylation and the binding of C/EBP activators, RNA polymerase II, and general transcription factors initially occurred at the inducible PPAR $\gamma$ 2 promoter in the absence of SWI/SNF function. However, the RNA polymerase and general transcription factors were subsequently lost from the promoter in cells expressing dominant negative SWI/SNF, explaining the inhibition of PPAR $\gamma$ 2 expression. To corroborate these data, we analyzed interactions at the PPAR $\gamma$ 2 promoter in differentiating pre-adipocytes. Changes in promoter structure, histone hyperacetylation, and binding of C/EBP activators, RNA polymerase II, and most general transcription factors preceded the interaction of SWI/SNF enzymes with the PPAR $\gamma$ 2 promoter. However, transcription of the PPAR $\gamma$ 2 gene occurred only upon subsequent association

of SWI/SNF and TFIIH with the promoter. Thus induction of the PPAR $\gamma$  nuclear hormone receptor during adipogenesis requires SWI/SNF enzymes to facilitate pre-initiation complex function.

## Introduction

Differentiation of adipocytes, as with all differentiation events, involves programmatic changes in gene expression patterns. Genes specifically expressed in adipocytes must be activated; the concerted action of several transcriptional regulators, including C/EBP $\alpha$ , C/EBP $\beta$ , and the nuclear hormone receptor PPAR $\gamma$ , controls these activation events via direct interaction with PPAR $\gamma$  and C/EBP binding sites in adipocyte-specific gene regulatory sequences [reviewed in (Debril et al., 2001; Rangwala and Lazar, 2000; Rosen and Spiegelman, 2000)]. Each of these regulators is expressed with different kinetics during adipocyte differentiation in culture, yet forced overexpression of any is sufficient to initiate adipogenic differentiation in fibroblast cells (Freytag et al., 1994; Tontonoz et al., 1994c; Wu et al., 1995b; Yeh et al., 1995). Function of the PPAR $\gamma$  regulator is especially critical, as many of the genes involved in adipogenesis as well as glucose homeostasis are activated by this nuclear hormone receptor. Since adipocyte specific genes are not expressed prior to differentiation, it is likely that the regulatory sequences controlling the expression of these genes are incorporated into a repressive chromatin structure that is refractory to gene expression. Eukaryotic cells have evolved two classes of enzymes that can alter chromatin structure to control accessibility to the transcriptional machinery. These include histone modifying enzymes, which post-translationally modify the N- and C-terminal domains of the individual histone proteins that comprise the nucleosome, and ATP-dependent chromatin remodeling enzymes, which alter structure by disrupting the histone:DNA contacts of the nucleosome, thereby altering nucleosome conformation and, in some cases, altering the position of the

histone octamer along the DNA [reviewed in (Narlikar et al., 2002; Strahl and Allis, 2000; Turner, 2002)].

The mammalian SWI/SNF family of ATP-dependent chromatin remodeling enzymes includes members containing either the Brg1 or Brm ATPase. Although the mammalian SWI/SNF enzymes share most of the same subunits, multiple forms of these enzymes exist; these are distinguished by the ATPase present, the presence of unique subunits, and/or the presence of tissue specific isoforms of common subunits (Olave et al., 2002; Sif et al., 2001; Wang et al., 1996a; Wang et al., 1996b). *In vitro* analyses of hBRM and different BRG1 containing enzymes reveal many similarities in chromatin remodeling assays (Sif et al., 2001). *In vivo*, however, clear differences in function likely exist. Brg1 knockout mice are embryonic lethal and heterozygotes are predisposed to tumors (Bultman et al., 2000). Brm knockout mice and cells, in contrast, show only modest proliferation differences compared to wild type (Reyes et al., 1998). Additionally, at the molecular level, chromatin immunoprecipitation (ChIP) analyses have revealed that Brg1 and Brm can be present on different promoters (Kadam and Emerson, 2003; Soutoglou and Talianidis, 2002), supporting the idea of differential functions.

Collectively, the literature reveals that Brg1 and/or Brm can physically interact with a number of different transcriptional regulatory proteins and these proteins have been localized by ChIP studies to the promoter sequences of a number of inducible genes during transcriptional activation. In particular, previous work has indicated a requirement for or a contribution by SWI/SNF enzymes for activation of cellular differentiation genes. Myeloid, erythrocyte, enterocyte, muscle and adipose cell differentiation events have been linked to the presence of functional SWI/SNF enzymes (Armstrong et al., 1998; de la Serna et al., 2001a; Kowenz-Leutz and Leutz, 1999;

Pedersen et al., 2001; Soutoglou and Talianidis, 2002) and, in a more limited number of cases, to the ability of SWI/SNF enzymes to alter chromatin structure at or near inducible promoters. In the case of adipogenesis, the factor C/EBP $\alpha$ , already known to physically interact with the RNA polymerase II associated general transcription factors TBP and TFIIB (Nerlov and Ziff, 1995), was shown to have the capability to interact with hBRM (Pedersen et al., 2001). Moreover, the domain mediating this interaction was critically required for the ability of C/EBP $\alpha$  to trans-differentiate fibroblasts into adipocyte-like cells. *In vitro*, the competency of PPAR $\gamma$  to activate *in vitro* transcription templates assembled into chromatin was dependent on a specific BRG1-containing SWI/SNF enzyme (Lemon et al., 2001). However, the nature of the role that SWI/SNF enzymes play in facilitating adipogenic gene expression remains to be determined.

Here, we explore the functional role of SWI/SNF enzymes during adipocyte differentiation by examining the activation of the PPAR $\gamma$  regulator itself. PPAR $\gamma$  mRNA is expressed from two distinct promoters that give rise to two distinct isoforms, termed PPAR $\gamma$ 1 and PPAR $\gamma$ 2 (Zhu et al., 1995). We focused on PPAR $\gamma$ 2 expression because in differentiating pre-adipocytes, PPAR $\gamma$ 2 is highly induced and is the predominant isoform in differentiated adipocytes (Saladin et al., 1999; Tontonoz et al., 1994a), while in undifferentiated and differentiated fibroblasts, PPAR $\gamma$ 1 expression was not observed (see below). We found that the SWI/SNF enzymes are critically required for trans-differentiation of fibroblasts along the adipogenic pathway. Temporal analyses of factor binding to the PPAR $\gamma$ 2 promoter revealed that the BRG1-based SWI/SNF enzymes did not facilitate activator binding to the promoter, but instead promoted pre-initiation complex function. Examination of PPAR $\gamma$ 2 activation during differentiation of committed pre-adipocyte cells confirmed that changes in chromatin structure, activator binding, and

assembly of multiple components of the preinitiation complex did not require SWI/SNF function. Nevertheless, activation of PPAR $\gamma$ 2 transcription did not occur until SWI/SNF and TFIID subsequently were brought to the promoter. Thus, using two different cellular models for adipocyte differentiation, we demonstrate that activation of the PPAR $\gamma$  regulator critically depends upon SWI/SNF enzymes, most likely by facilitating preinitiation complex assembly and function.

## Materials and Methods

**Plasmids.** The retrovirus encoding mouse PPAR $\gamma$ 2 (Tontonoz et al., 1994c) and cDNAs encoding rat C/EBP $\alpha$  (Landschulz et al., 1988) and mouse C/EBP $\beta$  (Cao et al., 1991) were kindly provided by Dr. Bruce Spiegelman. C/EBP $\alpha$  and C/EBP $\beta$  were subcloned into pBabe-Puro (Morgenstern and Land, 1990).

**Cell lines and differentiation methods.** The derivation and maintenance of the cell lines that express dominant-negative human BRG1 (B22 and B24), dominant negative human BRM (H17), and the Tet-VP16 regulator (Tet-VP16) were described (de La Serna et al., 2000). To infect cell lines, BOSC23 cells were cultured in 100 mm dishes and transfected at 80% confluence by FUGENE (Roche) with 10  $\mu$ g of pBabe-PPAR $\gamma$ 2, pBabe-C/EBP $\alpha$ , pBabe-C/EBP $\beta$  or the empty vector as described (Pear et al., 1993). Viral supernatants were harvested 48 hrs after transfection. Dishes (60mm) of B22, B24, H17, or Tet-VP16 cells at 50% confluence were infected with virus in DMEM containing 10% calf serum, 4  $\mu$ g/ml of polybrene and 2  $\mu$ g/ml tetracycline in a final volume of 5 ml. The corresponding cell lines were split 1:3 48 hrs after infection and placed under selection with 2  $\mu$ g/ml puromycin in the presence of tetracycline. Subsequently, each virally infected cell line was split 1:4 into media containing or lacking tetracycline. After 96 hours the media was changed. Plates designated to be undifferentiated received and continued to be maintained in DMEM plus 10% calf serum in the presence or absence of tetracycline while the plates designated for differentiation received DMEM plus 10% fetal calf serum plus cocktail containing 0.5 mM methylisobutylxanthine (Sigma), 1  $\mu$ M dexamethasone (Sigma), 5  $\mu$ g/ml insulin and 10

$\mu$ M troglitazone (Biomol) in the presence or absence of tetracycline for 48 hrs. The cells were subsequently maintained in DMEM plus 10% FCS with 5  $\mu$ g /ml insulin and re-fed every two days for up to 8 days.

3T3-L1 preadipocytes were purchased from ATTC and maintained in growth medium consisting of DMEM containing 10% calf serum and were induced to differentiate as described (Wu et al., 1995b).

**Oil Red O staining.** 60 mm dishes were washed twice with PBS and fixed with 10% buffered formalin for 30 min. The formalin was aspirated and the cells were stained for 1 hr in freshly diluted Oil Red O solution (Sigma), prepared by mixing 6 parts Oil Red O stock solution (0.5% Oil Red Oil in isopropanol) and 4 parts distilled water. The stain was removed; cells were washed 3 times with distilled water, and photographed.

**RNA Analysis.** RNA isolation and analysis by Northern blot was described (de la Serna et al., 2001c). Probes were derived from plasmids containing PPAR $\gamma$ , aP2, or adipsin cDNA (provided by B. Spiegelman) and were labeled by random priming. Washed blots were exposed to a PhosphorImager (Molecular Dynamics).

For RT-PCR, total RNA (3 micrograms) was reverse transcribed with MoMLV RT (Invitrogen). cDNA was amplified by PCR with Taq polymerase (Invitrogen; 2.5 units/reaction), 0.2 mM dNTPs, 8 ng/ $\mu$ l of each primer, and either 1mM (C/EBP $\alpha$ ), 1.5 mM (PPAR $\gamma$ ), or 2.5 mM (HPRT) MgCl<sub>2</sub>. The initial denaturation step was at 95 °C for 5 min and was followed by 25 cycles (PPAR $\gamma$  and C/EBP $\alpha$ ) or 27 cycles (HPRT). A cycle for PPAR $\gamma$  PCR consisted of denaturation for 30 sec, annealing for 40 sec at 68 °C, and extension at 72 °C for 30 sec. For C/EBP $\alpha$  it consisted of denaturation for 50 sec,



annealing for 55 sec at 66 °C, and extension at 72 °C for 50 sec. The final round of extension was for 5 min. The sequence of the primers were: 5'-GCATGGTGCCTTCGCTGATGC-3' and 5'-AGGCCTGTTGTAGAGCTGGGT-3' for PPAR $\gamma$ 2 (340 bp product); 5'-CCGGCCGCCTTCAACGAC-3' and 5'-CTCCTCGCGGGGCTCTTGT-3' for C/EBP $\alpha$  (288 bp product). HPRT and PPAR $\gamma$ 1 RT-PCRs were described (de la Serna et al., 2001c; Zhu et al., 1995).

Nuclear run-on analysis was performed as described previously by Schübeler and Bode (<http://juergenbode.gmxhome.de/t01176.htm>) on nuclei isolated from  $\sim 4 \times 10^6$  differentiating 3T3-L1 preadipocyte cells that were collected at days 0, 2, 4, 5, 6, and 7 after induction of differentiation. Hybond N+ membranes (Amersham) were prepared for hybridization as described (Ausubel et al., 1996). Immobilized DNAs included 0.1  $\mu$ g of genomic DNA from 3T3-L1 pre-adipocytes, 3  $\mu$ g EcoR1-linearized pBABE and pBABE-PPAR $\gamma$ 2, or 3  $\mu$ g of PCR product corresponding to nucleotides 181-531 of the mouse 36B4 cDNA, to nucleotides 111-641 of mouse *Gapdh*, or to *Hprt*. The HPRT PCR product was described (de la Serna et al., 2001c).

**Protein extracts and western analysis.** Isolation of protein and western blotting were described (de La Serna et al., 2000). Antibodies utilized included C/EBP $\beta$  (Santa Cruz, sc-7962), M2 anti-FLAG (Sigma), and PI-3 Kinase (Upstate, 06-496). Determination of the total Brg1 levels in Fig. II.4 was performed by scanning multiple film exposures and quantifying in ImageQuant (Molecular Dynamics).

**Accessibility assays.** Restriction enzyme and DNase I accessibility assays were performed as described (de la Serna et al., 2001a), except that buffer M contained 0.15 mM spermine and 0.5 mM spermidine. DNase I was purchased from Promega. Probes P1 and P2 were PCR fragments corresponding to -1579 to -1187 and -105 to +138 from the mouse PPAR $\gamma$ 2 promoter (Zhu et al., 1995).

**Chromatin Immunoprecipitation (ChIPs).** The procedure was adopted from the Upstate protocol. Cells at the indicated time points were fixed by adding 37% formaldehyde to a final concentration of 1% and incubated at 37° C for 10 minutes. Cross-linking was stopped by adding glycine to a final concentration of 0.125M. Cells were washed twice with cold PBS and collected in 1 ml PBS containing protease inhibitors. After centrifugation, the pellet of cells were resuspended in SDS-lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1) containing protease inhibitors and incubated on ice for 10 minutes. Cell lysates were sheared extensively by sonication (Ultrasonic processor from Cole and Parmer, 3mm tip at 80 Watts) on ice to obtain fragments from 200 to 600 bp, as revealed by ethidium bromide staining of aliquots run on agarose gels. Samples were centrifuged to pellet debris and an aliquot was taken for gel analysis and inputs. 100  $\mu$ g of soluble chromatin was diluted 10 times with IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167mM NaCl) containing protease inhibitors and precleared for 3 hours at 4°C with 50% slurry of protein A, G or L in TE (depending on the isotype of the antibody used) in the presence of 20  $\mu$ g sonicated salmon sperm DNA and 1 mg/ml BSA. After incubation, the beads were pelleted and the supernatant immunoprecipitated with antibodies of interest (see below) at 4°C overnight. Immune complexes were collected with 50% slurry of protein A, G or L containing 20  $\mu$ g sonicated salmon sperm DNA, and 1 mg/ml BSA in TE by incubating at 4°C for 1 hour. Sepharose beads were washed sequentially for 5 minutes at 4°C with wash 1 (0.1 % SDS, 1% Triton X-100, 2 mM

EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), wash 2 (wash 1 containing 500 mM NaCl), wash 3 (0.25M LiCl, 1% NP40, 1% sodium deoxycolate, 1mM EDTA, 10 mM Tris-HCl, pH 8.1) and finally twice with TE, pH 8.0. Immune complexes were eluted from the beads with 1% SDS in TE, pH 8.0, and protein-DNA cross-links were reversed by adding 200 mM NaCl and heating at 65°C overnight. After treatment with proteinase K, the samples were purified with QIAquick PCR purification Kit (Qiagen). One tenth of the immunoprecipitated DNA and 1% of the inputs DNA were analyzed by PCR.

Antibodies used included Brg1, Brm and Ini 1 (de La Serna et al., 2000), di-acetylated (K9K14) H3 (Upstate, 06-599), tetra-acetylated H4 (Upstate, 06-866), Pol-II CTD-Ser-5P (Covance), and the following Santa Cruz antibodies: C/EBP $\alpha$  (sc-9314), C/EBP $\beta$  (sc-7962), C/EBP $\delta$  (sc-151), TFIIB (sc-274), TBP (sc-273), TFIID (sc-293). When RNA polymerase II antibody was used, 10mM NaF was added to all buffers. PCRs were performed with Qiagen HotStart Taq master mix in the presence of 2 $\mu$ Ci  $\alpha$ -<sup>32</sup>P dATP under the following conditions: 94°C, 15 minutes, followed by 26 cycles ( $\beta$ -actin) or 27 cycles (PPAR $\gamma$ 2) of 94°C for 30 sec, then either 65°C for 40 sec ( $\beta$ -actin) or 49.5°C for 40 sec (PPAR $\gamma$ 2), then 72°C for 30 sec, followed by a 72°C extension for 5 min. PCR products were resolved in 8% polyacrilamide-1X TBE gels, dried and exposed to a PhosphorImager. Primers used were:  $\beta$ -actin: 5' (+31)GCTTCTTTGCAGCTCCTTCGT TG-3' and 5' (+135)TTTGACATGCCGGAGCCGTTGT-3' and PPAR $\gamma$  2 promoter 5' (-413) TACGTTTATCGGTGTTTCAT-3' and 5' (-247) TCTCGCCAGTGACCC-3'. Experiments utilizing RNA polymerase II or general transcription factor antibodies were also performed using primers spanning -216 to -20 of the PPAR $\gamma$ 2 promoter with identical results.

## Results

### **SWI/SNF enzymes are required for differentiation of fibroblasts along the adipogenic pathway**

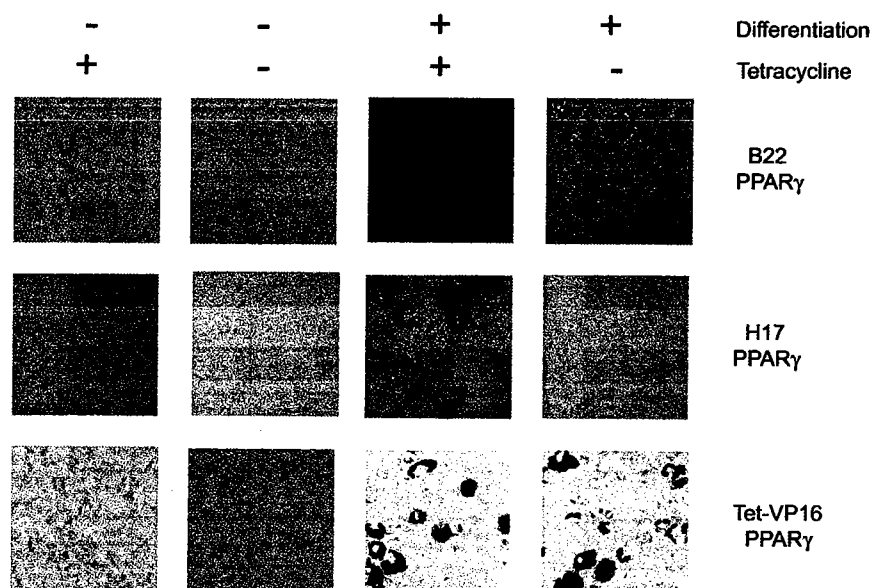
We previously described the B22 and H17 fibroblast cell lines that utilize the tet-regulatory system to inducibly express an ATPase-deficient, FLAG-tagged human allele of BRG1 (B22) or an ATPase-deficient, FLAG-tagged human allele of hBRM (H17). The parent for these cell lines, termed Tet-VP16, inducibly expresses only the tet-VP16 transactivator and thus serves as a control for our experiments. The mutant BRG1 and hBRM protein expressed in B22 and H17 cells are competent to associate with other endogenous subunits of SWI/SNF chromatin remodeling enzymes and can act as dominant negatives with regard to different inducible gene activation events (de La Serna et al., 2000; de la Serna et al., 2001a).

To determine the requirement for BRG1- and hBRM-based SWI/SNF enzymes in adipogenesis, we forced the differentiation of these cells in the presence (dominant negative OFF) or absence (dominant negative ON) of tetracycline by infection with retroviral vectors encoding the PPAR $\gamma$  nuclear hormone receptor or the C/EBP $\alpha$  or C/EBP $\beta$  activators. Cells infected with empty retroviral vector served as a control. Cells then were split into media containing or lacking tetracycline for 96 hours and subsequently were cultured for 7 days in the presence or absence of a differentiation regiment that included exposure to a differentiation cocktail (see methods) containing troglitazone, a synthetic PPAR $\gamma$  ligand. Adipocyte differentiation was shown by staining with Oil Red O, a lipophilic dye. In our hands, approximately 15-20% of the PPAR $\gamma$  infected cells stained with Oil Red O, which is similar to previous data for un-modified

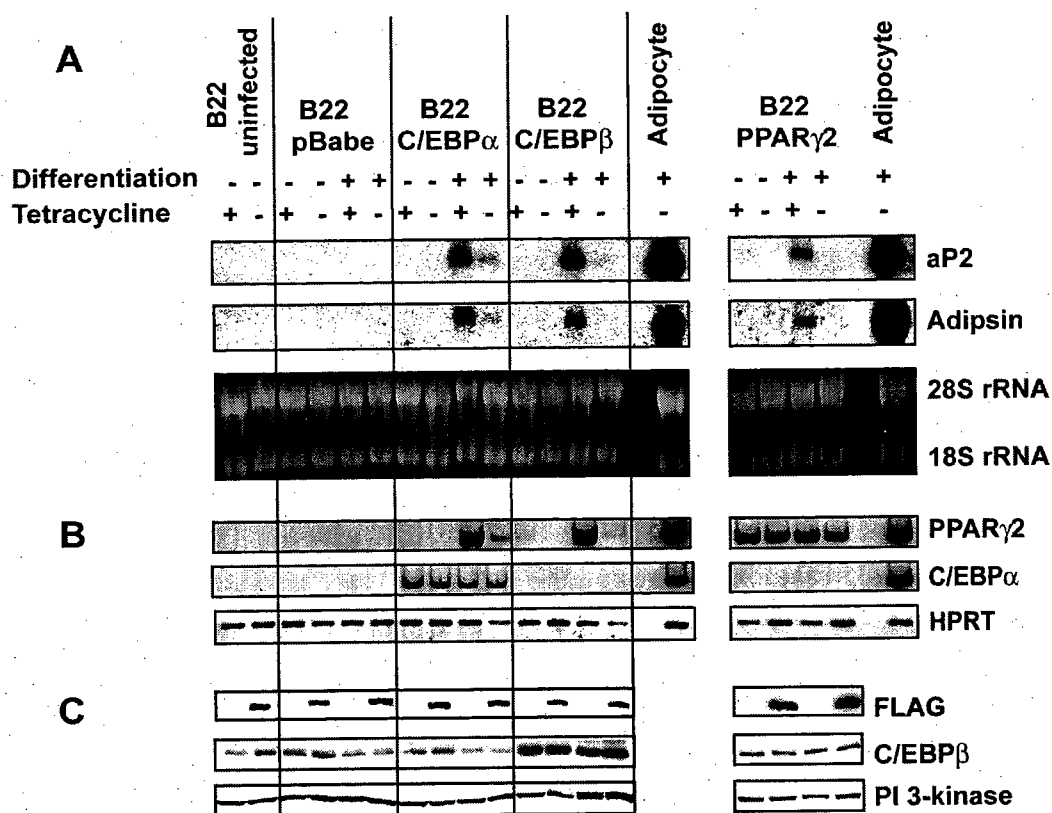
NIH3T3 cells infected with PPAR $\gamma$  (Tontonoz et al., 1994c). Tet-VP16 control cells stained positively when infected with the PPAR $\gamma$  retrovirus but not when infected with the empty vector (data not shown), and differentiation into adipocytes was dependent on exposure to the differentiation cocktail (Fig. II.1). The presence or absence of tetracycline made no difference, as expected. In contrast, B22 and H17 cells infected with the PPAR $\gamma$  retrovirus and cultured in the absence of tetracycline did not differentiate (Fig. II.1), suggesting that the expression of dominant negative BRG1 or hBRM interfered with the differentiation process. The same results were obtained for cells infected with either the C/EBP $\alpha$  or the C/EBP $\beta$  virus (data not shown).

**SWI/SNF enzymes are required for the activation of adipogenic genes as well as for activation of PPAR $\gamma$**

We analyzed the mRNA levels of two adipogenic marker genes, aP2 and adipsin, in cells expressing PPAR $\gamma$ , C/EBP $\alpha$ , or C/EBP $\beta$ . Cells infected with the PPAR $\gamma$  retrovirus and cultured in the presence of tetracycline and differentiation cocktail expressed both genes, while cells cultured in the absence of tetracycline produced dominant negative BRG1 and inhibited the expression of both genes (Fig. II.2A – right panel). Expression of PPAR $\gamma$  in each of the samples was monitored by RT-PCR (Fig. II.2B – right panel) and induction of dominant negative BRG1 was monitored by FLAG expression (Fig. II.2C – right panel). Other experiments demonstrated that the related B24 cell line, which also inducibly expresses dominant negative BRG1, and H17 cells, which inducibly express dominant negative hBRM, similarly inhibited expression of adipogenic marker genes in the presence of dominant negative protein (data not shown). Control Tet-VP16 cells infected with PPAR $\gamma$  encoding virus showed accumulation of both



**Figure II.1.** Expression of dominant-negative BRG1 or hBRM blocks the ability of PPAR $\gamma$ 2 to induce adipogenesis in fibroblast cells. B22 and H17 cells, which inducibly express ATPase-deficient, dominant-negative BRG1 or hBRM, respectively, upon removal of tetracycline, or Tet-VP16 cells, which induce only the Tet-VP16 regulator, were infected with a retroviral vector encoding PPAR $\gamma$ 2, grown in the presence or absence of tetracycline, and differentiated for 7 days in the presence or absence of differentiation cocktail (see Materials and Methods). Cells were fixed, stained with Oil Red O, and photographed.



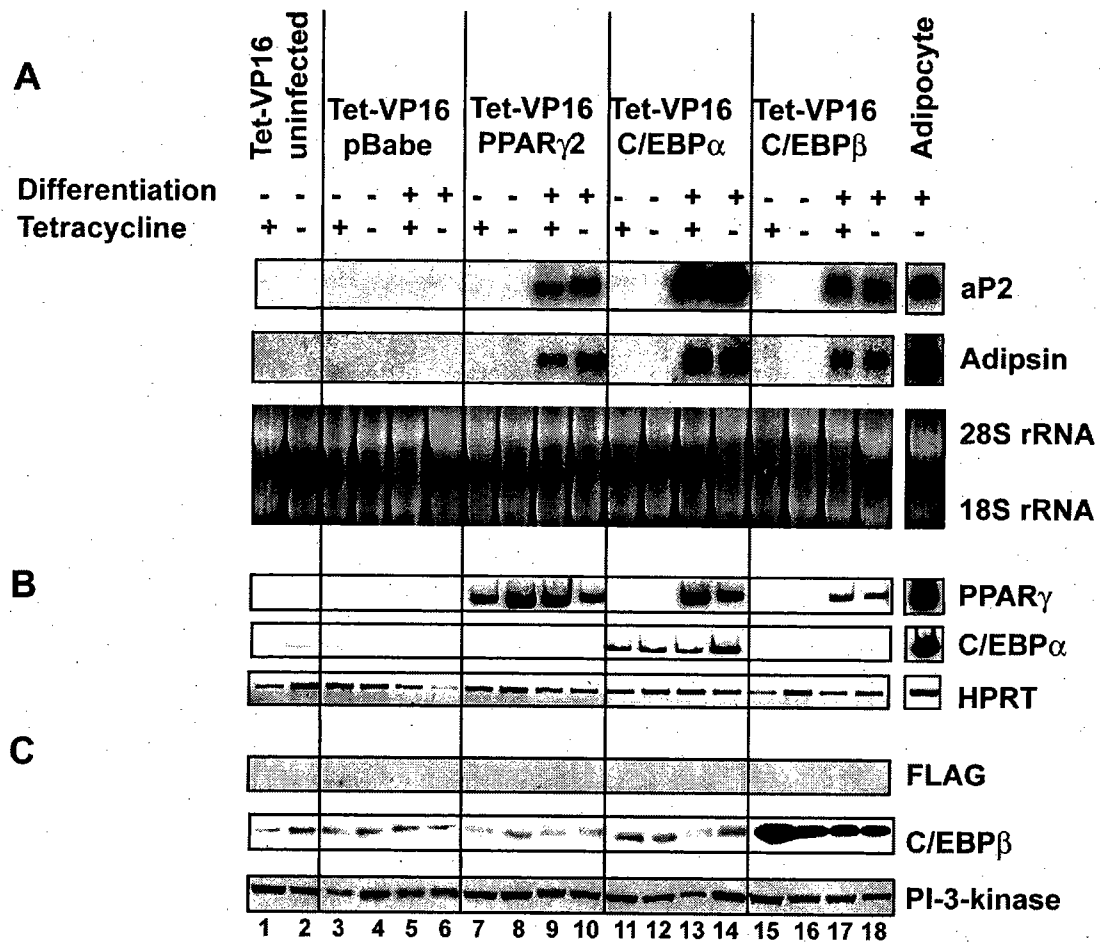
**Figure II.2.** Expression of dominant-negative BRG1 blocks the ability of PPAR $\gamma$ 2, C/EBP $\alpha$ , or C/EBP $\beta$  to induce adipogenesis in fibroblast cells. B22 cells were infected with retroviral vectors encoding PPAR $\gamma$ 2, C/EBP $\alpha$ , or C/EBP $\beta$ , grown in the presence or absence of tetracycline, and differentiated for 7 days in the presence or absence of differentiation cocktail. Samples in lanes marked Adipocyte were taken from 3T3-L1 preadipocytes differentiated for 7 days. (A) Northern blot showing levels of aP2 and adipsin mRNA. Ethidium bromide staining of rRNA is shown as a control. (B) RT-PCR showing levels of PPAR $\gamma$ 2 and C/EBP mRNA. HPRT mRNA levels are shown as a control. (C) Western blot showing levels of FLAG-tagged dominant-negative BRG1, C/EBP $\beta$ , and PI3-kinase.

aP2 and adipsin mRNAs, regardless of the presence or absence of tetracycline (Fig. II.3, lanes 9-10). The results demonstrate that expression of dominant negative BRG1 or hBRM interfered with the differentiation process by preventing expression of adipogenic genes. Since BRG1 and hBRM have only been found in cells in a large molecular weight complex associated with the other SWI/SNF subunits; presumably adipogenesis is also dependent on the activity of the SWI/SNF chromatin remodeling enzymes.

Adipogenesis also can be induced in culture via ectopic expression of the cellular transcription factors C/EBP $\alpha$  or C/EBP $\beta$  (Freytag et al., 1994; Wu et al., 1995b; Yeh et al., 1995). When B22 cells were infected with retrovirus expressing either C/EBP $\alpha$  or C/EBP $\beta$ , a similar block to aP2 and adipsin gene expression was observed in the presence of dominant-negative BRG1, though in cells expressing C/EBP $\alpha$ , the aP2 and adipsin mRNA levels were reduced 85-90% and were not completely absent (Fig. II.2A – left panel). Expression levels of C/EBP $\alpha$  (Fig. II.2B – left panel), C/EBP $\beta$  and dominant negative-BRG1 (Fig. II.2C – left panel) in these samples were monitored by RT-PCR or western blot. Similar results were obtained in H17 cells expressing dominant negative hBRM (data not shown). As anticipated, Tet-VP16 cells infected with C/EBP $\alpha$  or C/EBP $\beta$  encoding virus expressed both aP2 and adipsin mRNAs in both the presence and absence of tetracycline (Fig. II.3, lanes 13-14 and 17-18). Thus, SWI/SNF chromatin remodeling enzymes also are required for activation of the adipogenic gene program by C/EBP $\alpha$  or C/EBP $\beta$ .

Interestingly, cells induced to differentiate via expression of C/EBP $\alpha$  or C/EBP $\beta$  activated PPAR $\gamma$  expression in a SWI/SNF dependent manner (Fig. II.2B– left panel). This suggests that during differentiation promoted by C/EBP $\alpha$  or C/EBP $\beta$ , induction of



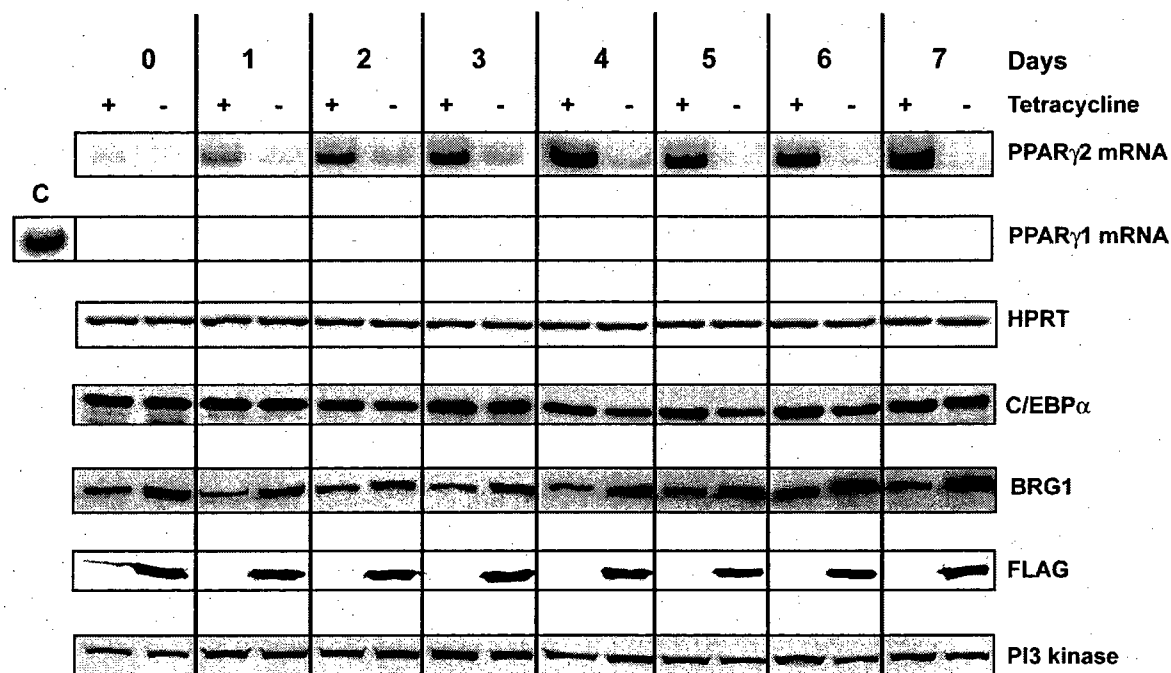


**Figure II.3.** PPAR $\gamma$ 2, C/EBP $\alpha$ , and C/EBP $\beta$  induce adipogenesis in Tet-VP16 control fibroblasts. Tet-VP16 cells were infected and manipulated as described for Fig. 2. (A) Northern blot showing levels of aP2 and adipsin mRNA. Ethidium bromide staining of rRNA is shown as a control. (B) RT-PCR showing levels of PPAR $\gamma$ 2 and C/EBP $\alpha$  mRNA. HPRT mRNA is shown as a control. (C) Western blot showing levels of FLAG-tagged dominant-negative BRG1, C/EBP $\beta$ , and PI3-kinase.

PPAR $\gamma$  – one of the earliest steps in differentiation – is dependent on the activity of BRG1 or BRM. This explains the significant decrease in aP2 and adipsin expression in these cells. However, the data presented here also indicate that SWI/SNF enzymes play a broader role in adipogenesis than just promoting induction of PPAR $\gamma$ , because when the induction of PPAR $\gamma$  is bypassed by providing PPAR $\gamma$  via retroviral infection, expression of the downstream adipogenic marker genes remained SWI/SNF dependent (Fig. II.2 – right panels). Thus the data indicate that SWI/SNF enzymes are required for both early and late gene activation events during adipocyte differentiation.

#### **Kinetics of PPAR $\gamma$ 2 expression in differentiating cells**

To further analyze the molecular events controlling activation of the adipogenic pathway, we initiated differentiation by infecting cells with C/EBP $\alpha$ , as in our hands we could observe that nearly 70% of cells differentiated and accumulated lipid droplets (data not shown). RT-PCR analysis of PPAR $\gamma$ 2 mRNA levels in cells grown in the presence of tetracycline showed a detectable accumulation on day 1 of differentiation, with robust levels present from days 2-7 (Fig. II.4). Low levels of PPAR $\gamma$ 2 transcripts were observed in cells grown in the absence of tetracycline. We could not detect PPAR $\gamma$ 1 mRNA in these cells. Western analyses confirmed that C/EBP $\alpha$  was present at equivalent levels in each sample (Fig. II.4). Analysis of FLAG-tagged protein levels indicated expression of the mutant BRG1 protein occurred in all samples grown in the absence of tetracycline. Re-probing of this blot for total Brg1 levels revealed only a 1.5 - 2.0 fold increase in levels of Brg1 in the cells expressing the dominant negative BRG1. This suggests that the levels of mutant Brg1 are only a fraction of the total Brg1 present in the cells yet still

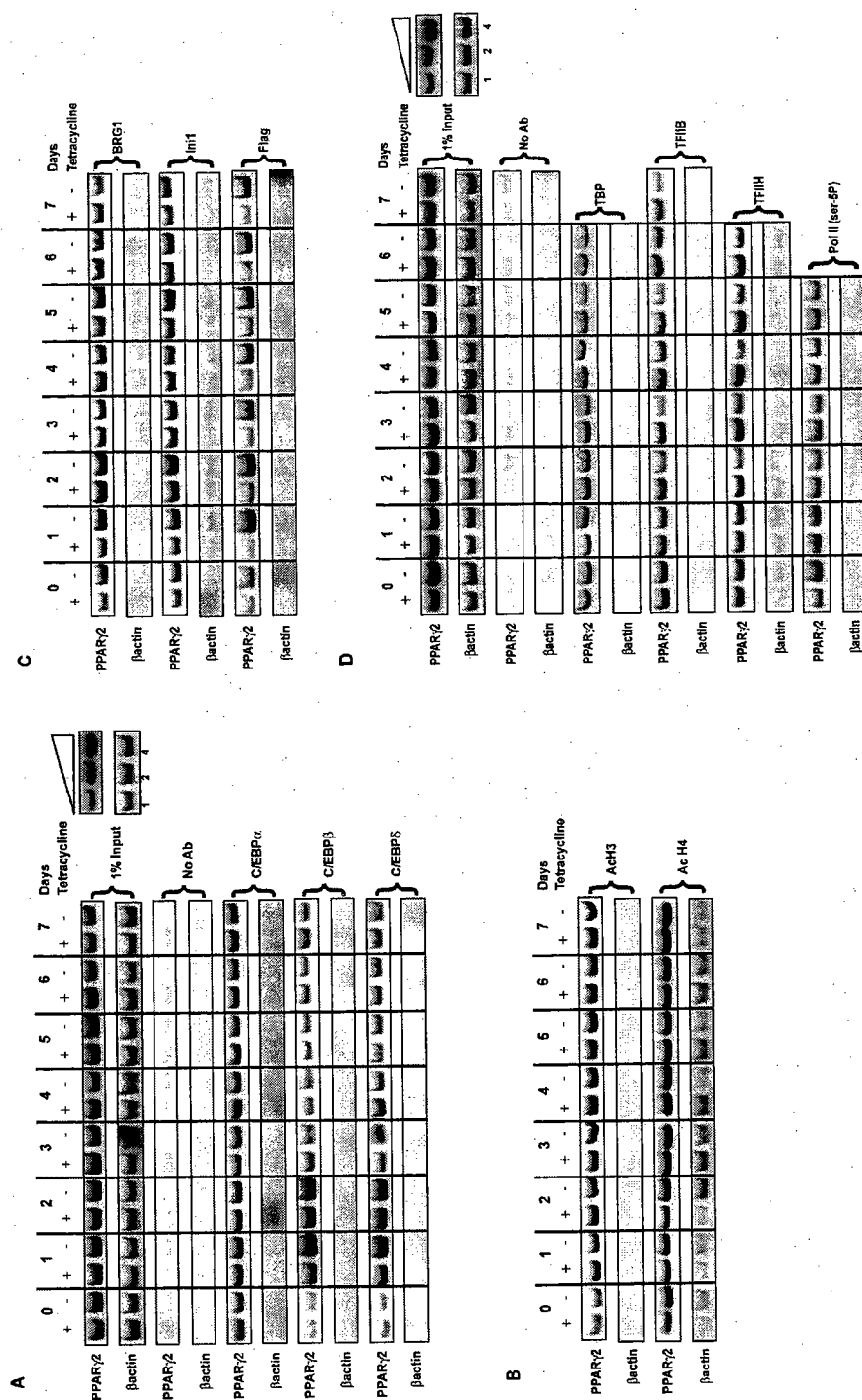


**Figure II.4.** Expression levels of adipogenic regulators during differentiation of fibroblast cells along the adipogenic pathway. B22 cells were infected with a retroviral vector encoding C/EBP $\alpha$ , grown in the presence or absence of tetracycline, and differentiated. Expression levels of adipogenic regulators are given as a function of time of differentiation. PPAR $\gamma$ 2 and PPAR $\gamma$ 1 transcript levels were measured by RT-PCR. HPRT levels are shown as a control. The lane marked C is a positive control from day 7 differentiated 3T3-L1 preadipocytes. C/EBP $\alpha$ , total Brg1, dominant negative, FLAG-tagged BRG1, and PI3-kinase levels were measured by Western blotting.

function as dominant negative proteins. Alternatively, expression of the mutant Brg1 reduces expression from the endogenous locus, perhaps for the purpose of maintaining a specific overall level of Brg1 protein. In either case, the results indicate that high levels of Brg1 overexpression are not occurring in the differentiating cells.

#### **Histone hyperacetylation and binding of C/EBP factors to the PPAR $\gamma$ 2 promoter is independent of Brg1-based SWI/SNF activity**

The mouse PPAR $\gamma$ 2 promoter has been defined but not well characterized functionally. Of interest, however, is the presence of multiple C/EBP binding sites in the promoter, including two half-consensus sites around -325 relative to the mRNA start site that contribute to activation of PPAR $\gamma$ 2 reporter genes (Elberg et al., 2000). To temporally analyze factor interactions at the endogenous PPAR $\gamma$ 2 promoter, we performed chromatin immunoprecipitation (ChIP) experiments and amplified either PPAR $\gamma$ 2 promoter sequences or  $\beta$ -actin sequences as a control. Because we forced differentiation by ectopically expressing C/EBP $\alpha$  before inducing differentiation, we were not surprised to see interaction of C/EBP $\alpha$  with the PPAR $\gamma$ 2 promoter at the initiation of the differentiation process and throughout the time course. This observation likely reflects and explains the capacity of C/EBP $\alpha$  to initiate the adipogenic gene expression program in non-adipogenic cells. The presence of dominant negative BRG1 had no or little effect on C/EBP $\alpha$  interactions (Fig. II.5A). In differentiating adipocytes, there is a temporal order to expression of the C/EBP family members, where C/EBP $\beta$  and C/EBP $\delta$  are rapidly induced, then are shut off over the first few days of differentiation.



**Figure II.5.** ChIP analysis of interactions on the PPAR $\gamma$ 2 promoter as a function of time of differentiation. B22 cells were infected with a retroviral vector encoding C/EBP $\alpha$ , grown in the presence or absence of tetracycline, and differentiated. One percent of input is shown for each experiment. Portions of the PPAR $\gamma$ 2 promoter or the  $\beta$ -actin 5' untranslated region and coding sequence were amplified from each sample. (A) Levels of interactions with C/EBP $\alpha$ ,  $\beta$ , and  $\delta$ . (B) Levels of interactions with tetra-acetylated H4 and K9-, K14-diacetylated H3. (C) Levels of interactions with total Brg1, dominant-negative, FLAG tagged BRG1, and Ini1. (D) Levels of interactions with TBP, TFIIIB, TFIIH, p89, and RNA polymerase II phosphorylated on Ser-5 of the CTD. Inset: linearity controls for PCR amplifications. Each ChIP was repeated in two to five independent experiments. The data shown in parts A, B, and C derive from a single differentiation experiment. ChIPs in part D derive from a different differentiation experiment.

Subsequently, C/EBP $\alpha$  is induced at about day 2-3 and is maintained during differentiation [reviewed in (Lane et al., 1999)]. We therefore examined interactions of C/EBP $\beta$  and C/EBP $\delta$  at the PPAR $\gamma$ 2 promoter. Somewhat surprisingly, both C/EBP $\beta$  and C/EBP $\delta$  showed a robust induction of occupancy of the promoter at days 1-2 of differentiation, despite the presence of ectopic C/EBP $\alpha$ , and regardless of the presence of dominant negative BRG1 (Fig. II.5A). Thus there appears to be a preference during the early stages of differentiation for interaction with C/EBP $\beta$  and C/EBP $\delta$ . In addition, binding of these factors does not require functional SWI/SNF enzymes.

There are a multitude of histone modifications and other ATP-dependent chromatin remodeling activities that may mediate C/EBP factor binding in the presence of mutant SWI/SNF enzymes. We examined di-acetylation of histone H3 on lysines 9 and 14 as well as tetra-acetylation of H4 (Fig. II.5B). Levels of tetra-acetylated H4 were high at the beginning of the differentiation process and remained constant throughout. In contrast, there was induction of di-acetylated H3 at day 1, concurrent with occupancy of the promoter by the C/EBP $\beta$  and C/EBP $\delta$  factors. Acetylation of H3 and H4 was unaffected by the presence of dominant negative BRG1.

#### **C/EBP factors initiate recruitment of SWI/SNF and RNA polymerase II associated general transcription factors to the promoter**

Previous work has demonstrated that both C/EBP $\alpha$  and C/EBP $\beta$  can physically interact with the SWI/SNF component hBRM in cells overexpressing both proteins (Kowenz-Leutz and Leutz, 1999; Pedersen et al., 2001). Additionally, C/EBP $\alpha$  interacts with the RNA polymerase II associated general transcription factors TFIIB and TBP (Nerlov and Ziff, 1995). These data have been used to argue that the C/EBP factors can

recruit SWI/SNF and general transcription factors to facilitate transcription. ChIP experiments against the FLAG-tagged ATPase-deficient BRG1 produced in B22 cells revealed that the mutant ATPase, when expressed, was present on the PPAR $\gamma$ 2 promoter as early as day 0 and maximally from day 1 through day 7 of differentiation (Fig. II.5C). Thus the presence of C/EBP $\alpha$  may be recruiting SWI/SNF to the promoter at or before the onset of differentiation, possibly reflecting the ability of these proteins to physically interact. ChIP analysis of Brg1 and the SWI/SNF component Ini1 revealed kinetics of promoter occupancy that were similar to each other and to FLAG-tagged mutant BRG1. Promoter occupancy in cells not expressing the dominant negative BRG1 (plus tetracycline) differed in that interaction of SWI/SNF components peaked at day 2, which coincides with full induction of PPAR $\gamma$ 2 transcription (Fig. II.4). The observed interaction of the mutant BRG1 with the promoter on day 0 may be due to the modest overexpression of total Brg1 in the cells expressing mutant BRG1 (Fig. II.4).

Analyses of phosphorylated RNA polymerase II and its associated general transcription factors, TBP, TFIIB, and TFIIF, indicated that each of these factors is present on the PPAR $\gamma$ 2 promoter on day 0, and occupancy in the presence of the dominant negative SWI/SNF complex is essentially unaffected on days 0 and 1 (Fig. II.5D). Thus SWI/SNF function is not required to assemble these factors onto the promoter in the presence of ectopic C/EBP $\alpha$ . However, the presence of the C/EBP factors, Brg1-based SWI/SNF enzyme, and these GTFs on the promoter are not sufficient to initiate transcription at day 0. Maximal induction of PPAR $\gamma$ 2 transcription in the presence of functional SWI/SNF enzymes occurs on day 2 (Fig. II.4). In contrast, starting on day 2 and continuing throughout the rest of the time course, the association of TBP, TFIIB, RNA polymerase II, and especially TFIIF is compromised in cells



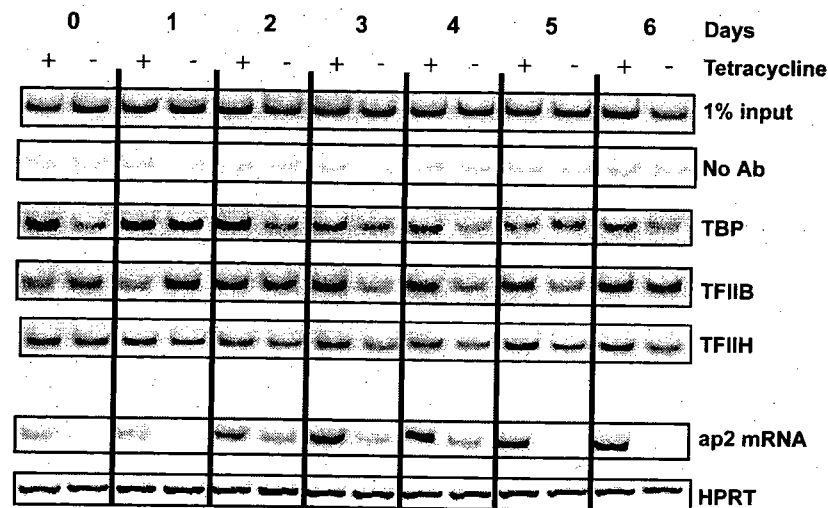
expressing the dominant negative BRG1 (Fig. II.5D). Western blot analysis of these GTFs and RNA polymerase II shows that the levels of these factors are not altered by differentiation or by expression of the dominant negative BRG1 (data not shown). Thus, on or about day 2 of differentiation, SWI/SNF function is necessary to maintain the preinitiation complex on the PPAR $\gamma$ 2 promoter and to promote transcription.

Next, we asked if SWI/SNF complexes use the same mechanism to regulate other adipogenic genes. We performed a series of ChIPs on the aP2 and adipsin promoters during adipogenesis and determined that recruitment of one or more general transcription factors was also compromised at the onset of gene expression (Fig. II.6).

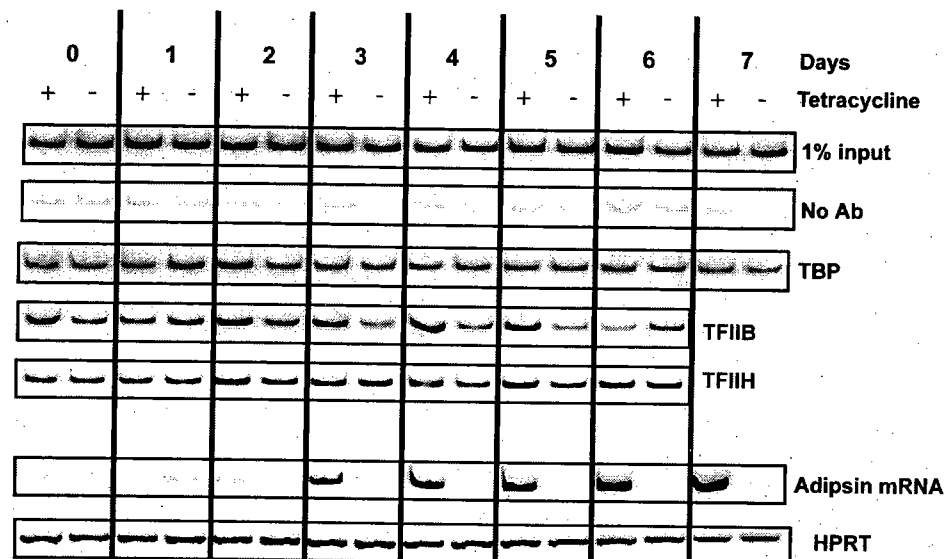
### **Examination of PPAR $\gamma$ 2 promoter accessibility and activation in differentiating 3T3-L1 pre-adipocytes**

Although forced differentiation of fibroblasts has been utilized for many years as a model system and has been essential in the identification and characterization of adipogenic regulatory proteins, we were concerned that the events leading to activation of the PPAR $\gamma$ 2 promoter under conditions where the C/EBP $\alpha$  activator was prematurely expressed might not precisely reflect the events that occurred during differentiation of a committed pre-adipocyte cell. We therefore examined PPAR $\gamma$ 2 activation in differentiating 3T3-L1 pre-adipocytes.

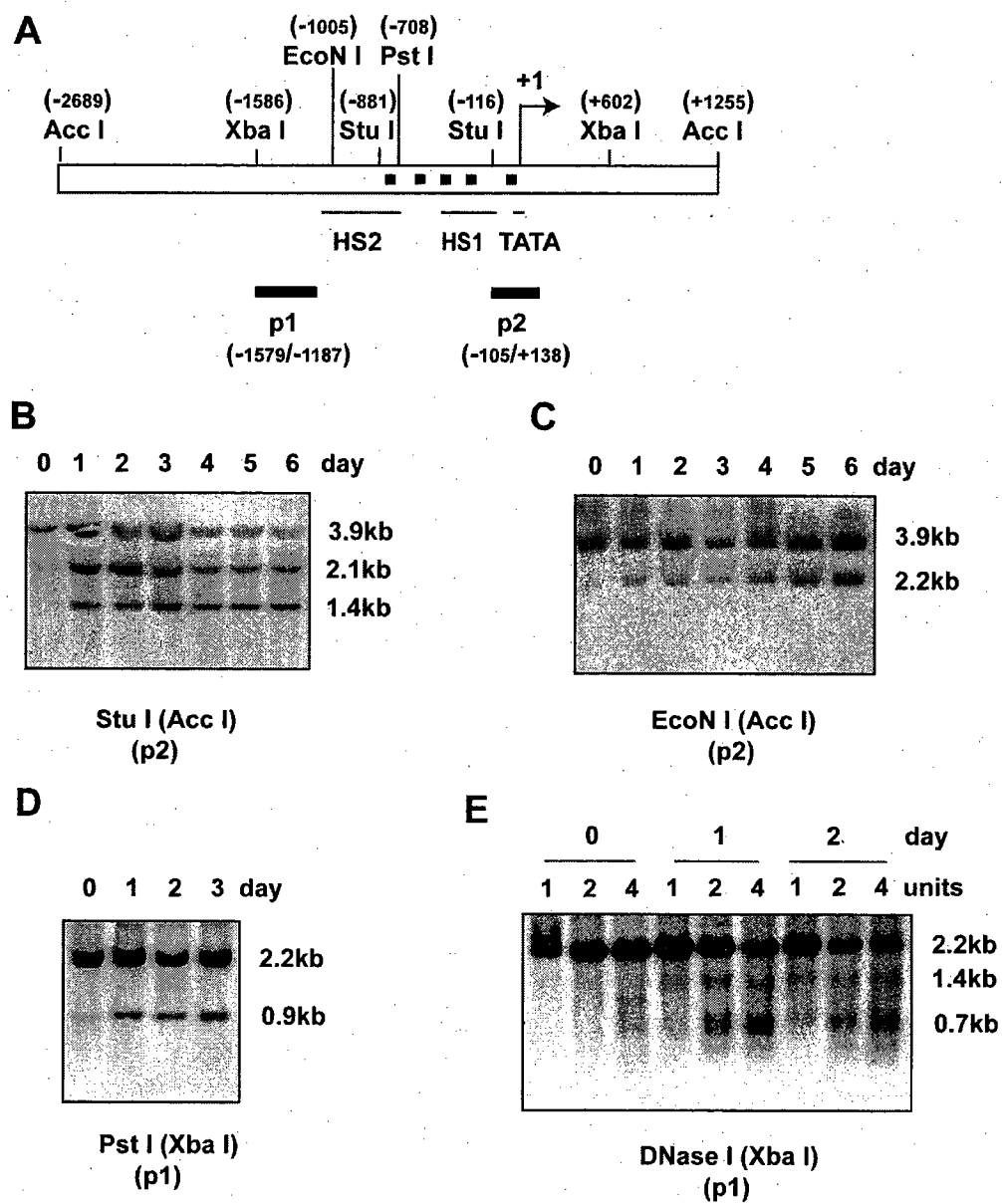
Initially, we examined changes in PPAR $\gamma$ 2 promoter structure as reflected by increases in nuclease accessibility. A schematic of the promoter is presented in Fig.II.7A. Examination of two *Stu*I sites, an *Eco*NI site and a *Pst*I site located between -100 and -1000 on the PPAR $\gamma$ 2 promoter revealed a dramatic increase in accessibility in



## Adipsin promoter



**Figure II.6.** ChIP analysis of interactions on the aP2 and adipsin promoters as a function of time of differentiation. B22 cells were infected with a retroviral vector encoding C/EBP $\alpha$ , grown in the presence or absence of tetracycline, and differentiated. One percent of input is shown for each experiment. It is shown the levels of interactions with TBP, TFIIB, and TFIH p89 and mRNA levels.



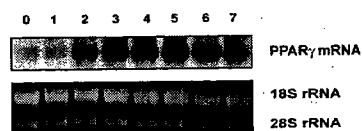
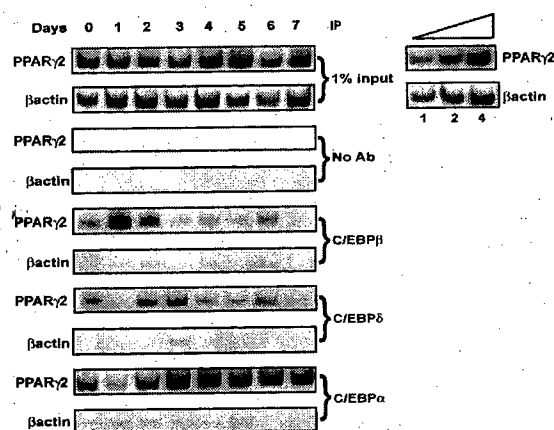
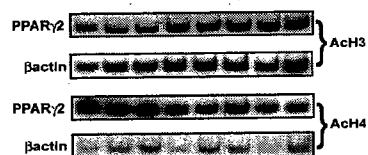
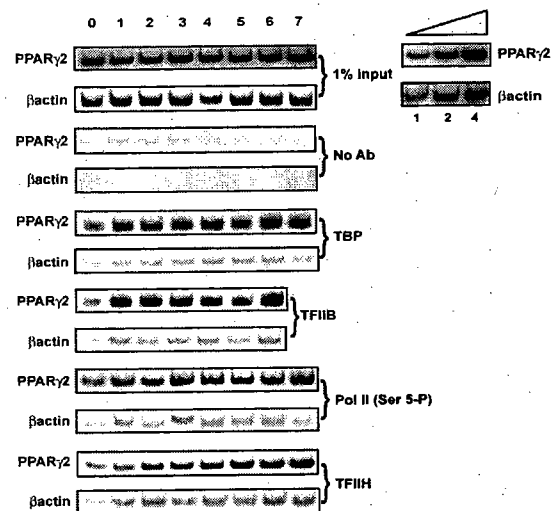
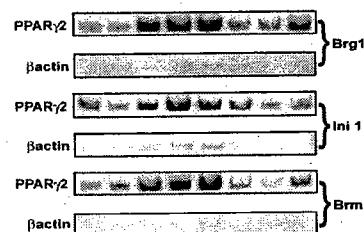
**Figure II.7.** Changes in PPAR $\gamma$ 2 promoter accessibility occur on day 1 of differentiation. 3T3-L1 preadipocytes were induced to differentiate, and nuclei were harvested at the indicated times for nuclease accessibility experiments. (A) Schematic of the PPAR $\gamma$ 2 promoter. The locations of relevant restriction enzyme sites are indicated, as are the previously reported DNase I hypersensitive sites (HS1 and HS2) (34). Black squares represent potential C/EBP binding sites. Black bars P1 and P2 indicate the fragments used as Southern blot probes. (B to D) Restriction enzyme accessibility of EcoNI, Stul, and PstI. The enzymes used for flanking digests are indicated in parentheses. (E) Accessibility of DNase I.

this region on day 1 of the differentiation process (Figs. II.7B-D), reflecting changes in promoter structure. A previous report (Ren et al., 2002) indicated that two DNase I hypersensitive sites exist on the PPAR $\gamma$ 2 promoter in undifferentiated 3T3-L1 cells (see Fig. II.7A). Our analysis indicated these DNase I hypersensitive sites were difficult to detect in undifferentiated, day 0 cells. However, by day 1, they were clearly visible, indicating that these hypersensitive sites are induced by differentiation (Fig. II.7E). Together the results clearly demonstrate a change in PPAR $\gamma$ 2 promoter structure on day 1 of 3T3-L1 differentiation, prior to induction of PPAR $\gamma$ 2 gene expression.

Northern analysis of PPAR $\gamma$  transcript levels revealed a robust induction of expression on day 2 of 3T3-L1 pre-adipocyte differentiation (Fig. II.8A), in agreement with prior studies (Tontonoz et al., 1994c). Thus, induction of the PPAR $\gamma$ 2 is temporally distinct from changes in promoter accessibility.

#### **Binding of C/EBP factors, hyperacetylated histones, RNA polymerase II and most general transcription factors precede the onset PPAR $\gamma$ 2 induction**

ChIP experiments to identify interactions of the C/EBP family of factors with the PPAR $\gamma$ 2 promoter showed an induction of C/EBP $\beta$  and C/EBP $\delta$  binding on days 1 and 2 of differentiation, respectively (Fig. II.8B), largely consistent with the expression patterns of these C/EBPs in differentiating 3T3-L1 cells and with the ChIP results from the forced fibroblast system (Fig. II.5A). Thus, interaction of C/EBP $\beta$  correlated temporally with changes in nuclease accessibility on the promoter. Over days 2 and 3, C/EBP $\beta$  and  $\delta$  disappeared from the promoter, concurrent with the appearance of C/EBP $\alpha$  (Fig. II.8B).

**A****B****C****D****E**

**Figure II.8.** (A) PPAR $\gamma$  expression levels in differentiating 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate. PPAR $\gamma$  expression levels were analyzed by Northern blotting. Ethidium bromide-stained 28S and 18S rRNA is shown as a control. (B to E) ChIP analysis of interactions on the PPAR $\gamma$ 2 promoter as a function of time of differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate and processed for ChIP assays at the indicated times. Portions of the PPAR $\gamma$ 2 promoter or the  $\beta$ -actin gene were amplified from each sample. (B) Levels of interactions with C/EBP $\alpha$ ,  $\beta$ , and  $\delta$ . (C) Levels of interactions with tetra-acetylated H4 and K9-, K14-diacetylated H3. (D) Levels of interactions with TBP, TFIIB, TFIIF p89, and Pol II phosphorylated on Ser-5 of the CTD. (E) Levels of interactions with Brg1, Brm, and Ini1. Inset: linearity controls for PCR amplifications. Each ChIP was repeated in three to five independent experiments. The data shown in parts B, C, and E derive from a single differentiation experiment. ChIPs in part D derive from a different differentiation experiment.

Thus in differentiating 3T3-L1 cells, the C/EBP binding sites in the PPAR $\gamma$ 2 promoter undergo a transition of factor occupancy from C/EBP $\beta$  to C/EBP $\alpha$ .

As in the C/EBP $\alpha$  forced fibroblast differentiation experiments, ChIP analysis of tetra-acetylated H4 indicated that levels were high prior to differentiation (Fig. II.8C). In contrast to the forced fibroblasts, di-acetylated H3 levels were present on the PPAR $\gamma$ 2 promoter at day 0, but increased on day 3 (Fig. II.8C). This increase clearly occurred after the onset of PPAR $\gamma$ 2 expression but correlated with the transition from binding of C/EBP $\beta$  and  $\delta$  to C/EBP $\alpha$ . Whether the change in di-acetylated H3 levels is a cause or effect of the transition to C/EBP $\alpha$  binding has not been determined. Di-acetylated H3 was also present at relatively high levels on the control  $\beta$ -actin sequences.

Examination of phosphorylated RNA polymerase II and associated general transcription factors by ChIP showed that RNA polymerase II, TBP, and TFIIB became associated with the promoter on day 1 of differentiation, prior to expression of PPAR $\gamma$ 2 (Fig. II.8D). Collectively, these data agree with the results obtained with the fibroblast differentiation model and indicate that much of the transcriptional machinery is present on the promoter prior to the onset of gene activation.

#### **PPAR $\gamma$ 2 expression is coincident with the binding of TFIIH and SWI/SNF components to the promoter**

In contrast to the other factors examined, the increase in association of TFIIH did not occur until day 2 of 3T3-L1 differentiation (Fig. II.8D). Similarly, association of the SWI/SNF components Brg1, Brm, and Ini1 occurred on day 2 (Fig. II.8E), coincident with the onset of PPAR $\gamma$ 2 transcription. Unlike RNA polymerase II and all of the general



transcription factors examined, the interaction of SWI/SNF subunits was transient, with only background levels present on the promoter after day 4. The Ini1 subunit is common to all SWI/SNF enzymes examined, but the Brg1 and Brm ATPases form distinct SWI/SNF enzymes, indicating that at least two distinct forms of the enzyme are present at the PPAR $\gamma$ 2 promoter at the onset of transcription. The data indicate that histone hyperacetylation, changes in promoter structure, C/EBP activator binding, and association of phosphorylated RNA polymerase II and multiple general transcription factors with the PPAR $\gamma$ 2 promoter occur over the first 24 hours of adipocyte differentiation but that these events are not sufficient to initiate transcription. PPAR $\gamma$ 2 expression is facilitated by association of TFIIH and SWI/SNF enzymes on day 2 of differentiation.

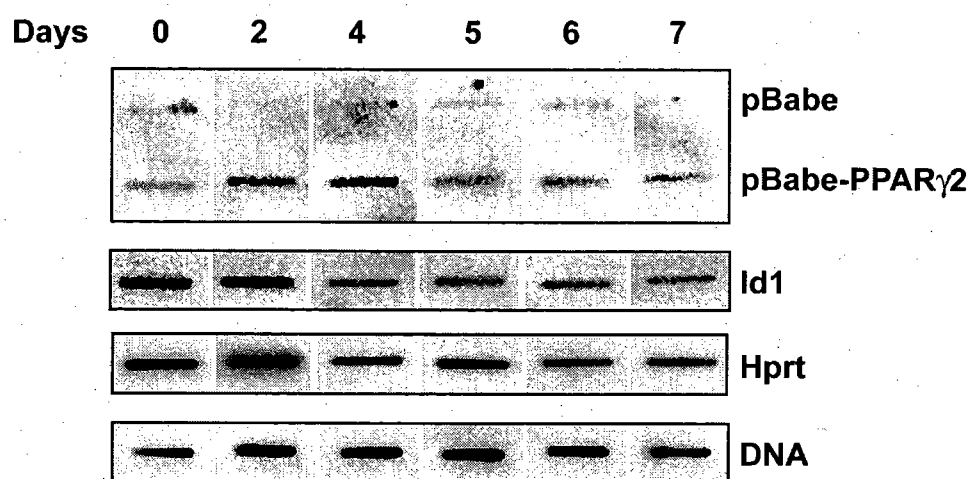
**A decrease in the rate of PPAR $\gamma$  transcription correlates with the dissociation of SWI/SNF enzyme components from the PPAR $\gamma$ 2 promoter**

The ChIP experiments in Figure 11.8E clearly demonstrate that SWI/SNF components were no longer stably associated with the PPAR $\gamma$ 2 promoter after day 4 of differentiation, even though the general transcription factors and C/EBP $\alpha$  remained. Analysis of stable mRNA levels indicated that PPAR $\gamma$  mRNA was abundant on days 5-7 (Figure 11.8A). Two possible explanations for these data exist. The promoter may achieve a stable structure that is permissive for continued transcription in the absence of SWI/SNF or the transcription of PPAR $\gamma$ 2 decreases or stops after day 4 and the PPAR $\gamma$  message observed on days 5-7 represents stable mRNAs produced on day 4 or earlier.

To distinguish between these possibilities, we performed a time course of nuclear run-on experiments. Linearized plasmid DNA containing the PPAR $\gamma$ 2 cDNA or empty

vector was immobilized on membranes and hybridized to radiolabeled run-on transcripts produced by nuclei isolated from differentiating 3T3-L1 cells on the days indicated. The data demonstrated induction of PPAR $\gamma$  transcription on day 2, continued transcription on day 4, but little transcription on days 5-7 (Figure II.9). Thus the rate of PPAR $\gamma$  transcription decreased between days 4 and 5 and correlated with the loss of SWI/SNF enzyme components on the PPAR $\gamma$ 2 promoter (Fig. II.8E). In contrast, the rate of Id1 transcription decreased as a function of differentiation (Fig. II.9), in agreement with previously published results from the differentiation of human preadipocyte cells (Moldes et al., 1997). We utilized non-specific hybridization to plasmid sequences and hybridization to 3T3-L1 genomic DNA as controls, as has been described (Cornelius et al., 1990; Long and Pekala, 1996; Stephens and Pekala, 1991; Waite et al., 2001), since we were unable to identify other genes that gave a constant rate of transcription over the differentiation time course. The rates of Hprt, 36B4, and Gapdh all decreased over the seven day time course (Fig. II.9 and data not shown).

We note that the immobilized template contained the entire PPAR $\gamma$ 2 cDNA, thus PPAR $\gamma$ 1 transcription would also have been detected. We were unable to detect run-on signal when only the short, 121 bp portion of the cDNA unique to PPAR $\gamma$ 2 (Zhu et al., 1995) was used for the hybridizations (data not shown). However, PPAR $\gamma$  mRNA at day 5 of 3T3-L1 differentiation and beyond is predominantly expressed from the PPAR $\gamma$ 2 promoter (Saladin et al., 1999; Tontonoz et al., 1994a). Thus the data most likely reflect a decrease in the rate of PPAR $\gamma$ 2 or in the rates of both PPAR $\gamma$ 2 and PPAR $\gamma$ 1 transcription.



**Figure II.9.** The rate of PPAR $\gamma$  transcription decreases after day 4 of 3T3-L1 differentiation. Nuclear run-on assays were performed on nuclei isolated from differentiating 3T3-L1 cells on the days indicated. Run-on transcripts were hybridized to 3T3-L1 genomic DNA, linearized pBABE vector, linearized pBABE containing the PPAR $\gamma$ 2 cDNA, or PCR products corresponding to Id1 or Hprt cDNAs.

## Discussion

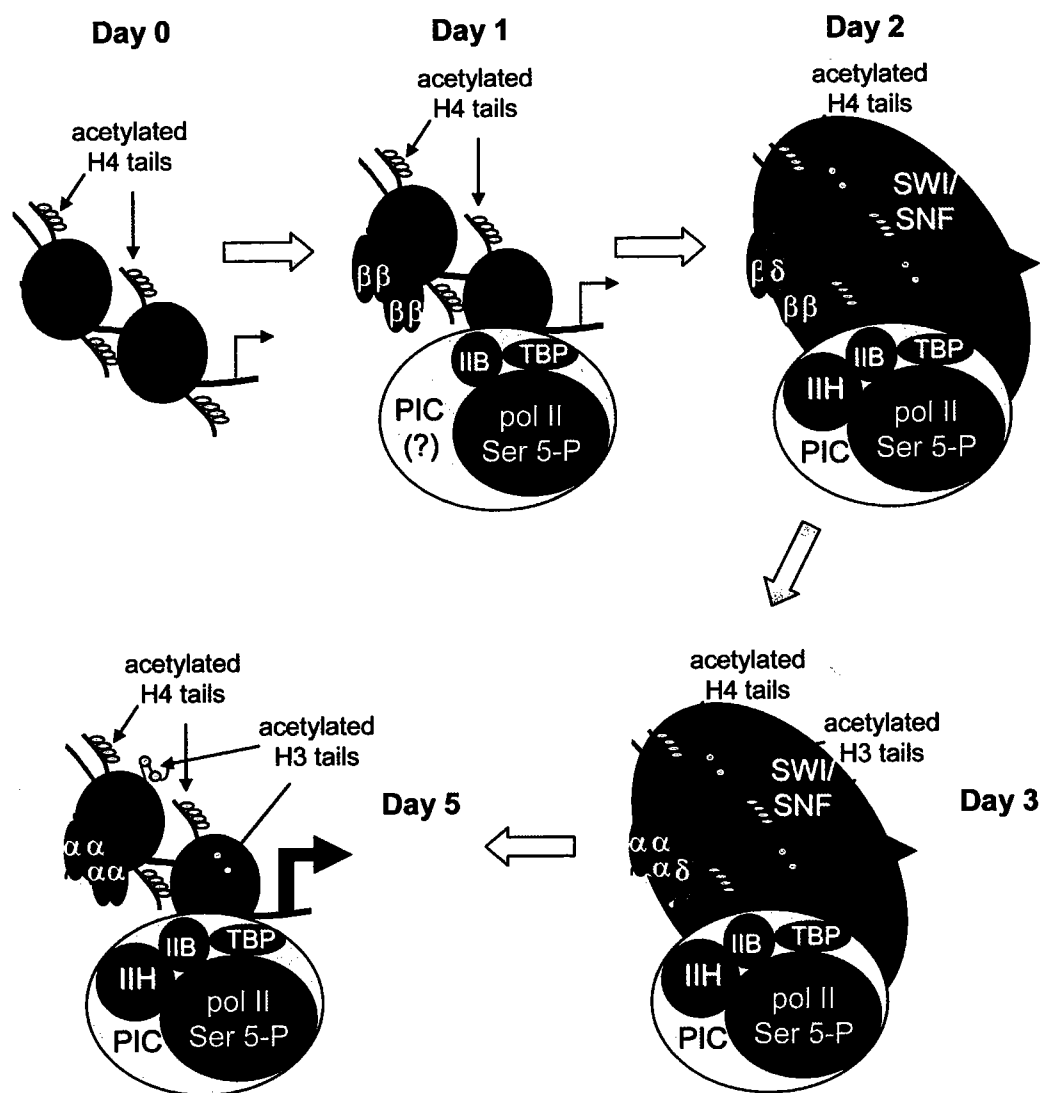
PPAR $\gamma$  is involved in adipocyte differentiation, insulin sensitivity and diabetes, atherosclerosis and the control of cell proliferation in some cancer cells [reviewed in (Berger and Moller, 2002; Rosen and Spiegelman, 2001)]. Consequently, its function has been the subject of intense investigation. Relatively little however, is known about the mechanisms controlling its expression. Here we utilized two different cellular models for adipocyte differentiation to temporally describe the molecular interactions that occur at the promoter of the inducible PPAR $\gamma$ 2 gene during adipocyte differentiation, with particular emphasis on the requirement for SWI/SNF chromatin remodeling enzymes. Through use of a differentiation system driven by introduction of the adipogenic regulator, C/EBP $\alpha$ , we demonstrate a requirement for SWI/SNF enzymes in the activation the PPAR $\gamma$  regulator as well as in the activation of adipogenic marker genes expressed later during differentiation. Moreover, these experiments revealed that this requirement for SWI/SNF enzymes was relatively late in the cascade of events leading to PPAR $\gamma$ 2 activation. Activator binding, RNA polymerase II and associated general transcription factors interactions at the promoter, and histone H3 and H4 acetylation occurred prior to and independently of SWI/SNF function. Instead, the data revealed a role for SWI/SNF enzymes in the function of the preinitiation complex components at the promoter at the time of transcriptional activation. Preliminary results on aP2 and adipsin promoters (Fig. II.6) support the idea that a similar mechanism operates on genes regulated by SWI/SNF.

Because of the inherent differences between forcing differentiation of fibroblasts into the adipocyte lineage and genuine pre-adipocyte differentiation, we analyzed differentiation of 3T3-L1 pre-adipocytes and confirmed both the general order of events

that occur during PPAR $\gamma$ 2 activation and a role for SWI/SNF enzymes in facilitating preinitiation complex function. We expect that the differences exhibited by the two systems reflects the ability of the C/EBP $\alpha$  activator to recruit general transcription factors and SWI/SNF enzymes (Pedersen et al., 2001) prematurely at the initiation of the forced differentiation program. Despite the differences, it is important to note the data from both systems are consistent with a need for SWI/SNF enzymes to promote the function of the preinitiation complex.

The order of events occurring in differentiating 3T3-L1 cells is diagrammed schematically in Fig. II.10. Acetylation of H4 occurs before the onset of differentiation, followed by concurrent changes in promoter accessibility, binding of the C/EBP $\beta$  and  $\delta$  activators, and assembly of polymerase II and most of the GTFs on day 1 of differentiation. Subsequently, on day 2, SWI/SNF enzymes and TFIIH associate with the promoter, indicating that the SWI/SNF enzymes likely facilitate assembly of the pre-initiation complex, thereby permitting PPAR $\gamma$ 2 transcription to commence. On day 3, there is both an increase in the levels of H3 acetylation and a transition from binding of C/EBP $\beta$  and  $\delta$  to binding of C/EBP $\alpha$ . Which event, if either is causal remains to be determined. Following day 4, the SWI/SNF enzymes disappear from the promoter and the rate of PPAR $\gamma$  transcription drops, indicating that the presence of SWI/SNF is required for continued transcription.

The data from differentiating 3T3-L1 pre-adipocytes indicate that both BRG1 and BRM are present on the PPAR $\gamma$ 2 promoter, suggesting that both complexes are contributing to function. Alternatively, the two complexes could be redundant in function,



**Figure II.10.** Schematic model of the temporal changes in factor interactions at the PPAR $\gamma$ 2 promoter during 3T3-L1 preadipocyte differentiation. Nucleosome positions are presented for illustrative purposes only.

and the presence of either SWI/SNF enzyme might be sufficient. Our studies using cell lines that inducibly express dominant negative BRG1 or BRM also suggest that both ATPases are required for PPAR $\gamma$ 2 activation and adipocyte differentiation. However, because the BRG1 and BRM SWI/SNF complexes share multiple subunits, it is possible that expression of one mutant ATPase deleteriously affects both complexes by sequestering subunits from the other, endogenous ATPase. Thus we cannot rigorously state at present whether BRG1, BRM or both are required for PPAR $\gamma$ 2 activation and adipocyte differentiation.

One of the interesting results from our studies is the demonstration that the C/EBP binding sites undergo a transition during the time course of differentiation from binding C/EBP $\beta$  and  $\delta$  to binding C/EBP $\alpha$ . Although the kinetics of expression for these factors has long supported this idea, this is the first documentation that such a transition occurs at a promoter expressed during adipogenesis. These results differ from previously published work that showed that C/EBP $\alpha$  and  $\delta$ , but not C/EBP $\beta$ , could bind to the C/EBP sites in the PPAR $\gamma$ 2 promoter in a gel shift study and could activate a transiently transfected PPAR $\gamma$ 2 reporter plasmid (Elberg et al., 2000). The differences between the studies may be attributed to the likelihood that the chromatin structure at the genomic locus differs from that on a transfected template and undergoes changes during the differentiation process that affect factor interactions.

Previous reports documenting the potential of C/EBP $\alpha$  and  $\beta$  to physically interact with BRM in cells transfected with both the C/EBP isoform and BRM suggested that these factors may recruit SWI/SNF enzymes (Kowenz-Leutz and Leutz, 1999; Pedersen et al., 2001). Our temporal analysis of factor interactions on the PPAR $\gamma$ 2 promoter during C/EBP $\alpha$  driven differentiation strongly suggests that targeting of

SWI/SNF by C/EBP $\alpha$  can occur. However, C/EBP $\alpha$  is prematurely present on the promoter in this differentiation system; the temporal differences in the appearance of the different C/EBP factors on the PPAR $\gamma$ 2 promoter in the differentiating 3T3-L1 (Agalioti et al., 2000) adipocytes suggest that in a more natural differentiation context, C/EBP $\beta$  and  $\delta$  may target SWI/SNF enzymes, which then later recruit C/EBP $\alpha$ .

Changes in nuclease accessibility and the binding of C/EBP factors, RNA polymerase II, and many of the GTFs on day 1 prior to the appearance of SWI/SNF enzymes on the PPAR $\gamma$ 2 promoter indicate that other factors must control the initial accessibility of these promoter sequences. Changes in H4 acetylation did not correlate with initial factor binding to the promoter. Changes in other histone modifications at the PPAR $\gamma$ 2 promoter have not been tested but potentially could mediate factor accessibility. Alternatively, a different ATP dependent remodeling enzyme(s) could alter chromatin structure and promote activator binding prior to SWI/SNF function. This hypothesis is supported by in vitro studies showing that ISWI containing chromatin remodeling enzymes facilitated stable interaction of RAR $\alpha$ :RXR on chromatin templates prior to SWI/SNF enzyme mediated stimulation of transcription (Dilworth et al., 2000). Finally, transcriptional regulators present on the promoter prior to differentiation, such as GATA-2 and 3 (Tong et al., 2000) and KLF2 (Banerjee et al., 2003), might influence chromatin structure in a manner that promotes the transition to an actively transcribing gene.

Our analysis of general transcription factor interactions on the PPAR $\gamma$ 2 promoter also revealed that serine 5 phosphorylated RNA polymerase II is present at the promoter before TFIIH. TFIIH contains a kinase activity that is capable of phosphorylating the RNA polymerase II CTD, however, the temporal order of factor appearance at the PPAR $\gamma$ 2 promoter suggests that either a different kinase is responsible for the CTD



phosphorylation or that TFIIF mediates CTD phosphorylation independently of promoter binding. Additionally, the presence of RNA polymerase II phosphorylated at serine 5 of the CTD raises the possibility that the polymerase may be transcriptionally engaged at day 1, and that the inclusion of SWI/SNF enzymes and TFIIF on day 2 promotes release of the polymerase and/or elongation. Multiple other genes are regulated at the level of transcriptional elongation, including hsp70, where elongation can be stimulated by SWI/SNF enzymes *in vitro* and *in vivo* (Brown et al., 1996; Corey et al., 2003).

The concurrent entry of SWI/SNF enzymes and TFIIF onto the PPAR $\gamma$ 2 promoter in differentiating 3T3-L1 cells suggests that SWI/SNF facilitates the interaction of TFIIF with the rest of the pre-initiation complex. Such a role for SWI/SNF enzymes has not previously been documented. However, the data presented here agree with and extend findings from temporal analyses of other mammalian promoters that show that (1) some form of histone hyperacetylation precedes association of SWI/SNF with the promoter, and (2) SWI/SNF enzymes work late in the activation of many genes, typically after some, if not most, of the components driving transcription have associated with the promoter (Agalioti et al., 2000; Lomvardas and Thanos, 2001; Martens et al., 2003; Soutoglou and Talianidis, 2002). Thus the data we present on PPAR $\gamma$ 2 activation and the preliminary results on aP2 and adipsin expression during adipocyte differentiation support a general model where SWI/SNF enzymes function subsequent to activator binding by completing or stabilizing pre-initiation complex formation and/or by promoting promoter clearance and elongation.

# CHAPTER III

The work presented in this chapter was published in Journal of Molecular Endocrinology  
(2006) volume 36: 139-151. Data presented in figure III.1 was generated by Hengyi  
Xiao, Ph.D.

## CHAPTER III

### TEMPORAL RECRUITMENT OF CCAAT/ENHANCER-BINDING PROTEINS TO EARLY AND LATE ADIPOGENIC PROMOTERS *IN VIVO*

#### Abstract

The C/EBP family of transcriptional regulators is critically important for the activation of adipogenic genes during differentiation. The C/EBP $\beta$  and  $\delta$  isoforms are rapidly induced upon adipocyte differentiation and are responsible for activating the adipogenic regulators C/EBP $\alpha$  and PPAR $\gamma$ 2, which together activate the majority of genes expressed in differentiating adipocytes. However, mitosis is required following the induction of adipogenesis, and the activation of C/EBP $\alpha$  and PPAR $\gamma$ 2 gene expression is delayed until cell division is underway. Previous studies have used electromobility shift assays (EMSAs) to suggest that this delay is due at least in part to a delay between the induction of C/EBP $\beta$  protein levels and the acquisition of DNA binding capacity by C/EBP $\beta$ . Here we used *in vivo* chromatin immunoprecipitation (ChIP) analysis of the C/EBP $\alpha$ , PPAR $\gamma$ 2, resistin, adiponectin, and leptin promoters to examine the kinetics of C/EBP protein binding to adipogenic genes in differentiating cells. In contrast to prior studies, we determined that C/EBP $\beta$  and  $\delta$  were bound to endogenous regulatory sequences controlling the expression of these genes within 1-4 hours of adipogenic induction. These results indicate that C/EBP $\beta$  and  $\delta$  bind not only to genes that are induced early in the adipogenic process but also to genes that are induced much later during differentiation, without a delay between induction of C/EBP protein levels and

DNA binding by these proteins. We also showed that each of the genes examined undergoes a transition *in vivo* from early occupancy by C/EBP $\beta$  and  $\delta$  to occupancy by C/EBP $\alpha$  at times that correlate with the induction of C/EBP $\alpha$  protein levels, demonstrating the generality of the transition during adipogenesis and indicating the binding of specific C/EBP isoforms does not correlate with timing of expression from each gene. We conclude that C/EBP family members bind to adipogenic genes *in vivo* in a manner that follows the induction of C/EBP protein synthesis.

## Introduction

The major transcription factor families involved as key regulators of adipocyte differentiation include the nuclear hormone receptor PPAR $\gamma$  and the CCAAT/enhancer-binding proteins (C/EBPs) (Camp et al., 2002; Darlington et al., 1998; Fajas et al., 1998; Lane et al., 1999; MacDougald and Mandrup, 2002; Morrison and Farmer, 2000; Rangwala and Lazar, 2000; Rosen et al., 2000). The C/EBP family members belong to the basic-leucine zipper (bZIP) class of transcription factors, and bind to specific DNA sequences as dimers with other C/EBPs [reviewed in (Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002)]. When adipocyte differentiation is induced in preadipocyte cell lines, C/EBP $\beta$  and  $\delta$  are rapidly and transiently induced (Cao et al., 1991; Yeh et al., 1995). These regulators act synergistically to modulate the expression of C/EBP $\alpha$  and PPAR $\gamma$ 2 via interaction with the C/EBP regulatory elements present in the proximal promoters of these genes (Christy et al., 1991; Clarke et al., 1997; Tang et al., 1999; Zhu et al., 1995). Subsequently, C/EBP $\alpha$  and PPAR $\gamma$ 2 play a prominent role regulating the expression of adipocyte genes necessary for the development of functional, mature adipocytes (Fajas et al., 1998; Lin and Lane, 1994; Rosen et al., 2000; Tontonoz et al., 1994c; Wu et al., 1999).

The essential role of the C/EBP proteins in adipocyte differentiation has been established. Ectopic expression of C/EBP $\beta$  or C/EBP $\alpha$  is able to force non-adipogenic cell lines to differentiate into adipocytes (Freytag et al., 1994; Wu et al., 1995b; Yeh et al., 1995). In contrast, expression of antisense C/EBP $\alpha$  RNA in preadipocyte cell lines prevents the differentiation program (Lin and Lane, 1992). Additionally, analysis of promoter regions of adipogenic genes as well as studies of knockout mice have demonstrated the involvement of this family of transcription factors in regulating

adipogenesis and other important physiological processes [reviewed in (Cornelius et al., 1994; Darlington et al., 1998; Gregoire et al., 1998; Lane et al., 1999; MacDougald and Lane, 1995b; Ramji and Foka, 2002; Rangwala and Lazar, 2000; Tanaka et al., 1997)].

Most regulatory sequences controlling the expression of adipocyte-specific genes contain at least one functional C/EBP binding site, from which transactivation is mediated by members of the C/EBP family [reviewed in (Cowherd et al., 1999; Gregoire et al., 1998; Hwang et al., 1997; Morrison and Farmer, 2000; Rangwala and Lazar, 2000)]. Work on the PPAR $\gamma$ 2 and C/EBP $\alpha$  promoters has focused on the role of C/EBP $\beta$  and  $\delta$  as primary inducers of the expression of key regulators. PPAR $\gamma$  are expressed by day 2 of the differentiation process, following one or two rounds of mitotic clonal expansion. The induction of C/EBP $\beta$  and  $\delta$  protein levels, however, occurs almost immediately after addition of differentiation inducers at the onset of differentiation (Cao et al., 1991; Darlington et al., 1998; Tang and Lane, 1999; Yeh et al., 1995). Thus, even though both C/EBP $\beta$  and  $\delta$  are expressed at high levels at the beginning of the differentiation program, the target genes C/EBP $\alpha$  and PPAR $\gamma$ 2 are not expressed until nearly two days later (Lane et al., 1999; Rosen et al., 2000). Previous work using electrophoretic mobility shift assay (EMSA) analysis (Tang and Lane, 1999) determined that the lag in C/EBP $\alpha$  expression is due to a delay in acquisition of C/EBP $\beta$  and  $\delta$  DNA binding activity, therefore pausing the transcriptional activation of the gene. The need for such a delay fits well with the numerous observations that C/EBP $\alpha$  is anti-mitotic in both pre-adipocytes as well other cell types (Lin et al., 1993; Timchenko et al., 1996; Umek et al., 1991; Wang et al., 2001).

The transcriptional activity of the C/EBP $\beta$  protein is regulated at several levels, including transcription, translation, association with other proteins, and posttranslational

modification, which includes the regulation of the phosphorylated state by multiple effectors. Multiple phosphorylation sites have been characterized on C/EBP $\beta$ , some of which result in attenuation or enhancement of DNA binding and transactivation activity [(Mahoney et al., 1992; Park et al., 2004a; Tang et al., 2005; Trautwein et al., 1993; Trautwein et al., 1994) and references therein]. At least some of these phosphorylation events occur almost simultaneously with induction of C/EBP $\beta$  levels, and it has been suggested that phosphorylation causes a conformational change in C/EBP from a repressor to an activator (Kowenz-Leutz et al., 1994).

We previously examined the temporal interactions of C/EBP family members, modified histones, and subunits of the SWI/SNF family of ATP dependent chromatin remodeling enzymes at the PPAR $\gamma$ 2 promoter as a function of adipocyte differentiation. In that study, we used chromatin immunoprecipitation assays to determine that C/EBP $\beta$  was bound at the PPAR $\gamma$ 2 promoter at 24 and 48 hours following the initiation of the differentiation program in both differentiating 3T3-L1 cells and in fibroblasts forced to differentiate into adipocytes by ectopic expression of C/EBP $\alpha$  (Salma et al., 2004). Continuation of these ChIP studies at earlier time points revealed that the C/EBP $\beta$  and  $\delta$  isoforms were found on the regulatory regions of numerous adipocyte specific genes in differentiating 3T3-L1 cells within a few hours of the onset of differentiation. These genes included PPAR $\gamma$ 2, C/EBP $\alpha$ , and several genes expressed later in the differentiation process. Thus the binding of C/EBP $\beta$  and  $\delta$  to adipogenic genes in cells correlates with the kinetics of C/EBP $\beta$  and  $\delta$  expression. In addition, binding of C/EBP $\beta$  and  $\delta$  was replaced at each of the loci by the binding of C/EBP $\alpha$ . C/EBP $\alpha$  binding was noted on each promoter between 20 and 48 hours post-differentiation, indicating that it was

associating with promoters as soon as it was expressed and that binding did not strictly correlate with the time at which the locus became transcriptionally active. We have concluded that the binding of C/EBP transcription factors to regulatory sequences controlling the expression of adipogenic genes in vivo occurs rapidly and without significant delay following the induction of each isoform during adipogenic differentiation.



## Materials and methods

**Cell lines and differentiation methods.** 3T3-L1 preadipocytes were purchased from the American Type Culture Collection (ATCC), maintained in growth medium consisting of Dulbecco's minimum essential medium containing 10% calf serum, and induced to differentiate as described previously (Wu et al., 1995b). Cells were collected at 0, 1, 2, 4, 8, 12, 16, 20, and 24 hours and then at 24 hour intervals for 7 days after addition of differentiation cocktail (Salma et al., 2004) for Western blot, RT-PCR and ChIP analysis. In experiments where 3T3-L1 cells overexpressed C/EBP $\beta$ , cells were infected with pBABE retrovirus containing C/EBP $\beta$  or empty vector as described previously (Salma et al., 2004; Tontonoz et al., 1994c). Generation of retrovirus was previously described (Pear et al., 1993; Salma et al., 2004). Samples were collected at 0, 4, 24, 48 and 168 hours in the presence or absence of differentiation cocktail for Western blots, RT-PCR and ChIP analysis.

**Electrophoretic Mobility Shift Assays (EMSA).** Nuclear extracts isolated from 3T3-L1 cells differentiated in the presence or absence of cocktail were prepared as described (Hasegawa et al., 1997). The binding reaction contained 6  $\mu$ g of nuclear extract and 5 fmol of  $^{32}$ P-labeled double stranded oligonucleotide probe corresponding to the region from -343 to -306 bp from the mRNA start site in the mouse PPAR $\gamma$ 2 promoter. This region contains a functional C/EBP binding site (Clarke et al., 1997; Zhu et al., 1995). Binding reactions contained 10 mM Tris-HCl (pH 7.5), 5% glycerol, 50 mM NaCl, 0.5 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, and 0.5 mg/ml of Poly (dl-dC) Poly (dl-dC) in a volume of 10  $\mu$ l. Reactions were incubated for 30 minutes at room temperature and separated electrophoretically on 4% non-denaturing polyacrylamide gels made with 0.5 x Tris-

Borate-EDTA buffer. Some reactions were preincubated for 10 minutes with 1  $\mu$ l of IgG or anti-C/EBP $\beta$  antibody (Santa Cruz, sc-7962) prior to addition of the labeled oligonucleotides. The sequence of the oligonucleotide probe was: 5'-TAAAAAGCAATCAATATTGAACAATCTCTGCTCTGGTAA-3'.

**RNA analysis.** RNA isolation and analysis by Northern blotting was described previously (de la Serna et al., 2001c). Probes were derived from plasmids containing PPAR $\gamma$  (provided by B. Spiegelman) and 36B4 (obtained by RT-PCR) and labeled by random priming. Washed blots were exposed to a PhosphorImager (Molecular Dynamics). For reverse transcriptase (RT)-PCR, total RNA (3  $\mu$ g) was reverse transcribed with Moloney murine leukemia virus RT (Invitrogen). cDNA was amplified by PCR with QIAGEN HotStart Taq master mix in the presence of 2  $\mu$ Ci of [ $\alpha^{32}$ P]dATP. The sequences of the primers were as follows: 5'-CCG GCC GCC TTC AAC GAC-3' and 5'-CTC CTC GCG GGG CTC TTG TTT-3' for C/EBP $\alpha$  (288 bp product); 5'-GAA CTG AGT TGT GTC CTG CT-3' and 5'-TGC ACA CTG GCA GTG ACA-3' for resistin (340 bp product); 5'-GAT CAA TGA CAT TTC ACA CA-3' and 5'-GGA CGC CAT CCA GGC TCT CT-3' for leptin (281bp product); 5'-CAG TGG ATC TGA CGA CAC CA-3' and 5'-CGA ATG GGT ACA TTG GGA AC-3' for adiponectin (433bp product) and 5'-CTC CAA GCA GAT GCA GCA GA-3' and 5'-TCA ATG GTG CCT CTG GAG AT-3' for the ribosomal phosphoprotein 36B4 (351 bp). The PCR conditions for leptin and 36B4 were: 95°C, 15 minutes, followed by 24 cycles of: 95°C, 30s; 62°C, 40s; 72°C, 30 s and a final round of extension for 5 minutes. The PCR conditions for adiponectin were the same, except that the number of cycles was 20. For resistin, the conditions were the same as for leptin except that the annealing temperature was 58°C. For C/EBP $\alpha$  the PCR

conditions were: 95°C, 15 minutes, followed by 25 cycles of: 95°C, 50 s; 66°C, 55 s; 72°C, 50 s and a final round of extension for 5 minutes.

**Protein extracts and Western analysis.** Isolation of protein and Western blotting have been described (de la Serna et al., 2001a). Antibodies utilized included the following from Santa Cruz: C/EBP $\alpha$  (sc-61), C/EBP $\beta$  (sc-7962), C/EBP $\delta$  (sc-151) and Cyclin A (sc-596). Phosphatidylinositol (PI) 3-kinase antibody (06-496) was obtained from Upstate.

**Chromatin Immunoprecipitation.** The ChIP procedure was adopted from the Upstate protocol and was performed as described in (Salma et al., 2004). One-tenth of the immunoprecipitated DNA and 1% of the input DNA were analyzed by PCR. Antibodies used included Santa Cruz antibodies: C/EBP $\alpha$  (sc-61), C/EBP $\beta$  (sc-7962), and C/EBP $\delta$  (sc-151). PCRs were performed with QIAGEN Hot Start Taq master mix in the presence of 2  $\mu$ Ci of [ $\alpha$ -32P] dATP under the following conditions: a pre-heating at 94°C for 15 minutes, followed by 24-30 cycles of 94°C for 30 s, 62°C for 40 s (except for PPAR $\gamma$ 2 which was 49.5°C), 72°C for 30 s, followed by a 72°C extension for 5 minutes. PCR products were resolved in 6-8% polyacrylamide-1X Tris-borate-EDTA gels, dried, and exposed to a PhosphorImager. Primers used were the following:  $\beta$ -actin, 5'(+31) GCTTCTTTGCAGCTCCTTCGTTG-3' and 5'(+135)TTTGACATGCCGGAGCCGTTGT-3' (Rayman et al., 2002); PPAR $\gamma$ 2 promoter, 5'(-413) TACGTTTATCGGTGTTTCAT-3' and 5'(-247) TCTCGCCAGTGACCC-3'; upstream region of PPAR $\gamma$ 2 promoter 5'(-1871) GGGCGTTAAACACAATCCT-3' and 5' (-1707) TCTCTTCCTCCTTCCCTTCC-3'; C/EBP $\alpha$  promoter, 5' (-315) TGACTTAGAGGCTTAAAGGA-3' and 5' (-32)

CGGGGACCGCTTTTATAGAG-3'; resistin promoter, 5'(-177)

CACCATGGTCCCTGGTGTTA-3' and 5' (+26) CTCAGTTCTGGGTATTAGCTC-3';

adiponectin promoter, 5'(-272) ATTGTCCTTACCCTTGCCCC-3' and 5'(-15) and, leptin

promoter 5'(-323) GCCTTCTGTAGCCTCTTGCT-3' and 5'(-22)

GCTCCATGCCCTGCCTGC-3'. Representative experiments from at least three

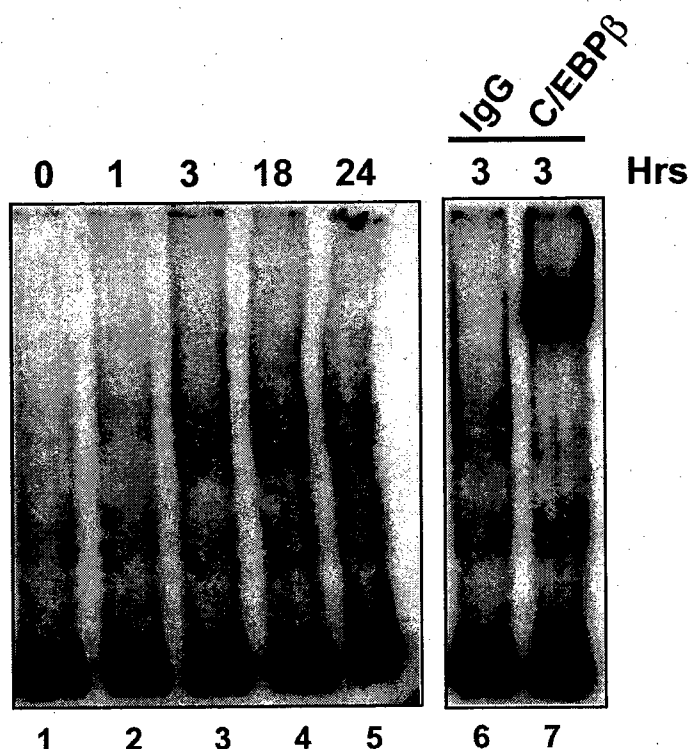
independent experiments are shown.

## Results

### ***In vitro* binding of C/EBP $\beta$ at a C/EBP site in the PPAR $\gamma$ 2 promoter occurs as early as 3 hours post-differentiation.**

While investigating the role of C/EBP isoforms in the activation of adipogenic genes, we examined *in vitro* binding of C/EBP $\beta$  at a C/EBP regulatory element present in the PPAR $\gamma$ 2 promoter by EMSA during a short time course of 3T3-L1 pre-adipocytes induced to differentiate into adipocytes. We found that C/EBP $\beta$  present in nuclear extracts prepared from cells differentiated for 3, 18, and 24 hours was able to bind to a C/EBP site in the PPAR $\gamma$ 2 promoter (Fig. III.1, lanes 3-5). Confirmation that the shifted band in the EMSA was C/EBP $\beta$  was demonstrated by the appearance of a supershifted band upon addition of C/EBP $\beta$  antibody to the reaction while addition of purified IgG had no effect (Fig. III.1, lanes 6 and 7). The results reveal that the C/EBP $\beta$  regulator has the capacity to bind to DNA as early as 3 hours following the induction of differentiation. In addition, we observed that the apparent level of C/EBP $\beta$  binding under the reaction conditions used did not increase between 3 and 24 hours post-differentiation (Fig. III.1, lanes 3-5).

These results diverge somewhat from data published previously where the *in vitro* binding activity of C/EBP $\beta$  was delayed until 12 to 16 hours following the stimulation of adipogenic differentiation (Tang and Lane, 1999). We note that the previous report demonstrated that C/EBP $\beta$  binding could be observed at 4 hours post-differentiation but that binding was significantly induced at the 12-16 hour time points. Possibly, this dissimilarity in results is due to differences in reaction conditions, or perhaps due to the



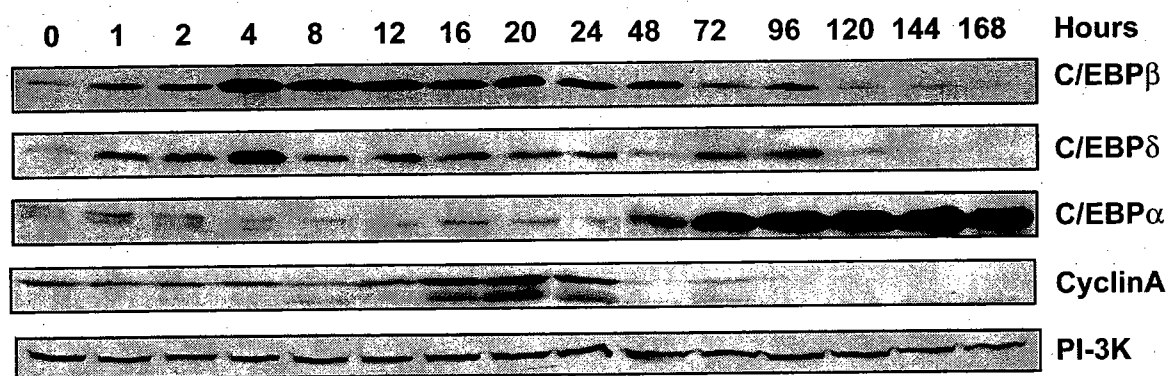
**Figure III.1.** *In vitro* binding of C/EBP $\beta$  to a C/EBP site in the PPAR $\gamma$ 2 promoter occurs as early as 3 h following the induction of 3T3-L1 adipocyte differentiation. EMSA was performed using nuclear extracts prepared from preadipocytes (day 0) or from differentiating preadipocytes at 1, 3, 18, and 24 h after the addition of the differentiation cocktail (lanes 1–5). The double-stranded,  $^{32}$ P end-labeled oligonucleotide probe encoded the C/EBP binding site between –343 and –306 relative to the start site of PPAR $\gamma$ 2 transcription. Supershift experiments were performed by adding purified IgG from pre-immune serum (lane 6) or antibodies against C/EBP $\beta$  (lane 7).

use of different oligonucleotide probes. In the previous study, EMSA was performed using a C/EBP site from the C/EBP $\alpha$  promoter, while our experiment utilized a probe that contained a C/EBP binding site from the PPAR $\gamma$ 2 promoter. Nevertheless, the results presented in Figure III.1 raised a question about the timing of C/EBP $\beta$  binding to adipogenic regulatory sequences. To better address this issue, we decided to perform ChIP experiments at different times following the induction of adipocyte differentiation in 3T3-L1 cells in order to examine the binding of C/EBP isoforms *in vivo*.

#### **Kinetics of C/EBPs expression in differentiating 3T3-L1**

Because the EMSA data presented in Figure III.1 indicated a potential difference with previously published studies, we first performed a series of control experiments to analyze the expression levels of C/EBP $\beta$ ,  $\delta$  and  $\alpha$  during adipocyte differentiation of 3T3-L1 cells by Western blot in order to eliminate the possibility that our results might be due to differences in the experimental handling of the differentiating 3T3-L1 cells. It has been well established that initiating differentiation of 3T3-L1 preadipocytes activates a cascade of gene expression events. Among the initial events are the rapid induction of C/EBP $\beta$  and  $\delta$ , which are stimulated by components of the differentiation cocktail, followed by the induction of C/EBP $\alpha$  on the second day of differentiation (Wu et al., 1996; Yeh et al., 1995). Western blot analyses corroborate that induced protein levels of C/EBP $\beta$  and  $\delta$  were detectable at 1 hour, reached a maximum at 4 hour, and were declining at 48 and 120 hours, respectively (Fig. III.2). Expression of C/EBP $\alpha$  occurred later; significant induction began at about 48 hours post-differentiation and expression was maintained throughout the time course (Fig. III.2).

Fig. 2



**Figure III.2.** Western blot analysis of transcriptional and cell cycle regulators during differentiation of 3T3-L1 cells. Protein extracts were prepared from differentiating cells at the indicated times and the kinetics of protein expression for the indicated proteins were determined by western blot analysis. PI-3K was used as a loading control.



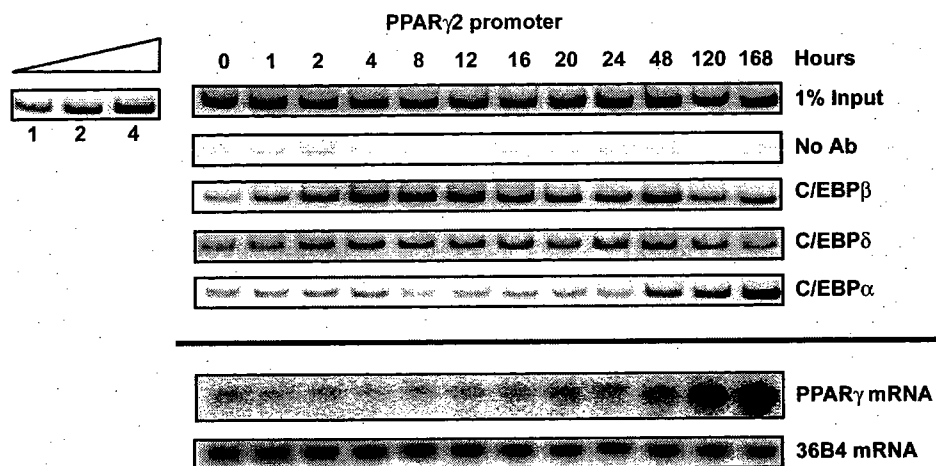
To corroborate that the cells began the mitotic expansion phase, we performed a western blot with antibodies directed to cyclin A (Fig. III.2). As was expected, cyclin A levels were increased between 16-24 hours post-differentiation, indicating that the cells had entered the cell cycle at 16 hour and had exited by approximately 48 hour. All of the data presented in Figure III.2 indicate that the differentiation of the 3T3-L1 cells occurred as expected and confirm previously published results (Morrison and Farmer, 1999; Tang et al., 2003a; Tang et al., 2003b). Thus we have demonstrated the integrity of the 3T3-L1 cells and the differentiation protocol used for this and subsequent experiments.

***In vivo* recruitment of C/EBP $\beta$ ,  $\delta$  and  $\alpha$  to the PPAR $\gamma$ 2 and C/EBP $\alpha$  promoters during adipogenesis**

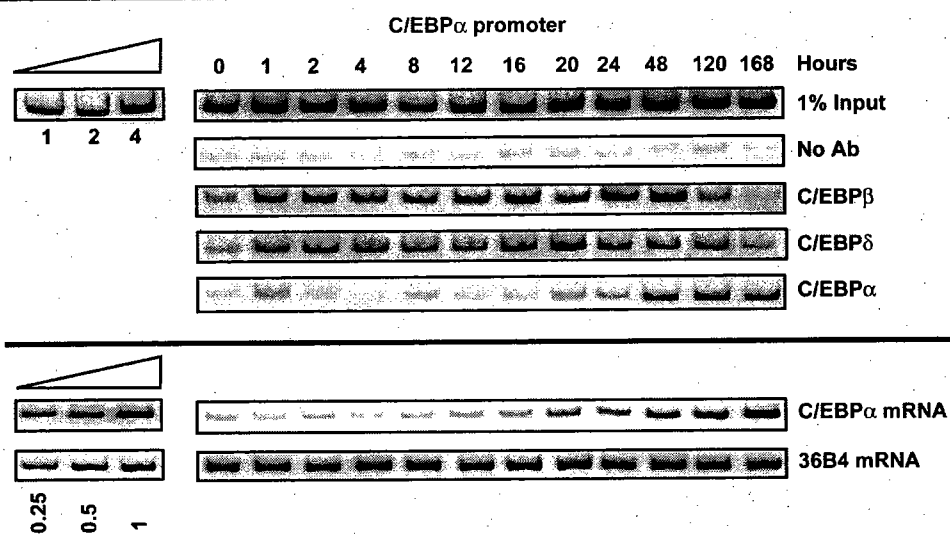
The results presented in Fig. III.1 show that C/EBP $\beta$  was able to bind to one of the C/EBP binding sites on the PPAR $\gamma$ 2 promoter at times that correlated with the induction of C/EBP $\beta$  levels. To determine whether binding occurs on endogenous adipocyte promoters at early times after the induction of adipocyte differentiation, we performed ChIP experiments and temporally analyzed binding, not only of C/EBP $\beta$ , but also of C/EBP $\delta$  and C/EBP $\alpha$ , to specific adipocyte promoters. First, we chose to evaluate binding at the C/EBP $\alpha$  and the PPAR $\gamma$ 2 promoters, since these two essential adipogenic regulators are expressed early during differentiation, on day 2 (Figs. III.3A and B).

Regulation of the C/EBP $\alpha$  and the PPAR $\gamma$ 2 promoters by C/EBP family members has been previously characterized (Christy et al., 1991; Clarke et al., 1997; Elberg et al., 2000; Tang et al., 1999; Tang and Lane, 1999; Tang et al., 2004; Yang and Chow, 2003; Zhu et al., 1995). The C/EBP $\alpha$  proximal promoter contains a C/EBP regulatory element

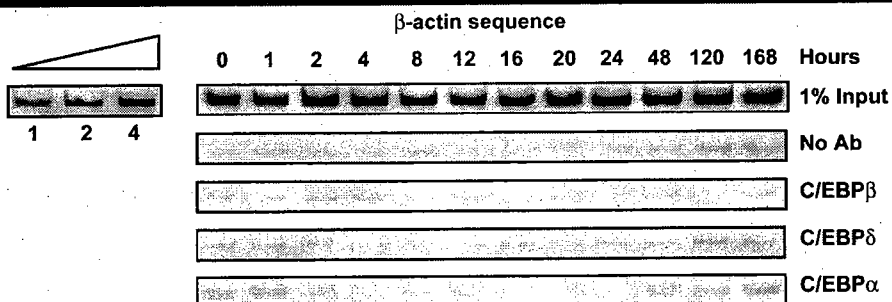
3A



3B



3C



**Figure III.3.** ChIP assays reveal that C/EBP $\beta$  binding to the PPAR $\gamma$ 2 and C/EBP promoters occurs *in vivo* within 2 h of the induction of 3T3-L1 adipocyte differentiation. ChIP assays were performed at the indicated time-points using the indicated antibodies and amplified for the PPAR $\gamma$ 2 promoter (A, top panel), the C/EBP $\alpha$  promoter (B, top panel), or the  $\beta$ -actin 5' untranslated region and coding region (C); 1% of each input is shown. A twofold titration (number of  $\mu$ l indicated) of input from the 0 h sample is shown on the left to demonstrate linearity of the PCR reactions. (A, bottom panel) Duplicate samples were used to prepare RNA and a Northern blot showing the levels of PPAR $\gamma$  and 36B4 mRNA at each time-point is shown. (B, bottom panel) C/EBP $\alpha$  and 36B4 mRNA levels were determined by RT-PCR. A twofold titration (number of  $\mu$ l indicated) of input reverse transcribed RNA from the day 7 sample is shown on the left to demonstrate linearity of the PCR reactions. Ab, antibody.

at -187 relative to the transcriptional start site that mediates transactivation by C/EBPs. The PPAR $\gamma$ 2 promoter contains two previously characterized C/EBP recognition elements at -340 bp and -327 bp relative to the transcriptional start site in addition to other potential sites that diverge from the C/EBP consensus. As shown in Fig. III.3A, recruitment of C/EBP $\beta$  as well as C/EBP $\delta$  at the C/EBP regulatory element on the PPAR $\gamma$ 2 promoter was induced in a manner consistent with the protein expression patterns of both proteins (Fig. III.2), with binding of both proteins apparent 2 hours after the onset of differentiation. Subsequently, C/EBP $\beta$  and C/EBP $\delta$  were replaced by C/EBP $\alpha$ , which initiated binding to the promoter at 48 hours and was maintained throughout the time course. These results confirm the transition of binding from C/EBP $\beta/\delta$  to C/EBP $\alpha$  at this promoter *in vivo* (Salma et al., 2004; Tang et al., 2004).

Analysis of the C/EBP $\alpha$  promoter revealed the same pattern found at the PPAR $\gamma$ 2 promoter, except that binding occurred even earlier following differentiation (Fig. III.3B). C/EBP $\beta$  and C/EBP $\delta$  were bound as early as 1 hour post-differentiation and remained present until 48 hour and 120 hours respectively. Induction of C/EBP $\alpha$  binding to the C/EBP $\alpha$  promoter began at 48 hrs as reported previously (Tang et al., 2004). Therefore, the transition in binding of the C/EBP isoforms that was observed at the PPAR $\gamma$ 2 promoter also occurred at the C/EBP $\alpha$  promoter.

The appearance and disappearance of binding of the different C/EBP isoforms at the PPAR $\gamma$ 2 and C/EBP $\alpha$  promoters at different times post-differentiation indicate specificity of binding. No antibody controls provide further evidence for specific binding (Figs. III.3A and B). As an additional control, we analyzed C/EBP factor interactions at the  $\beta$ -actin locus (Fig. III.3C) and at sequences 1.8 kb upstream of the PPAR $\gamma$ 2 start site

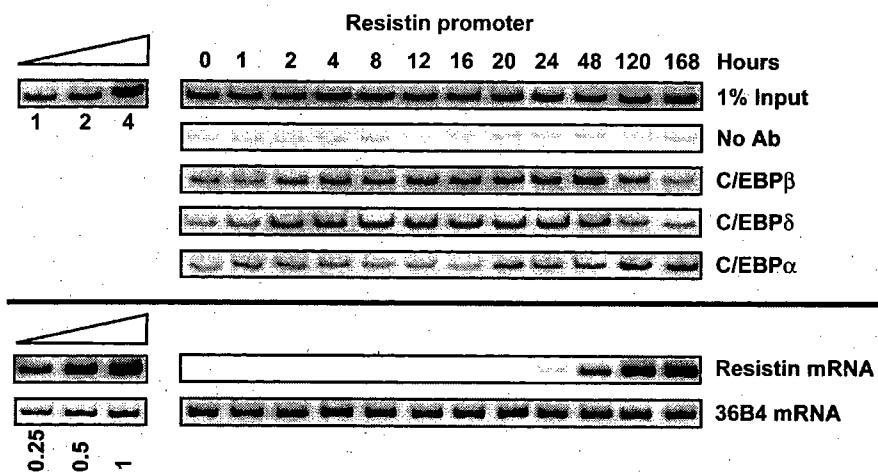
(data not shown). Neither  $\beta$ -actin sequences nor sequences upstream of the PPAR $\gamma$ 2 promoter were immunoprecipitated by any of the antibodies used in the ChIP procedure.

***In vivo* recruitment of C/EBP $\beta$ ,  $\delta$  and  $\alpha$  to additional adipogenic gene promoters during adipogenesis**

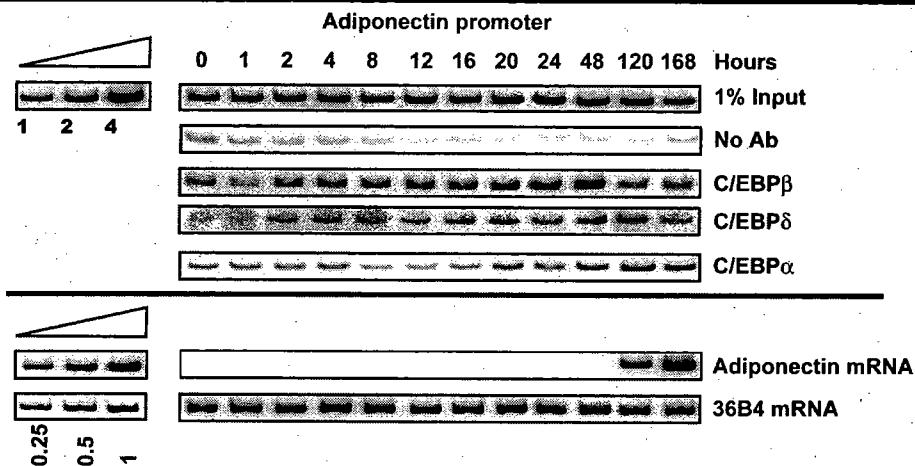
We next examined if C/EBP $\beta$ ,  $\delta$  and  $\alpha$  are recruited to the resistin, adiponectin and leptin promoters. These adipocyte secreted peptides, collectively referred to as adipocytokines, have generated considerable interest since they are important regulators of body mass and their misregulation may play a role in obesity (Miner, 2004). The proximal promoters of these genes contain C/EBP binding sites that are necessary for expression. The resistin promoter contains a C/EBP site at -56 relative to the transcriptional start site, adiponectin contains two identified C/EBP sites at -775 and -264, and two potential binding sites at -117 and -73, and leptin has three consensus C/EBP binding sites at nucleotides -55, -211, and -292. The activity of these C/EBP sites has been confirmed by reporter assays for each of these genes (de la Brousse et al., 1996; Hartman et al., 2002; Hwang et al., 1996; Park et al., 2004b).

Binding of C/EBP $\beta$  and  $\delta$  to the C/EBP site at the resistin proximal promoter was evident at 2-4 hours post-differentiation and did not decline until after 48 hours (Fig. III.4A). A modest increase in binding of C/EBP $\alpha$  was observed from 20 to 48 hour, and was further increased at 120 hour (Fig. III.4A). These results indicate that C/EBP $\beta$  and  $\delta$  bind early after differentiation to the resistin promoter and suggest that all three C/EBP isoforms play a role regulating this adipocyte gene. It was previously demonstrated that C/EBP $\alpha$  binds specifically to the C/EBP element on the resistin promoter that is essential

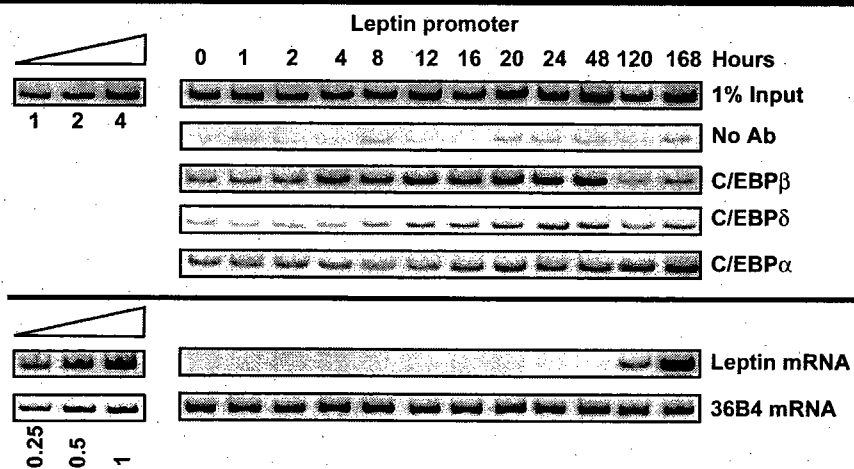
4A



4B



4C



**Figure III.4.** ChIP assays show that C/EBP $\beta$  binding to the adipocytokine promoters occurs *in vivo* within 4 h of the induction of 3T3-L1 adipocyte differentiation. ChIP assays were performed at the indicated time-points using the indicated antibodies and amplified for the resistin promoter (A, top panel), the adiponectin promoter (B, top panel), or the leptin promoter (C, bottom panel); 1% of each input is shown. A twofold titration (number of  $\mu$ l indicated) of input from the day 0 sample is shown on the left to demonstrate linearity of the PCRs. Duplicate samples were used to prepare RNA at each time-point and mRNA levels for resistin (A, bottom panel), adiponectin (B, bottom panel), and leptin (C, bottom panel) as determined by RT-PCR are shown. 36B4 mRNA levels are shown as a control. A twofold titration (number of  $\mu$ l indicated) of input reverse transcribed RNA from the day 7 sample is shown on the left to demonstrate linearity of the PCRs. Ab, antibody.

for expression (Hartman et al., 2002), however, a transition in binding of these regulators has not been previously demonstrated.

The adiponectin promoter contains two C/EBP sites at -775 and -264 and two potential binding sites at -117 and -73, however, the C/EBP element at -264 and the potential C/EBP sites at -117 and -73 confer promoter activity as defined in transient promoter studies, EMSA, DNase I footprinting, and ChIP assays (Park et al., 2004b; Seo et al., 2004). Consequently, we performed ChIPs in the region of the proximal promoter that contains these C/EBP sites. The analysis of binding of C/EBP $\beta$ , C/EBP $\delta$  and C/EBP $\alpha$  to the adiponectin promoter showed a pattern nearly identical to that observed for the resistin promoter, despite the fact that adiponectin expression initiated later than resistin expression (Fig. III.4B). C/EBP $\beta$  and  $\delta$  were bound to the promoter at 2 hours and maintained until 48 hours. Definitive binding of C/EBP $\alpha$  was present at 48 hours, although a modest increase was noticed at 20-24 hours.

Finally, we assessed the recruitment of the C/EBP members on the leptin promoter. The proximal promoter contains three consensus C/EBP binding sites. DNase I footprint analysis, reporter gene assays, and EMSA studies have demonstrated that one of these C/EBP sites, located at -53 relative to the transcriptional start site, is functional (de la Brousse et al., 1996; Hwang et al., 1996; Mason et al., 1998). However, because the C/EBP sites are near each other, we designed PCR primers to amplify a region of the proximal promoter containing all three sites. The recruitment of C/EBP $\beta$  occurred at 4 hours and remained relatively constant until 48 hours (Fig. III.4C). In contrast, C/EBP $\delta$  was detectable from 12 hours to 48 hours. Binding of C/EBP $\alpha$  was observed on the promoter at 20 hours, but was not robust until 120 hours post-differentiation, which coincides with the start of leptin mRNA accumulation. These results

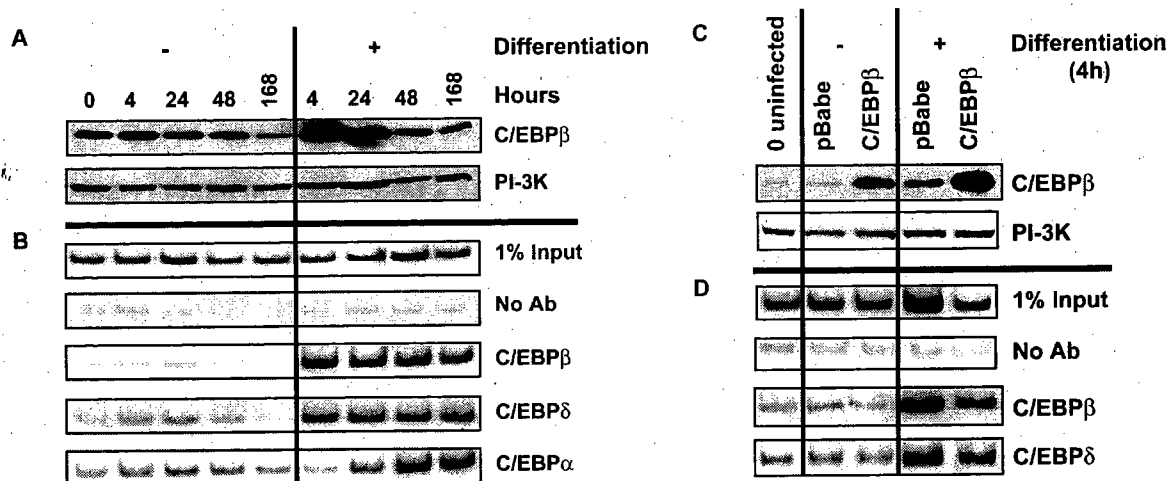


indicate that C/EBP $\beta$  and  $\delta$  bind quickly after the induction of differentiation to adipogenic promoters that are not expressed until much later in the differentiation process. Furthermore, the transition from C/EBP $\beta$  and  $\delta$  to C/EBP $\alpha$  binding on these promoters also occurred prior to gene expression, suggesting that C/EBP factor interactions with adipogenic promoters is independent of the time at which gene expression is initiated.

**Overexpression of C/EBP $\beta$  is not sufficient to promote C/EBP protein binding to the PPAR $\gamma$ 2 promoter in differentiating 3T3-L1 cells**

The detection of C/EBP $\beta$  on adipogenic promoters *in vivo* within a few hours of the onset of adipogenic stimulation caused us to evaluate whether over-expression of C/EBP $\beta$  in 3T3-L1 pre-adipocytes would be sufficient to induce binding of C/EBP $\beta$  to the regulatory sequences examined. 3T3-L1 pre-adipocytes were infected with a retroviral vector expressing C/EBP $\beta$  and were allowed to reach confluence, but no differentiation cocktail was added. Instead, cells were maintained in 10% calf serum as a confluent plate, and C/EBP binding was assessed at 0, 4, 24, 48 and 168 hours. Despite the ectopic expression of C/EBP $\beta$  (Fig. III.5A), no binding of C/EBP $\beta$ ,  $\delta$ , or  $\alpha$  was observed at the PPAR $\gamma$ 2 promoter, whereas control plates treated with differentiation cocktail showed C/EBP $\beta$  and  $\delta$  binding to the PPAR $\gamma$ 2 promoter within 4 hours post-differentiation and C/EBP $\alpha$  binding by 48 hours post-differentiation (Fig. III.5B).

The data suggest that overexpression of C/EBP $\beta$  in cells is not sufficient to promote C/EBP $\beta$  binding to adipogenic promoters in the absence of differentiation cocktail. A potential caveat to this conclusion is that ectopic expression did not provide a



**Figure III.5.** Overexpression of C/EBP $\beta$  in undifferentiated 3T3-L1 preadipocytes is not sufficient to induce the binding of C/EBP factors at the PPAR $\gamma$ 2 promoter. Subconfluent 3T3-L1 preadipocytes were infected with either pBabe retrovirus or pBabe retrovirus encoding C/EBP $\beta$ . For differentiation, cells were placed in fresh media lacking or containing the differentiation cocktail, and samples were harvested at the indicated times for (A and C) Western blot analysis or (B and D) ChIP analysis. Ab, antibody.

high enough level of C/EBP $\beta$  protein to surpass a threshold level of C/EBP $\beta$  protein necessary to achieve binding. We note that the inoculum of pBABE-C/EBP $\beta$  retrovirus used in these experiments is the same as we have previously used to trans-differentiate fibroblast lines into adipocyte-like cells; thus the levels of C/EBP $\beta$  provided are sufficient to reprogram cells of a different lineage (Salma et al., 2004). However, to more directly address this concern, we compared the levels of C/EBP $\beta$  protein present in uninfected cells and in cells infected with pBABE-C/EBP $\beta$  or with the pBABE empty retrovirus that were differentiated in the presence or absence of differentiation cocktail for 4 hours. A Western blot (Fig. III.5C) demonstrates that cells infected with the C/EBP $\beta$  virus contained greater levels of C/EBP $\beta$  protein in both the presence and absence of differentiation cocktail (compare lane 2 to lane 3 and lane 5 to lane 6), as expected. We also observed that C/EBP $\beta$  levels in vector infected cells that were differentiated in the presence of cocktail are lower than C/EBP $\beta$  levels in the pBABE-C/EBP $\beta$  infected cells that were differentiated in the absence of cocktail (compare lanes 4 and 3). The levels of C/EBP $\beta$  protein in pBABE infected cells treated with differentiation cocktail were sufficient to permit C/EBP $\beta$  binding to the PPAR $\gamma$ 2 promoter, whereas higher levels of C/EBP $\beta$  in the C/EBP $\beta$  infected cells differentiated in the absence of cocktail were not (Fig. III.5D, compare lanes 4 and 3). The results exclude the possibility that insufficient levels of C/EBP $\beta$  were present in the cells not treated with differentiation cocktail. The C/EBP $\beta$  protein undergoes a number of post-translational modifications that are associated with the induction of adipogenesis; it is likely that such modifications are induced by addition of the differentiation cocktail and are necessary to promote rapid

binding to adipogenic gene regulatory sequences *in vivo*. Thus simple overexpression of C/EBP $\beta$  is not sufficient to induce C/EBP $\beta$  binding in 3T3-L1 pre-adipocytes.

## Discussion

The C/EBP family of transcription factors is widely expressed and is a key regulator of a variety of target genes important in physiological events, including energy metabolism, inflammation, hematopoiesis, cellular proliferation, and differentiation (Darlington et al., 1998; Kovacs et al., 2003; Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foka, 2002; Rosen et al., 2000). Of note is the essential role C/EBP family members play during adipogenesis [reviewed in (Darlington et al., 1998; Lane et al., 1999)]. Almost immediately upon induction of adipogenesis, the C/EBP family members  $\beta$  and  $\delta$  are induced in a manner dependent on several signal transduction cascades that result in phosphorylation of these proteins [(Bezy et al., 2005; Park et al., 2004a; Tang et al., 2005) and references therein]. However, the activation of the early adipogenic regulators, PPAR $\gamma$ 2 and C/EBP $\alpha$ , which are dependent on C/EBP $\beta$  and  $\delta$ , does not occur until day 2 of the differentiation process. During the first two days, cells undergo one or two rounds of mitosis, a process termed mitotic clonal expansion (Bernlohr et al., 1985; Cornelius et al., 1994; MacDougald and Lane, 1995b). Thus, the transcriptional activation potential of the C/EBP $\beta$  and  $\delta$  proteins are masked or repressed until clonal expansion commences.

Over the past several years, data have accumulated that suggest that the binding capacity of C/EBP $\beta$  for its cognate binding site is delayed 12-20 hours post-induction of adipocyte differentiation (Lane et al., 1999; Tang et al., 2005; Tang et al., 1999; Tang et al., 2003a). This observation fits well with the need to delay expression of C/EBP $\alpha$ , which has anti-mitotic properties (Lin et al., 1993; Timchenko et al., 1996; Umek et al., 1991; Wang et al., 2001), until clonal expansion occurs. Moreover, the kinetics of DNA binding activation fit well with the kinetics of other events that occur at this time, including

the appearance of phosphorylated Rb and the localization of C/EBP $\beta$  to pericentric heterochromatin, which contains numerous C/EBP binding sites in the satellite DNA sequences (Tang and Lane, 1999). How this change in sub-nuclear distribution relates to gene expression has not been well established, but the observation raises the possibility that localization of proteins to specific nuclear compartments contributes to the complexity of adipocyte gene regulation.

In the course of examining the activation of the PPAR $\gamma$ 2 promoter during adipogenesis, we noted that *in vitro* binding of C/EBP $\beta$  to a C/EBP binding site in the PPAR 2 promoter did not appear to change significantly between 3 and 24 hours post-differentiation (Fig. III.1). Given the wide range of variable conditions that can affect protein binding in a gel shift assay, we initially did not view this as contradictory to the existing models explaining the delay in activation of C/EBP $\alpha$  and PPAR $\gamma$ 2 gene expression. However, ChIP assays, which specifically detect *in vivo* protein:DNA interactions at endogenous loci, clearly demonstrated that C/EBP $\beta$  and C/EBP $\delta$  were capable of binding to both the C/EBP $\alpha$  and the PPAR $\gamma$ 2 promoter at very early times post differentiation. Moreover, C/EBP $\beta$  and  $\delta$  also could bind at early times to adipocyte specific promoters that do not begin to transcribe until much later in the differentiation process. Our results do not alter the original conclusion that mechanisms exist during the time of mitotic clonal expansion to delay activation of C/EBP $\alpha$  and PPAR $\gamma$ 2 gene expression and the target genes that they subsequently activate. Instead they indicate that the rate-limiting step is not the interaction of the C/EBP $\beta$  protein with binding sites at the endogenous target gene promoters.

Numerous possibilities to restrict the transcriptional activating properties of C/EBP $\beta$  exist even if the protein is DNA bound. The exact isoform of C/EBP $\beta$  that is

bound could influence the transcriptional potential. Interaction between C/EBP $\beta$  and repressor proteins (Ron and Habener, 1992; Tang et al., 1999) would not necessarily be restricted to solution interactions; repression could occur via interactions at promoter sequences as shown previously (Mo et al., 2004). DNA-bound C/EBP $\beta$  could be and likely is still subject to post-translational modifications, including phosphorylation, acetylation and sumoylation (Eaton and Sealy, 2003; Kim et al., 2002; Park et al., 2004a; Tang et al., 2005; Xu et al., 2003), that may modulate transcriptional capacity. C/EBP $\beta$  bound loci could remain transcriptionally silent because other activators, RNA polymerase II, or RNA polymerase II-associated general transcription factors have not been synthesized, are spatially restricted, have not yet undergone the appropriate post-translational modification, or cannot bind in the absence of specific chromatin modifications or alterations. In support of this last possibility, Wiper-Bergeron *et al* (2003) showed that histone deacetylase 1 (HDAC1) could affect the acetylation status of the C/EBP $\alpha$  promoter in a manner regulated by glucocorticoids (Wiper-Bergeron et al., 2003). Finally, C/EBP $\beta$ -bound loci may change position within the nucleus, becoming associated or disassociated with specific sub-nuclear structures such as pericentric heterochromatin or splicing domains. None of these possibilities are mutually exclusive, and it is likely that multiple mechanisms are acting cooperatively to control the timing of expression for each individual target gene.

We also note that in other cell types, C/EBP $\beta$ -mediated activation of endogenous target genes can occur without induction of C/EBP $\beta$  levels or induction of post-translational modifications. Lipopolysaccharide-mediated induction of C/EBP $\beta$  target genes in B cell-derived lines lacking C/EBP proteins could be accomplished by constitutive expression of the bZIP domain of C/EBP $\beta$  (Hu et al., 2000). More recently,

C/EBP $\beta$  binding to and subsequent activation of target genes in lipopolysaccharide stimulated macrophages were shown to occur prior to induction of C/EBP $\beta$  protein levels and in the absence of induced C/EBP $\beta$  phosphorylation or nuclear translocation (Bradley et al., 2003). Thus different mechanisms likely control C/EBP $\beta$  function in different cell types.

The *in vivo* binding capacity of C/EBP $\beta$  and  $\delta$  to adipogenic genes as early as 1 hr post-differentiation seems likely to be modulated by components of the cocktail of differentiation. ChIP assays performed with 3T3-L1 over-expressing C/EBP $\beta$  in the absence of differentiation inducers showed that this regulator is not recruited to the PPAR $\gamma$ 2 and other promoters (Fig. III.5). This result shows that the physical presence of C/EBP $\beta$  alone is insufficient to be recruited to specific promoters. Further studies will be required to determine the nature of modifications required for both C/EBP $\beta$  and  $\delta$  to rapidly bind to adipogenic regulatory sequences following differentiation signaling as well as the relative importance of C/EBP $\beta$  homodimers and C/EBP $\beta$  and  $\delta$  heterodimers.

Limited data on the binding of C/EBP $\beta$  and  $\delta$  to gene promoters other than C/EBP $\alpha$  and PPAR $\gamma$ 2 exist. In differentiating 3T3-L1 cells, C/EBP $\beta$  binds to the aP2 promoter between 24-72 hours post-differentiation (Tang et al., 2004), and a recent report that shows both C/EBP $\beta$  and  $\delta$  on the adiponectin promoter in mouse adipose tissue (Park et al., 2004b). These data raise the question of why C/EBP $\beta$  and  $\delta$  are bound to such promoters when evidence strongly suggests that the genes are not activated until later times when C/EBP $\alpha$  has replaced C/EBP $\beta$  and  $\delta$  at these loci. We speculate that the presence of C/EBP $\beta$  may be part of the process by which C/EBP $\alpha$  is recruited to adipogenic promoters. Alternatively, or perhaps, additionally, all of the



adipogenic loci undergo structural changes at the onset of differentiation, and the binding of C/EBP $\beta$  to these loci serves as a mark for subsequent gene activation.

The data also clearly demonstrate that the C/EBP binding sites on each of the genes undergo a transition in binding from C/EBP $\beta$  and  $\delta$  to C/EBP $\alpha$  *in vivo*. We and others previously demonstrated that this occurred on the PPAR $\gamma$ 2 promoter during adipogenesis (Salma et al., 2004; Tang et al., 2004). From the data presented here, we predict that this transition occurs on all adipocyte genes containing C/EBP binding sites with similar kinetics, indicating the importance of the transition and a role for C/EBP $\beta$  and  $\delta$  in both early and late events of the differentiation program. We note that the binding of C/EBP $\beta$  and  $\delta$  correlated with the induction of overall cellular levels of C/EBP $\beta$  and  $\delta$ . Similarly, C/EBP $\beta$  and  $\delta$  were replaced on each promoter between 24 and 48 hours post-differentiation, the time at which C/EBP $\alpha$  protein levels begin to be induced. Thus the binding of the specific C/EBP proteins to adipocyte specific genes *in vivo* does not correlate with the time of target gene expression but instead occurs rapidly after the induction of C/EBP protein levels.

# CHAPTER IV

## CHAPTER IV

### GENERAL DISCUSSION

#### Role of SWI/SNF in adipogenesis

During cellular differentiation the expression of a group of genes is activated sequentially to establish the tissue-specific phenotype. Gene activation requires that specific transcription factors bind to the control regions to facilitate the interaction of additional factors, such as chromatin modifying enzymes that are involved in the decondensation of the chromatin, which in turn facilitates the recruitment of the basal transcription machinery to the core promoter to initiate transcription.

The major transcription factors that control the adipogenic process include the C/EBP protein family and the nuclear hormone receptor PPAR $\gamma$ , which act in concert to regulate the expression of adipocyte-specific genes. The action of both transcription factors requires modification of the chromatin structure at the adipocyte specific loci, however, prior to this work, no studies of chromatin remodeling had been described. The main focus of this thesis was to study the function of chromatin remodeling enzymes, in particular the ATP-dependent chromatin remodeling enzymes, during adipocyte differentiation. For these studies, we utilized cell lines that inducibly express mutant versions of SWI/SNF enzymes (de La Serna et al., 2000). Adipocyte differentiation was induced in these cells by forced expression of three main regulators of adipogenesis, C/EBP $\beta$ , C/EBP $\alpha$  or PPAR $\gamma$ . The results of these studies, which were presented in chapter II, clearly demonstrated that BRG1 and hBRM activities are required in adipogenesis mediated by C/EBP $\beta$ , C/EBP $\alpha$  or PPAR $\gamma$  activators.

Analysis of the expression of the late adipocyte genes aP2 and adipsin showed that BRG1 or BRM are required for the induction of these genes. Furthermore, overexpression of either C/EBP $\alpha$  or C/EBP $\beta$  activated PPAR $\gamma$  expression in a SWI/SNF dependent way. These results revealed the requirements of SWI/SNF in both early (inducing PPAR $\gamma$ ) and late (inducing aP2 and adipsin) gene activation. In contrast, dominant negative BRG1 or BRM did not affect the expression of endogenous C/EBP $\beta$  and C/EBP $\delta$ , suggesting that SWI/SNF is not required for expression of these genes. Another master regulator in adipogenesis is C/EBP $\alpha$ , which is regulated by other members of the C/EBP family and by PPAR $\gamma$ . However, the role for SWI/SNF in C/EBP $\alpha$  expression could not be determined using the dominant negative cell lines that over express C/EBP $\beta$  or PPAR $\gamma$  because NIH-3T3 cells are deficient in C/EBP $\alpha$  (Wu et al., 1998). Future studies will address the role of SWI/SNF enzymes in the regulation of C/EBP $\alpha$  by utilizing Swiss-3T3 cells, a cell line that expresses high levels of C/EBP $\alpha$ . By silencing the expression of either BRG1 or BRM in Swiss-3T3 cells, using small interfering RNA, we will determine if there is an effect on C/EBP $\alpha$  expression.

### **Mechanisms by which SWI/SNF activates PPAR $\gamma$**

Our next question was to understand how SWI/SNF activity regulates adipocyte gene expression. Biochemical studies have indicated that SWI/SNF alters nucleosome structure and may also promote the binding of transcription factors like GAL4, and some of the general transcription factors like TBP/TFIIA (Cote et al., 1998; Cote et al., 1994; Imbalzano et al., 1994; Imbalzano et al., 1996; Kwon et al., 1994). Few studies have addressed this question in differentiating cells by examining the temporal binding of

different classes of chromatin remodeling enzymes, transcriptional regulators, and RNA polymerase II factors to the promoter DNA during gene activation (Agalioti et al., 2000; Martens et al., 2003; Soutoglou and Talianidis, 2002). Using our unique cell lines that inducibly express mutant SWI/SNF enzymes, we were able to specifically address the role of SWI/SNF in facilitating promoter accessibility to various factors to adipogenic loci.

Because PPAR $\gamma$  is the master regulator of adipocyte differentiation, it has become the focus of numerous studies (Rangwala and Lazar, 2004; Rosen and Spiegelman, 2001). The dependency of PPAR $\gamma$ 2 expression on SWI/SNF activity prompted us to evaluate this promoter. The temporal analysis of the binding of various classes of transcription factors to this promoter was analyzed by the chromatin immunoprecipitation assay (ChIP). From the studies in cell lines that express dominant negative BRG1, it is evident that SWI/SNF activity is required for the continuous stable association of the preinitiation complex and/or for promoting promoter clearance and elongation. Preinitiation complex formation was not affected by the presence of dominant negative BRG1 in these cells, but interestingly, at the onset of transcription, coincidently with SWI/SNF binding, the assembly of RNA polymerase II, and general transcription factors fell apart. These observations strongly suggest the SWI/SNF may facilitate promoter clearance.

In addition, SWI/SNF may be involved in facilitating preinitiation complex formation since TFIIH is recruited to the PPAR $\gamma$ 2 promoter in 3T3-L1 cells at the same time as Brg1, Brm and Ini1. The binding occurred at the onset of PPAR $\gamma$ 2 transcription. Phosphorylated RNA polymerase II and associated general transcription factors, TBP and TFIIB are associated with the promoter by day one.

Transcription in eukaryotes involves a coordinated cycle of events that consists of assembly of the pre-initiation complex, initiation, promoter clearance, elongation and termination. Although, the transition between these events is poorly understood, the hyperphosphorylation of the C-terminal domain (CTD) of RNA polymerase II triggers promoter clearance and thereby defines the initiation to the elongation transition. It is thought that the hyperphosphorylated CTD domain of RNA polymerase II disrupts the links to the pre-initiation machinery and establishes new contacts with the elongation factors, which leads to movement of RNA polymerase II through the chromatin (promoter clearance) and transcript elongation (Svejstrup, 2004). Since the disassembly of the preinitiation complex and RNA polymerase II occurred at the onset of transcription in dominant negative BRG1 cells, it implies that SWI/SNF may facilitate promoter clearance either by recruiting various factors or by facilitating RNA polymerase II to move through chromatin. Further experiments will be required to explore this mechanism.

Evidence from other reports also implies this mechanism when activities of ATP-dependent chromatin remodeling enzymes are involved. Early studies on the *hsp70* gene have suggested that the remodeling of nucleosomes near the promoter region by SWI/SNF activities is crucial to release the paused polymerase and allow elongation to proceed (Brown et al., 1996; Corey et al., 2003). Additional studies in *Drosophila* have shown a high correlation between distribution of BRM and transcriptionally active chromatin on the polytene chromosome of *Drosophila*. Interestingly, loss of BRM function resulted in decreased association of RNA polymerase II with the salivary gland chromosomes, suggesting a role of BRM in recruitment of the preinitiation complex and RNA polymerase II (Armstrong et al., 2002). Recently, it was shown that the *Drosophila*

trithorax group gene, kismet (kis), which is related to the members of the SWI/SNF and CHD1 families, associates with transcriptionally active chromatin. Studies with non functional Kis confirmed that the presence of elongating RNA polymerase II and the elongation factors SPT6 and CHD1 are considerably reduced on polytene chromosomes (Srinivasan et al., 2005).

### **Role of SWI/SNF regulating the expression of adipocyte specific genes**

Subsequently, we asked if a similar mechanism as observed at the PPAR $\gamma$ 2 promoter also extends to other adipogenic genes that are regulated by SWI/SNF activity. A series of ChIPs was performed on aP2 and adipsin promoters using dominant negative cell lines overexpressing C/EBP $\alpha$ . Preliminary data showed that in the presence of dominant negative BRG1, the recruitment of one or more of the general transcription factors, TFIIB, TFIIF, and/or TBP was compromised at the onset of gene expression. That possibly explains the down regulation of both genes (Fig. II.6). These results support that this might be a general mechanism of SWI/SNF mediated transcriptional activation.

### **SWI/SNF role in altering chromatin structure**

Previous studies demonstrated that SWI/SNF alters chromatin structure, as evidenced by an increase in accessibility at the promoter regions. However, our results indicate that changes associated with chromatin structure at the PPAR $\gamma$ 2 promoter in 3T3-L1 correlated neither with SWI/SNF recruitment nor with PPAR $\gamma$ 2 induction, indicating that factors other than SWI/SNF, such as ISWI complexes, may be involved in this process. It is possible that a subtle change in the remodeling of a positioned

nucleosome close to the start site at the promoter may require SWI/SNF function. This could be evaluated by fine mapping of the nucleosome positions during differentiation. Further investigations, using dominant negative BRG1 expressing cell lines, could clarify this concern. Moreover, neither the overall acetylation status at the promoter regions nor the binding sites of transcriptional factors was influenced by the presence of dominant negative SWI/SNF. But this does not exclude the involvement of SWI/SNF in some other histone specific modifications.

#### ***In vitro* analysis of SWI/SNF mediated transcriptional activation**

To determine if SWI/SNF enzymes play a critical role in the assembly, stabilization, and function of the preinitiation complex at the PPAR $\gamma$ 2 promoter, whether its activity is simply involved in remodeling of nucleosomes, or the combination of both, a molecular approach such as *in vitro* transcription on DNA templates immobilized on paramagnetic particles, could be considered for future experiments. By reconstituting the transcriptional events such as initiation, elongation, and termination on a naked or nucleosomal reconstituted template, this method allows an in depth analysis of the factors involved in the transcription of a particular gene. These experiments potentially will allow us to measure intermediates in PIC formation and elongation activity caused by blocking the ATPase activity of SWI/SNF enzymes.

#### **C/EBP transcription factors at the PPAR $\gamma$ 2 promoter**

The analysis of the binding of the C/EBP family of transcription factors to the PPAR $\gamma$ 2 promoter was important for several reasons. Many studies have supported the essential role of C/EBP transcription factors in adipogenesis and in the regulation of



PPAR $\gamma$ 2 expression (Clarke et al., 1997; Freytag et al., 1994; Lin and Lane, 1994; Wu et al., 1995b; Yeh et al., 1995). Analysis of the murine PPAR $\gamma$ 2 promoter identified two C/EBP recognition elements relative to the transcriptional start site that directly modulate transcription of PPAR $\gamma$ 2 (Clarke et al., 1997; Elberg et al., 2000; Zhu et al., 1995).

Physical interaction between C/EBP factors and components of the SWI/SNF complex and the general transcription factors is indicative of their possible role in recruitment of these activities to the promoter to mediate transcriptional activation (Kowenz-Leutz and Leutz, 1999; Nerlov and Ziff, 1995; Pedersen et al., 2001). Recently it has also been demonstrated that the C/EBPs recruit different mediator complexes to the target gene promoters depending on the phosphorylation status of the C/EBP $\beta$  MAPK site. The differential interactions mediate gene transcription or repression (Mo et al., 2004).

By ChIP analysis we demonstrated the temporal and sequential recruitment of C/EBP family members to PPAR $\gamma$ 2 promoter during differentiation of 3T3-L1 cells, where C/EBP $\beta$  and  $\delta$  bind first and then are substituted by C/EBP $\alpha$ . This binding pattern was consistent with the expression pattern of these C/EBPs. The pattern of binding of C/EBP $\beta$  and  $\delta$  did not change in C/EBP  $\alpha$  overexpressing cells in the presence of dominant negative BRG1, although C/EBP $\alpha$  remained bound to the promoter during the differentiation process.

Changes in chromatin structure measured by nuclease accessibility correlated with binding of C/EBP $\beta$  and  $\delta$  and general transcription factors. Based on early reports, we propose that C/EBP $\beta$  and  $\delta$  heterodimers are responsible for the recruitment of the preinitiation complex to the promoter at very early time points following the initiation of the differentiation program (Kowenz-Leutz and Leutz, 1999; Pedersen et al., 2001). In that study, the authors demonstrated that the transactivation element (TEIII) in C/EBP $\alpha$

and the conserved region (CR1) of C/EBP $\beta$  function as SWI/SNF binding domains. Ectopic expression of a C/EBP $\alpha$  mutant lacking TEIII in NIH-3T3 cells prevented adipogenesis. Additionally, ectopic expression of C/EBP $\alpha$  mutants in TE I and TEII, interfered with TFIIB and TBP recruitment and consequently inhibited adipogenesis (Kowenz-Leutz and Leutz, 1999; Pedersen et al., 2001). This observation suggests that C/EBP $\alpha$  or C/EBP $\beta$  may recruit SWI/SNF and general transcription factors to promoters. If this assumption is correct, then ectopic expression of C/EBP mutants in NIH-3T3 cells as discussed above should prevent such an interaction, resulting in the absence of SWI/SNF at the promoter and gene expression.

What causes the recruitment of SWI/SNF to the promoter? Does the transition in binding of C/EBP factors from  $\beta/\delta$  to  $\alpha$  initiate SWI/SNF recruitment? or does C/EBP $\alpha$  itself or in concert with C/EBP $\beta$  and/or  $\delta$  bring SWI/SNF activities to the promoter? Alternatively, is a posttranslational modification of a C/EBP member at the promoter required to bring SWI/SNF activity? There are still many questions that need to be answered in order to really establish the role of the C/EBPs in adipogenesis (see below). The scenario is complex since C/EBPs interact with a number of coactivators and repressors. In addition, C/EBP activity is controlled by a number of post-translational modifications. Nevertheless, it is clear from these studies that C/EBP family members play a pivotal role during PPAR $\gamma$ 2 activation, targeting to the promoter a series of coactivators which in concert modulate its expression.

Another important aspects of the functional activity of C/EBP family members in adipogenesis came from the studies of the double C/EBP $\beta$  and  $\delta$  knockout mice, C/EBP $\beta$ (-/-) C/EBP $\delta$ (-/-). These mice had a decreased adipose tissue mass and levels of some adipocyte markers were either completely inhibited or moderately down

regulated, even though protein levels of the master regulators, PPAR $\gamma$  and C/EBP $\alpha$ , were unaffected (Tanaka et al., 1997). These results are suggestive of a possible role of C/EBP $\beta$  and C/EBP $\delta$  in the regulation of late adipocyte genes. Likewise, we determined by ChIP assays that C/EBP $\beta$  and  $\delta$  are recruited to a series of adipocyte genes, including adipocyte transcription factors, and the results were presented in chapter III.

### **Kinetics of C/EBP protein binding to adipocyte genes during differentiation**

We temporally analyzed, in a short time course of differentiation in 3T3-L1 cells, the *in vivo* recruitment of C/EBP $\beta$  and  $\delta$  and  $\alpha$  to the C/EBP $\alpha$ , PPAR $\gamma$ , resistin, adiponectin and leptin promoters. C/EBP $\alpha$  and PPAR $\gamma$  promoters were considered as positive controls, since it was known that C/EBP factors interact *in vivo* with the C/EBP sites of both promoters (Rochford et al., 2004; Salma et al., 2004; Tang et al., 2004). All of the C/EBP isoforms  $\alpha$ ,  $\beta$  and  $\delta$  in this study play a role in regulating the expression of PPAR $\gamma$ 2, C/EBP $\alpha$ , resistin, adiponectin, and leptin. Binding of each of the C/EBPs undergoes a transition from C/EBP $\beta$  and  $\delta$  to  $\alpha$ . This transitional binding may play a pivotal role since it occurs in all of the adipocyte genes evaluated, including glut4 and ap2 promoters. It occurs prior to gene expression and it is independent of the time at which gene expression is initiated. The recruitment of the earlier expressed C/EBP $\beta$  and  $\delta$  occurred as early as 1 hour and correlated with induction of these activators. The same occurred with C/EBP $\alpha$  which is expressed later, between 24 to 48 hours.

Thus, from our results presented in chapter III, we conclude that C/EBP family members,  $\alpha$ ,  $\beta$  and  $\delta$  bind to adipocyte genes *in vivo* in a manner that follows the induction of C/EBP protein synthesis, without any delay, unlike previously published

reports (Lane et al., 1999; Tang et al., 2005; Tang and Lane, 1999; Tang et al., 2003a). A question that arises from these studies is whether overexpression of any of these proteins is sufficient to promote C/EBP binding to adipocyte gene promoters. When C/EBP $\beta$  was overexpressed in 3T3-L1 cells, no binding occurred on the PPAR $\gamma$ 2 promoter and other promoters in the absence of differentiation cocktail. As was mentioned earlier, C/EBP $\beta$  undergoes a series of posttranslational modifications such as phosphorylation, which are induced by the components of the differentiation cocktail, and these modifications modulate both its transcriptional activity and DNA binding capacity. The same result was obtained when C/EBP $\beta$  was overexpressed in NIH-3T3 cells. In contrast, overexpression of C/EBP $\alpha$  in NIH-3T3 cells is sufficient to promote binding to promoters, even in the absence of differentiation cocktail; however, gene activation does not occur unless cocktail is added. Unlike C/EBP $\beta$ , C/EBP $\alpha$  is only phosphorylated at three sites during adipogenesis; these phosphorylations cause conformational changes but do not affect transactivation activities and most likely DNA binding capacity [Reviewed in (Otto and Lane, 2005)]. Future experiments could contemplate the analysis of such modifications and how differentially they modulate each member of the family.

Binding of C/EBP $\beta$  and  $\delta$  on these promoters suggest a fundamental role for these proteins at early time points during differentiation, including early and late adipocyte genes. C/EBP $\beta$  and  $\delta$  interact with the same pattern of binding at regulatory sequences in adipocyte genes other than C/EBP $\alpha$  and PPAR $\gamma$ 2. Our results could explain the observations in the double knockout mice C/EBP $\beta$ (-/-).C/EBP $\delta$ (-/-) (Tanaka et al., 1997). aP2 expression in these double knockout mice is downregulated indicating that full induction requires the concerted activity of all of the members of the C/EBP

family involved in adipogenesis. Mouse embryonic fibroblasts (MEFs) isolated from the double mutant did not differentiate in culture since expression of PPAR $\gamma$ 2 and C/EBP $\alpha$  did not occur. Interestingly, when PPAR $\gamma$ 2 was introduced into these C/EBP $\beta$ (-/-).C/CPB $\delta$ (-/-) fibroblasts, they differentiated in the presence of inducers. However, expression of C/EBP $\alpha$ , Glut4, adipsin, and insulin receptor substrate 2 (IRS-2) was lower than in the wild type MEFs. These results also suggested a role for C/EBP $\beta$  and  $\delta$  in the regulation of genes such as Glut4 and IRS-2 that are involved in insulin-signaling (Yamamoto et al., 2002). Therefore, full induction of these genes requires the concerted activity of all of the C/EBP family members.

The binding of C/EBP $\beta$  and  $\delta$  at early time points from 1 to 48 hours before gene induction seemed to be essential since it occurred on all of the adipocyte genes studied. These proteins are on the promoter but there is no transcription. It is not clear why they are on the promoter so early and for such a long period of time. What are they regulating? What are the mechanisms during mitotic clonal expansion that cause the delay of the activation of all of the genes studied? The answer to these questions would increase our understanding of many aspects of the functional activity of these transcription factors and the establishment of interaction with other proteins that in concert modulate the whole adipogenic process. It is likely that crosstalk between the cell cycle regulators and the factors controlling adipogenesis (Fajas, 2003) is established through the C/EBP family members. It is well known that C/EBP $\beta$  during mitotic clonal expansion (Tang et al., 2003a; Tang et al., 2003b; Zhang et al., 2004b) and the recruitment of C/EBP $\alpha$  induces proliferation arrest and terminal differentiation (Muller et al., 1999). Numerous proteins and signaling pathways that are active during the cell

cycle stimulate or inhibit the activity of C/EBP proteins, perhaps by modulating the state of phosphorylation, or acetylation.

We speculate that early association of C/EBP $\beta$  and  $\delta$  to the regulatory elements of all of the genes evaluated may be functionally "marking" these genes for subsequent gene activation. The requirements of the master regulator PPAR $\gamma$  are crucial in this process. A recent study showed that C/EBP $\beta$  binds to the C/EBP $\alpha$  promoter, but in the absence of PPAR $\gamma$  is associated with HDAC1 (Zuo et al., 2006). Under these conditions C/EBP $\alpha$  expression did not take place unless PPAR $\gamma$  and its ligand are present. PPAR $\gamma$  and ligand dislodge HDAC1 from the C/EBP $\alpha$  promoter releasing the repression state (Zuo et al., 2006). It is possible that the presence of C/EBP $\beta$  and  $\delta$  at early time points during differentiation recruits HDAC or other repressive activity to the promoters "awaiting" the expression of the master regulators. Then, repression is removed when PPAR $\gamma$  is present and expression commences.

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# APPENDIX A

# Temporal Recruitment of Transcription Factors and SWI/SNF Chromatin-Remodeling Enzymes during Adipogenic Induction of the Peroxisome Proliferator-Activated Receptor $\gamma$ Nuclear Hormone Receptor

Nunciada Salma,<sup>1</sup> Hengyi Xiao,<sup>1</sup> Elisabetta Mueller,<sup>2</sup> and Anthony N. Imbalzano<sup>1\*</sup>

*Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655,<sup>1</sup> and Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115<sup>2</sup>*

Received 23 September 2003/Returned for modification 5 December 2003/Accepted 18 March 2004

**The peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) regulates adipogenesis, lipid metabolism, and glucose homeostasis, and roles have emerged for this receptor in the pathogenesis and treatment of diabetes, atherosclerosis, and cancer. We report here that induction of the PPAR $\gamma$  activator and adipogenesis forced by overexpression of adipogenic regulatory proteins is blocked upon expression of dominant-negative BRG1 or hBRM, the ATPase subunits of distinct SWI/SNF chromatin-remodeling enzymes. We demonstrate that histone hyperacetylation and the binding of C/EBP activators, polymerase II (Pol II), and general transcription factors (GTFs) initially occurred at the inducible PPAR $\gamma$ 2 promoter in the absence of SWI/SNF function. However, the polymerase and GTFs were subsequently lost from the promoter in cells expressing dominant-negative SWI/SNF, explaining the inhibition of PPAR $\gamma$ 2 expression. To corroborate these data, we analyzed interactions at the PPAR $\gamma$ 2 promoter in differentiating preadipocytes. Changes in promoter structure, histone hyperacetylation, and binding of C/EBP activators, Pol II, and most GTFs preceded the interaction of SWI/SNF enzymes with the PPAR $\gamma$ 2 promoter. However, transcription of the PPAR $\gamma$ 2 gene occurred only upon subsequent association of SWI/SNF and TFIID with the promoter. Thus, induction of the PPAR $\gamma$  nuclear hormone receptor during adipogenesis requires SWI/SNF enzymes to facilitate preinitiation complex function.**

Differentiation of adipocytes, as with all differentiation events, involves programmatic changes in gene expression patterns. Genes specifically expressed in adipocytes must be activated; the concerted action of several transcriptional regulators, including C/EBP $\alpha$ , C/EBP $\beta$ , and the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), controls these activation events via direct interaction with PPAR $\gamma$  and C/EBP binding sites in adipocyte-specific gene-regulatory sequences (reviewed in references 14, 33, and 36). Each of these regulators is expressed with different kinetics during adipocyte differentiation in culture, yet forced overexpression of any is sufficient to initiate adipogenic differentiation in fibroblast cells (17, 45, 50, 51). Function of the PPAR $\gamma$  regulator is especially critical, since many of the genes involved in adipogenesis, as well as glucose homeostasis, are activated by this nuclear hormone receptor. Since adipocyte-specific genes are not expressed prior to differentiation, it is likely that the regulatory sequences controlling the expression of these genes are incorporated into a repressive chromatin structure that is refractory to gene expression. Eukaryotic cells have evolved two classes of enzymes that can alter chromatin structure to control accessibility to the transcriptional machinery. These include histone-modifying enzymes, which posttransla-

tionally modify the N-terminal and C-terminal domains (CTDs) of the individual histone proteins that comprise the nucleosome, and ATP-dependent chromatin-remodeling enzymes, which alter structure by disrupting the histone:DNA contacts of the nucleosome, thereby altering nucleosome conformation and, in some cases, altering the position of the histone octamer along the DNA (reviewed in references 28, 42, and 46).

The mammalian SWI/SNF family of ATP-dependent chromatin-remodeling enzymes includes members containing either the Brg1 or Brm ATPase. Although the mammalian SWI/SNF enzymes share most of the same subunits, multiple forms of these enzymes exist; these are distinguished by the ATPase present, the presence of unique subunits, and/or the presence of tissue-specific isoforms of common subunits (30, 39, 48, 49). In vitro analyses of hBRM- and different BRG1-containing enzymes reveal many similarities in chromatin remodeling assays (39). In vivo, however, clear differences in function likely exist. Brg1 knockout mice are embryonic lethal, and heterozygotes are predisposed to tumors (7). Brm knockout mice and cells, in contrast, show only modest proliferation differences compared to the wild type (35). Additionally, at the molecular level, chromatin immunoprecipitation (ChIP) analyses have revealed that Brg1 and Brm can be present on different promoters (18, 40), supporting the idea of differential functions.

Collectively, the literature reveals that Brg1 and/or Brm can physically interact with a number of different transcriptional regulatory proteins, and these proteins have been localized by

\* Corresponding author. Mailing address: Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Ave. North, Worcester, MA 01655. Phone: (508) 856-1029. Fax: (508) 856-5612. E-mail: anthony.imbalzano@umassmed.edu.

ChIP studies to the promoter sequences of a number of inducible genes during transcriptional activation. In particular, previous work has indicated a requirement for or a contribution by SWI/SNF enzymes for activation of cellular differentiation genes. Myeloid, erythrocyte, enterocyte, muscle, and adipose cell differentiation events have been linked to the presence of functional SWI/SNF enzymes (2, 12, 19, 32, 40) and, in a more limited number of cases, to the ability of SWI/SNF enzymes to alter chromatin structure at or near inducible promoters. In the case of adipogenesis, the factor C/EBP $\alpha$ , already known to physically interact with the polymerase II (Pol II)-associated general transcription factors TBP and TFIIB (29), was shown to have the capability to interact with hBRM (32). Moreover, the domain mediating this interaction was critically required for the ability of C/EBP $\alpha$  to transdifferentiate fibroblasts into adipocyte-like cells. In vitro, the competency of PPAR $\gamma$  to activate in vitro transcription templates assembled into chromatin was dependent on a specific BRG1-containing SWI/SNF enzyme (22). However, the nature of the role that SWI/SNF enzymes play in facilitating adipogenic gene expression remains to be determined.

Here we explore the functional role of SWI/SNF enzymes during adipocyte differentiation by examining the activation of the PPAR $\gamma$  regulator itself. PPAR $\gamma$  mRNA is expressed from two distinct promoters that give rise to two distinct isoforms, termed PPAR $\gamma$ 1 and PPAR $\gamma$ 2 (52). We focused on PPAR $\gamma$ 2 expression because in differentiating preadipocytes, PPAR $\gamma$ 2 is highly induced and is the predominant isoform in differentiated adipocytes (38, 44), while in undifferentiated and differentiated fibroblasts, PPAR $\gamma$ 1 expression was not observed (see below). We found that the SWI/SNF enzymes are critically required for transdifferentiation of fibroblasts along the adipogenic pathway. Temporal analyses of factor binding to the PPAR $\gamma$ 2 promoter revealed that the BRG1-based SWI/SNF enzymes did not facilitate activator binding to the promoter but instead promoted preinitiation complex (PIC) function. Examination of PPAR $\gamma$ 2 activation during differentiation of committed preadipocyte cells confirmed that changes in chromatin structure, activator binding, and assembly of multiple components of the PIC did not require SWI/SNF function. Nevertheless, activation of PPAR $\gamma$ 2 transcription did not occur until SWI/SNF and TFIIB subsequently were brought to the promoter. Thus, using two different cellular models for adipocyte differentiation, we demonstrate that activation of the PPAR $\gamma$  regulator critically depends upon SWI/SNF enzymes, most likely by facilitating PIC formation and function.

#### MATERIALS AND METHODS

**Plasmids.** The retrovirus encoding mouse PPAR $\gamma$ 2 (45) and cDNAs encoding rat C/EBP $\alpha$  (20) and mouse C/EBP $\beta$  (8) were kindly provided by Bruce Spiegelman. C/EBP $\alpha$  and C/EBP $\beta$  were subcloned into pBabe-Puro (27).

**Cell lines and differentiation methods.** The derivation and maintenance of the cell lines that express dominant-negative human BRG1 (B22 and B24), dominant-negative hBRM (H17), and the Tet-VP16 regulator (Tet-VP16) were described previously (11). To infect cell lines, BOSC23 cells were cultured in 100-mm-diameter dishes and transfected at 80% confluence by FUGENE (Roche) with 10  $\mu$ g of pBabe-PPAR $\gamma$ 2, pBabe-C/EBP $\alpha$ , pBabe-C/EBP $\beta$ , or the empty vector as described previously (31). Viral supernatants were harvested 48 h after transfection. Dishes (60 mm) of B22, B24, H17, or Tet-VP16 cells at 50% confluence were infected with virus in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 4  $\mu$ g of polybrene/ml, and 2  $\mu$ g of tetracycline/ml in a final volume of 5 ml. The corresponding cell lines were split 1:3

48 h after infection and placed under selection with 2  $\mu$ g of puromycin/ml in the presence of tetracycline. Subsequently, each virally infected cell line was split 1:4 into media containing or lacking tetracycline. After 96 h the medium was changed. Plates designated to be undifferentiated received and continued to be maintained in DMEM plus 10% calf serum in the presence or absence of tetracycline, while the plates designated for differentiation received DMEM plus 10% fetal calf serum plus cocktail containing 0.5 mM methylisobutylxanthine (Sigma), 1  $\mu$ M dexamethasone (Sigma), 5  $\mu$ g of insulin/ml, and 10  $\mu$ M troglitazone (Biomol) in the presence or absence of tetracycline for 48 h. The cells were subsequently maintained in DMEM plus 10% fetal calf serum with 5  $\mu$ g of insulin/ml and refed every 2 days for up to 8 days.

3T3-L1 preadipocytes were purchased from the American Type Culture Collection, maintained in growth medium consisting of DMEM containing 10% calf serum, and induced to differentiate as described previously (50).

**Oil Red O staining.** Sixty-millimeter-diameter dishes were washed twice with phosphate-buffered saline (PBS) and fixed with 10% buffered formalin for 30 min. The formalin was aspirated, and the cells were stained for 1 h in freshly diluted Oil Red O solution (Sigma), prepared by mixing six parts Oil Red O stock solution (0.5% Oil Red O in isopropanol) and four parts distilled water. The stain was removed, and cells were washed three times with distilled water and photographed.

**RNA analysis.** RNA isolation and analysis by Northern blotting was described previously (13). Probes were derived from plasmids containing PPAR $\gamma$ ,  $\alpha$ P2, or adipin cDNA (provided by B. Spiegelman) and were labeled by random priming. Washed blots were exposed to a PhosphorImager (Molecular Dynamics).

For reverse transcriptase (RT)-PCR, total RNA (3  $\mu$ g) was reverse transcribed with Moloney murine leukemia virus RT (Invitrogen). cDNA was amplified by PCR with Taq polymerase (Invitrogen; 2.5 U/reaction), 0.2 mM deoxynucleoside triphosphates, 8 ng of each primer/ $\mu$ l, and either 1 mM (C/EBP $\alpha$ ), 1.5 mM (PPAR $\gamma$ ), or 2.5 mM hypoxanthine phosphoribosyltransferase (HPRT) MgCl $_2$ . The initial denaturation step was at 95°C for 5 min and was followed by 25 cycles (PPAR $\gamma$  and C/EBP $\alpha$ ) or 27 cycles (HPRT). A cycle for PPAR $\gamma$  PCR consisted of denaturation for 30 s, annealing for 40 s at 68°C, and extension at 72°C for 30 s. For C/EBP $\alpha$ , it consisted of denaturation for 50 s, annealing for 55 s at 66°C, and extension at 72°C for 50 s. The final round of extension was for 5 min. The sequences of the primers were as follows: 5'-GCATGGTGCCTTCGCTGATG C-3' and 5'-AGGCCTGTTGTAGAGCTGGGT-3' for PPAR $\gamma$ 2 (340-bp product), and 5'-CCGGCCGCTTCAACGAC-3' and 5'-CTCCTCGCGGGGCTC TTGTTT-3' for C/EBP $\alpha$  (288-bp product). HPRT and PPAR $\gamma$ 1 RT-PCRs were described previously (13, 52).

Nuclear run-on analysis was performed as described previously by Schübeler and Bode (<http://juergenbode.gmxhome.de/t01176.htm>) on nuclei isolated from  $\sim 4 \times 10^6$  differentiating 3T3-L1 preadipocyte cells that were collected at days 0, 2, 4, 5, 6, and 7 after induction of differentiation. Hybond N+ membranes (Amersham) were prepared for hybridization as described previously (3). Immobilized DNAs included 0.1  $\mu$ g of genomic DNA from 3T3-L1 preadipocytes, 3  $\mu$ g of EcoRI-linearized pBABE and pBABE-PPAR $\gamma$ 2, or 3  $\mu$ g of PCR product corresponding to nucleotides 181 to 531 of the mouse 36B4 cDNA, to nucleotides 111 to 641 of mouse *Gapdh*, or to *Hprt*. The HPRT PCR product has been described (13).

**Protein extracts and Western analysis.** Isolation of protein and Western blotting have been described (11). Antibodies utilized included C/EBP $\beta$  (Santa Cruz, sc-7962), M2 anti-FLAG (Sigma), and phosphatidylinositol (PI) 3-kinase (06-496; Upstate). Determination of the total Brg1 levels (see Fig. 4) was performed by scanning multiple film exposures and quantifying in ImageQuant (Molecular Dynamics).

**Accessibility assays.** Restriction enzyme and DNase I accessibility assays were performed as described previously (12), except that buffer M contained 0.15 mM spermine and 0.5 mM spermidine. DNase I was purchased from Promega. Probes P1 and P2 were PCR fragments corresponding to -1579 to -1187 and -105 to +138 from the mouse PPAR $\gamma$ 2 promoter (52).

**ChIPs.** The ChIP procedure was adopted from the Upstate protocol. Cells at the indicated time points were fixed by adding 37% formaldehyde to a final concentration of 1% and incubated at 37°C for 10 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M. Cells were washed twice with cold PBS and collected in 1 ml of PBS containing protease inhibitors. After centrifugation, the pellet of cells was resuspended in sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1]) containing protease inhibitors and incubated on ice for 10 min. Cell lysates were sheared extensively by sonication (Ultrasonic processor from Cole and Parmer; 3-mm tip at 80 W) on ice to obtain fragments from 200 to 600 bp, as revealed by ethidium bromide staining of aliquots run on agarose gels. Samples were centrifuged to pellet debris, and an aliquot was taken for gel analysis and inputs. One

hundred micrograms of soluble chromatin was diluted 10 times with IP buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris [pH 8.1], 167 mM NaCl) containing protease inhibitors and precleared for 3 h at 4°C with 50% slurry of protein A, G, or L in Tris-EDTA (TE) (depending on the isotype of the antibody used) in the presence of 20  $\mu$ g of sonicated salmon sperm DNA and 1 mg of bovine serum albumin/ml. After incubation, the beads were pelleted, and the supernatant was immunoprecipitated with antibodies of interest (see below) at 4°C overnight. Immune complexes were collected with 50% slurry of protein A, G, or L containing 20  $\mu$ g of sonicated salmon sperm DNA and 1 mg of bovine serum albumin/ml in TE by incubation at 4°C for 1 h. Sepharose beads were washed sequentially for 5 min at 4°C with wash 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), wash 2 (wash 1 containing 500 mM NaCl), and wash 3 (0.25 M LiCl, 1% NP-40, 1% sodium deoxycolate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]), and finally were washed twice with TE (pH 8.0). Immune complexes were eluted from the beads with 1% SDS in TE (pH 8.0), and protein-DNA cross-links were reversed by adding 200 mM NaCl and heating at 65°C overnight. After treatment with proteinase K, the samples were purified with the QIAquick PCR purification kit (QIAGEN). One-tenth of the immunoprecipitated DNA and 1% of the input DNA were analyzed by PCR.

Antibodies used included Brg1, Brm, and Inr1 (11), diacetylated (K9K14) H3 (06-599; Upstate), Tetra-acetylated H4 (06-866; Upstate), Pol II CTD-Ser-5P (Covance), and the following Santa Cruz antibodies: C/EBP $\alpha$  (sc-9314), C/EBP $\beta$  (sc-7962), C/EBP $\delta$  (sc-151), TFIIB (sc-274), TBP (sc-273), and TFIIF (sc-293). When Pol II antibody was used, 10 mM NaF was added to all buffers. PCRs were performed with QIAGEN HotStart *Taq* master mix in the presence of 2  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]dATP under the following conditions: 94°C, 15 min, followed by 26 cycles ( $\beta$ -actin) or 27 cycles (PPAR $\gamma$ ) of 94°C for 30 s, and then either 65°C for 40 s ( $\beta$ -actin) or 49.5°C for 40 s (PPAR $\gamma$ ), followed by 72°C for 30 s, followed by a 72°C extension for 5 min. PCR products were resolved in 8% polyacrylamide-1 $\times$  Tris-borate-EDTA gels, dried, and exposed to a PhosphorImager. Primers used were the following:  $\beta$ -actin, 5' (+31)GCTTCCTTGCAGCTCCTTCGTTG-3' and 5'(+135)TTTGACATGCCGAGCCGTTGT-3'; and PPAR $\gamma$ 2 promoter, 5'(-413) TACGTTTATCGGTGTTTCAT-3' and 5'(-247) TCTCGC CAGTGACCC-3'. Experiments utilizing Pol II or GTF antibodies were also performed using primers spanning -216 to -20 of the PPAR $\gamma$ 2 promoter with identical results.

## RESULTS

**SWI/SNF enzymes are required for differentiation of fibroblasts along the adipogenic pathway.** We previously described the B22 and H17 fibroblast cell lines that utilize the tet-regulatory system to inducibly express an ATPase-deficient, FLAG-tagged human allele of BRG1 (B22) or an ATPase-deficient, FLAG-tagged human allele of BRM (H17). The parent for these cell lines, termed Tet-VP16, inducibly expresses only the tet-VP16 transactivator and thus serves as a control for our experiments. The mutant BRG1 and hBRM proteins expressed in B22 and H17 cells are competent to associate with other endogenous subunits of SWI/SNF chromatin-remodeling enzymes and can act as dominant negatives with regard to different inducible gene activation events (11, 12).

To determine the requirement for BRG1- and hBRM-based SWI/SNF enzymes in adipogenesis, we forced the differentiation of these cells in the presence (dominant negative off) or absence (dominant negative on) of tetracycline by infection with a retroviral vector encoding the PPAR $\gamma$  nuclear hormone receptor or the C/EBP $\alpha$  or C/EBP $\beta$  activator. Cells infected with empty retroviral vector served as a control. Cells then were split into medium containing or lacking tetracycline for 96 h and subsequently were cultured for 7 days in the presence or absence of a differentiation regimen that included exposure to a differentiation cocktail (see Materials and Methods) containing troglitazone, a synthetic PPAR $\gamma$  ligand. Adipocyte differentiation was shown by staining with Oil Red O, a lipophilic

dye. In our hands, approximately 15 to 20% of the PPAR $\gamma$ -infected cells stained with Oil Red O, which is similar to previous data for unmodified NIH 3T3 cells infected with PPAR $\gamma$  (45). Tet-VP16 control cells stained positively when infected with the PPAR $\gamma$  retrovirus but not when infected with the empty vector (data not shown), and differentiation into adipocytes was dependent on exposure to the differentiation cocktail (Fig. 1). The presence or absence of tetracycline made no difference, as expected. In contrast, B22 and H17 cells infected with the PPAR $\gamma$  retrovirus and cultured in the absence of tetracycline did not differentiate (Fig. 1), suggesting that the expression of dominant-negative BRG1 or hBRM interfered with the differentiation process. The same results were obtained for cells infected with either the C/EBP $\alpha$  or the C/EBP $\beta$  virus (data not shown).

**SWI/SNF enzymes are required for the activation of adipogenic genes as well as for activation of PPAR $\gamma$ .** We analyzed the mRNA levels of two adipogenic marker genes, aP2 and adipsin, in cells expressing PPAR $\gamma$ , C/EBP $\alpha$ , or C/EBP $\beta$ . Cells infected with the PPAR $\gamma$  retrovirus and cultured in the presence of tetracycline and differentiation cocktail expressed both genes, while cells cultured in the absence of tetracycline produced dominant-negative BRG1 and inhibited the expression of both genes (Fig. 2A, right panel). Expression of PPAR $\gamma$  in each of the samples was monitored by RT-PCR (Fig. 2B, right panel), and induction of dominant-negative BRG1 was monitored by FLAG expression (Fig. 2C, right panel). Other experiments demonstrated that the related B24 cell line, which also inducibly expresses dominant-negative BRG1, and H17 cells, which inducibly express dominant-negative hBRM, similarly inhibited expression of adipogenic marker genes in the presence of dominant-negative protein (data not shown). Control Tet-VP16 cells infected with PPAR $\gamma$ -encoding virus showed accumulation of both aP2 and adipsin mRNAs, regardless of the presence or absence of tetracycline (Fig. 3, lanes 9 and 10). The results demonstrate that expression of dominant-negative BRG1 or hBRM interfered with the differentiation process by preventing expression of adipogenic genes. Since BRG1 and hBRM have been found only in cells in a large-molecular-weight complex associated with the other SWI/SNF subunits, presumably adipogenesis is also dependent on the activity of the SWI/SNF chromatin-remodeling enzymes.

Adipogenesis also can be induced in culture via ectopic expression of the cellular transcription factor C/EBP $\alpha$  or C/EBP $\beta$  (17, 50, 51). When B22 cells were infected with retrovirus expressing either C/EBP $\alpha$  or C/EBP $\beta$ , a similar block to aP2 and adipsin gene expression was observed in the presence of dominant-negative BRG1, though in cells expressing C/EBP $\alpha$ , the aP2 and adipsin mRNA levels were reduced 85 to 90% and were not completely absent (Fig. 2A, left panel). Expression levels of C/EBP $\alpha$  (Fig. 2B, left panel), C/EBP $\beta$ , and dominant-negative BRG1 (Fig. 2C, left panel) in these samples were monitored by RT-PCR or Western blotting. Similar results were obtained in H17 cells expressing dominant-negative hBRM (data not shown). As anticipated, Tet-VP16 cells infected with C/EBP $\alpha$ - or C/EBP $\beta$ -encoding virus expressed both aP2 and adipsin mRNAs in both the presence and absence of tetracycline (Fig. 3, lanes 13 to 14 and 17 to 18). Thus, SWI/SNF chromatin-remodeling enzymes also are re-



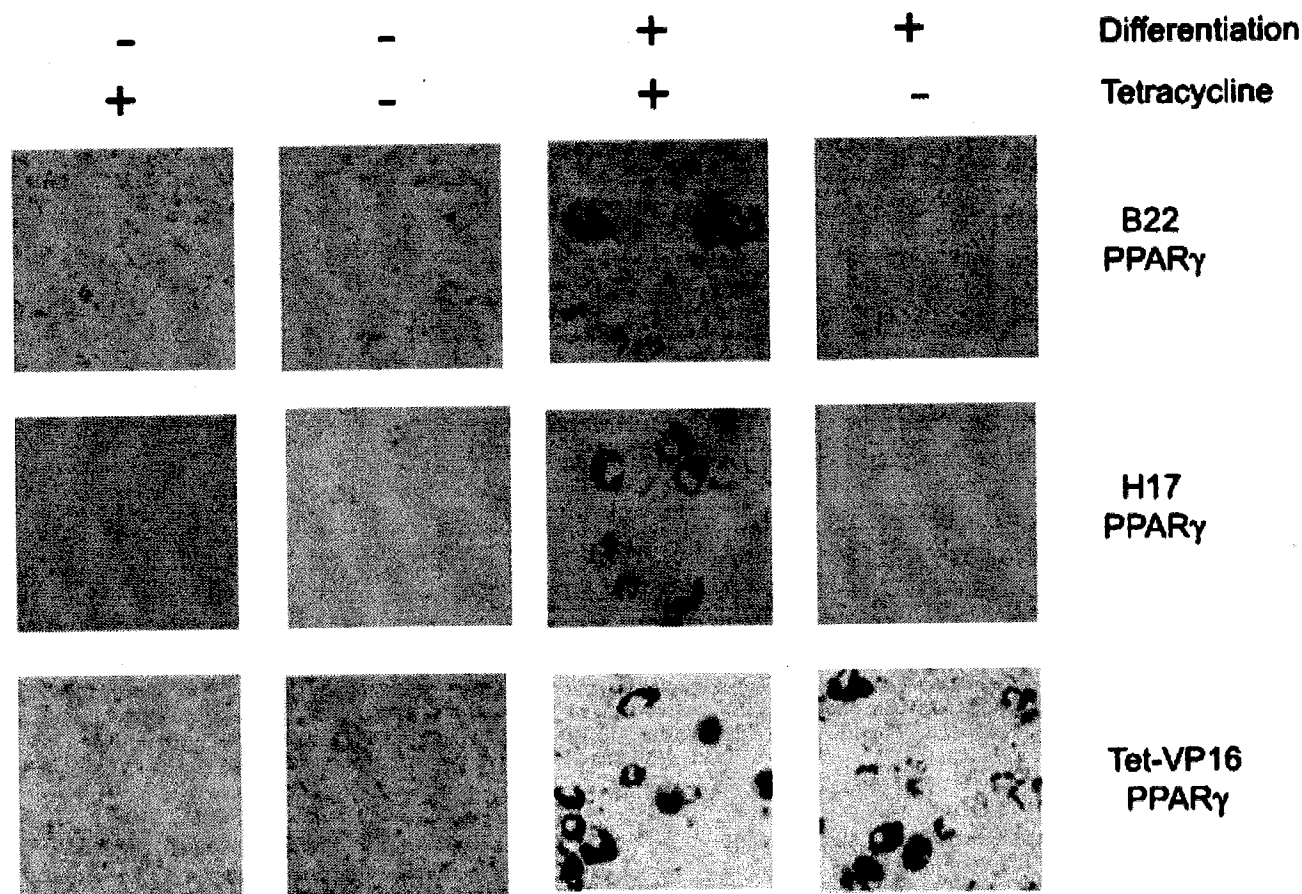


FIG. 1. Expression of dominant-negative BRG1 or hBRM blocks the ability of PPAR $\gamma$ 2 to induce adipogenesis in fibroblast cells. B22 and H17 cells, which inducibly express ATPase-deficient, dominant-negative BRG1 or hBRM, respectively, upon removal of tetracycline, or Tet-VP16 cells, which induce only the Tet-VP16 regulator, were infected with a retroviral vector encoding PPAR $\gamma$ 2, grown in the presence or absence of tetracycline, and differentiated for 7 days in the presence or absence of differentiation cocktail (see Materials and Methods). Cells were fixed, stained with Oil Red O, and photographed.

quired for activation of the adipogenic gene program by C/EBP $\alpha$  or C/EBP $\beta$ .

Interestingly, cells induced to differentiate via expression of C/EBP $\alpha$  or C/EBP $\beta$  activated PPAR $\gamma$  expression in a SWI/SNF-dependent manner (Fig. 2B, left panel). This suggests that during differentiation promoted by C/EBP $\alpha$  or C/EBP $\beta$ , induction of PPAR $\gamma$ —one of the earliest steps in differentiation—is dependent on the activity of BRG1 or BRM. This explains the significant decrease in aP2 and adipsin expression in these cells. However, the data presented here also indicate that SWI/SNF enzymes play a broader role in adipogenesis than just promoting induction of PPAR $\gamma$ , because when the induction of PPAR $\gamma$  is bypassed by providing PPAR $\gamma$  via retroviral infection, expression of the downstream adipogenic marker genes remained SWI/SNF dependent (Fig. 2, right panels). Thus, the data indicate that SWI/SNF enzymes are required for both early and late gene activation events during adipocyte differentiation.

**Kinetics of PPAR $\gamma$ 2 expression in differentiating cells.** To further analyze the molecular events controlling activation of the adipogenic pathway, we initiated differentiation by infecting cells with C/EBP $\alpha$ , since we could observe that nearly 70%

of cells differentiated for 1 week would accumulate lipid droplets (data not shown). RT-PCR analysis of PPAR $\gamma$ 2 mRNA levels in cells grown in the presence of tetracycline showed a detectable accumulation on day 1 of differentiation, with robust levels present from days 2 to 7 (Fig. 4). Low levels of PPAR $\gamma$ 2 transcripts were observed in cells grown in the absence of tetracycline. We could not detect PPAR $\gamma$ 1 mRNA in these cells. Western analyses confirmed that C/EBP $\alpha$  was present at equivalent levels in each sample (Fig. 4). Analysis of FLAG-tagged-protein levels indicated that expression of the mutant BRG1 protein occurred in all samples grown in the absence of tetracycline. Reprobing of this blot for total Brg1 levels revealed only a 1.5- to 2.0-fold increase in levels of Brg1 in the cells expressing the dominant-negative BRG1. This suggests that the levels of mutant Brg1 are only a fraction of the total Brg1 present in the cells, yet they still function as dominant-negative proteins. Alternatively, expression of the Brg1 mutant reduces expression from the endogenous locus, perhaps for the purpose of maintaining a specific overall level of Brg1 protein. In either case, the results indicate that high levels of Brg1 overexpression are not occurring in the differentiating cells.

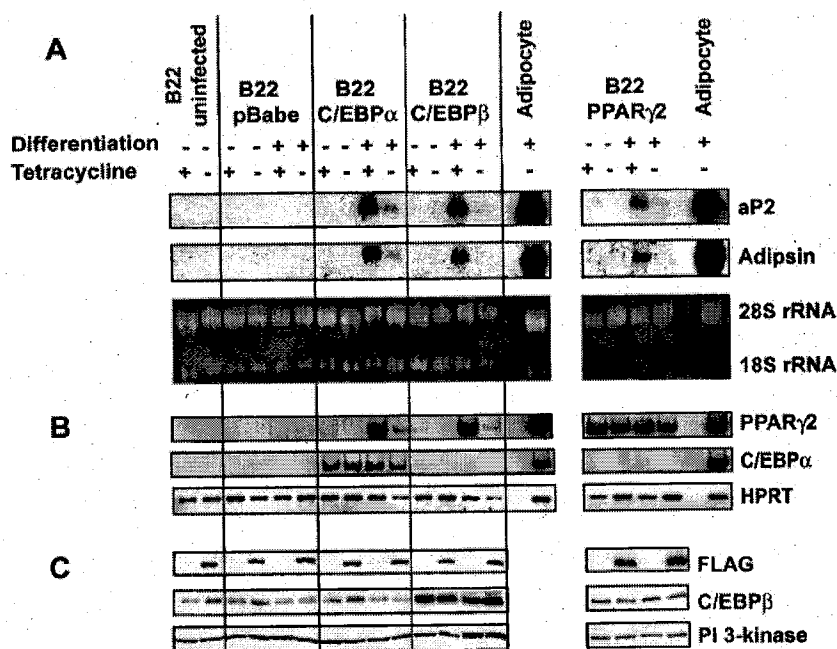


FIG. 2. Expression of dominant-negative BRG1 blocks the ability of PPAR $\gamma$ 2, C/EBP $\alpha$ , or C/EBP $\beta$  to induce adipogenesis in fibroblast cells. B22 cells were infected with retroviral vectors encoding PPAR $\gamma$ 2, C/EBP $\alpha$ , or C/EBP $\beta$ , grown in the presence or absence of tetracycline, and differentiated for 7 days in the presence or absence of differentiation cocktail. Samples in lanes marked Adipocyte were taken from 3T3-L1 preadipocytes differentiated for 7 days. (A) Northern blot showing levels of aP2 and adipsin mRNA. Ethidium bromide staining of rRNA is shown as a control. (B) RT-PCR showing levels of PPAR $\gamma$ 2 and C/EBP $\alpha$  mRNA. HPRT mRNA levels are shown as a control. (C) Western blot showing levels of FLAG-tagged dominant-negative BRG1, C/EBP $\beta$ , and PI3-kinase.

**Histone hyperacetylation and binding of C/EBP factors to the PPAR $\gamma$ 2 promoter are independent of Brg1-based SWI/SNF activity.** The mouse PPAR $\gamma$ 2 promoter has been defined but not well characterized functionally. Of interest, however, is the presence of multiple C/EBP binding sites in the promoter, including two half-consensus sites around -325 relative to the mRNA start site that contribute to activation of PPAR $\gamma$ 2 reporter genes (16). To temporally analyze factor interactions at the endogenous PPAR $\gamma$ 2 promoter, we performed ChIP experiments and amplified either PPAR $\gamma$ 2 promoter sequences or  $\beta$ -actin sequences as a control. Because we forced differentiation by ectopically expressing C/EBP $\alpha$  before inducing differentiation, we were not surprised to see interaction of C/EBP $\alpha$  with the PPAR $\gamma$ 2 promoter at the initiation of the differentiation process and throughout the time course. This observation likely reflects and explains the capacity of C/EBP $\alpha$  to initiate the adipogenic gene expression program in nonadipogenic cells. The presence of dominant-negative BRG1 had no or little effect on C/EBP $\alpha$  interactions (Fig. 5A). In differentiating adipocytes, there is a temporal order to expression of the C/EBP family members, where C/EBP $\beta$  and C/EBP $\delta$  are rapidly induced and then are shut off over the first few days of differentiation. Subsequently, C/EBP $\alpha$  is induced at about day 2 to 3 and is maintained during differentiation (reviewed in reference 21). We therefore examined interactions of C/EBP $\beta$  and C/EBP $\delta$  with the PPAR $\gamma$ 2 promoter. Somewhat surprisingly, both C/EBP $\beta$  and C/EBP $\delta$  showed a robust induction of occupancy of the promoter at days 1 to 2 of differentiation, despite the presence of ectopic C/EBP $\alpha$  and regardless of the presence of dominant-negative BRG1 (Fig. 5A). Thus, there

appears to be a preference during the early stages of differentiation for interaction with C/EBP $\beta$  and C/EBP $\delta$ . In addition, binding of these factors does not require functional SWI/SNF enzymes.

There are a multitude of histone modifications and other ATP-dependent chromatin-remodeling activities that may mediate C/EBP factor binding in the presence of mutant SWI/SNF enzymes. We examined diacetylation of histone H3 on lysines 9 and 14 as well as tetra-acetylation of H4 (Fig. 5B). Levels of tetra-acetylated H4 were high at the beginning of the differentiation process and remained constant throughout. In contrast, there was induction of diacetylated H3 at day 1, concurrent with occupancy of the promoter by the C/EBP $\beta$  and C/EBP $\delta$  factors. Acetylation of H3 and H4 was unaffected by the presence of dominant-negative BRG1.

**C/EBP factors initiate recruitment of SWI/SNF and Pol II-associated GTFs to the promoter.** Previous work has demonstrated that both C/EBP $\alpha$  and C/EBP $\beta$  can physically interact with the SWI/SNF component hBRM in cells overexpressing both proteins (19, 32). Additionally, C/EBP $\alpha$  interacts with the Pol II-associated general transcription factors (GTFs) TFIIB and TBP (29). These data have been used to argue that the C/EBP factors can recruit SWI/SNF and GTFs to facilitate transcription. ChIP experiments with the FLAG-tagged ATPase-deficient BRG1 produced in B22 cells revealed that the mutant ATPase, when expressed, was present on the PPAR $\gamma$ 2 promoter as early as day 0 and maximally from day 1 through day 7 of differentiation (Fig. 5C). Thus, the presence of C/EBP $\alpha$  may be recruiting SWI/SNF to the promoter at or before the onset of differentiation, possibly reflecting the abil-

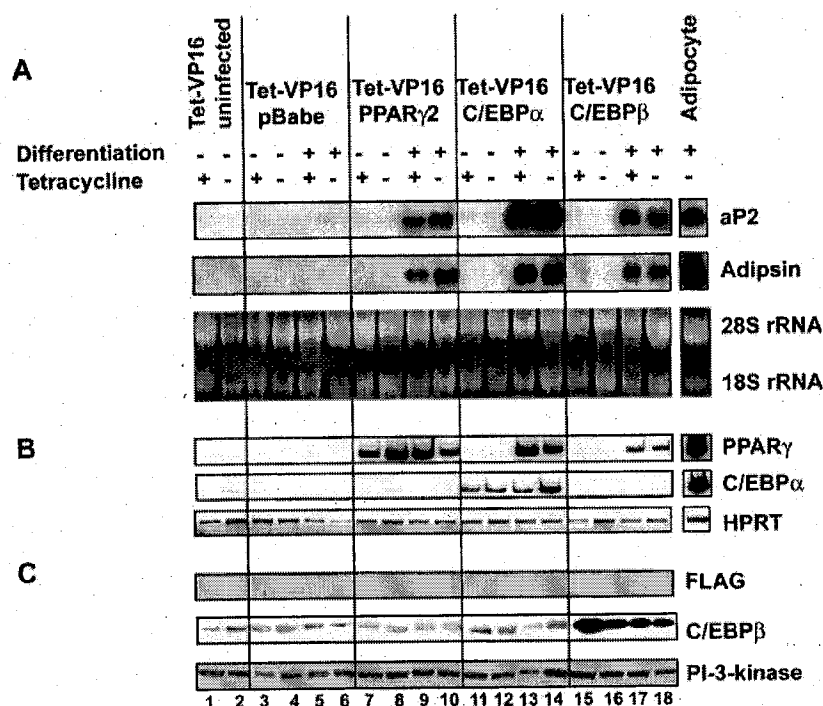


FIG. 3. PPAR $\gamma$ 2, C/EBP $\alpha$ , and C/EBP $\beta$  induce adipogenesis in Tet-VP16 control fibroblasts. Tet-VP16 cells were infected and manipulated as described for Fig. 2. (A) Northern blot showing levels of aP2 and adipsin mRNA. Ethidium bromide staining of rRNA is shown as a control. (B) RT-PCR showing levels of PPAR $\gamma$ 2 and C/EBP $\alpha$  mRNA. HPRT mRNA is shown as a control. (C) Western blot showing levels of FLAG-tagged dominant-negative BRG1, C/EBP $\beta$ , and PI3-kinase.

ity of these proteins to physically interact. ChIP analysis of Brg1 and the SWI/SNF component In11 revealed kinetics of promoter occupancy that were similar to each other and to FLAG-tagged mutant BRG1. Promoter occupancy in cells not expressing the dominant-negative BRG1 (plus tetracycline) differed in that interaction of SWI/SNF components peaked at

day 2, which coincides with full induction of PPAR $\gamma$ 2 transcription (Fig. 4). The observed interaction of the mutant BRG1 with the promoter on day 0 may be due to the modest overexpression of total Brg1 in the cells expressing mutant BRG1 (Fig. 4).

Analyses of phosphorylated RNA Pol II and its associated

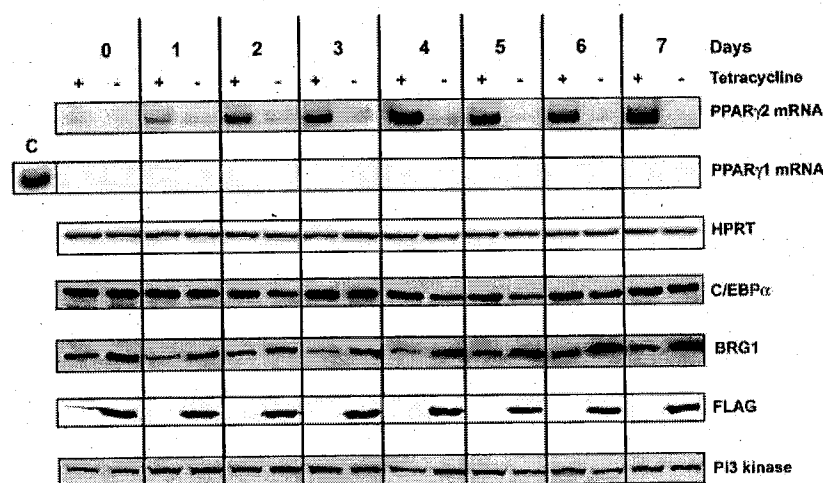


FIG. 4. Expression levels of adipogenic regulators during differentiation of fibroblast cells along the adipogenic pathway. B22 cells were infected with a retroviral vector encoding C/EBP $\alpha$ , grown in the presence or absence of tetracycline, and differentiated. Expression levels of adipogenic regulators are given as a function of time of differentiation. PPAR $\gamma$ 2 and PPAR $\gamma$ 1 transcript levels were measured by RT-PCR. HPRT levels are shown as a control. The lane marked C is a positive control from day 7 differentiated 3T3-L1 preadipocytes. C/EBP $\alpha$ , total Brg1, dominant-negative, FLAG-tagged BRG1, and PI3-kinase levels were measured by Western blotting.

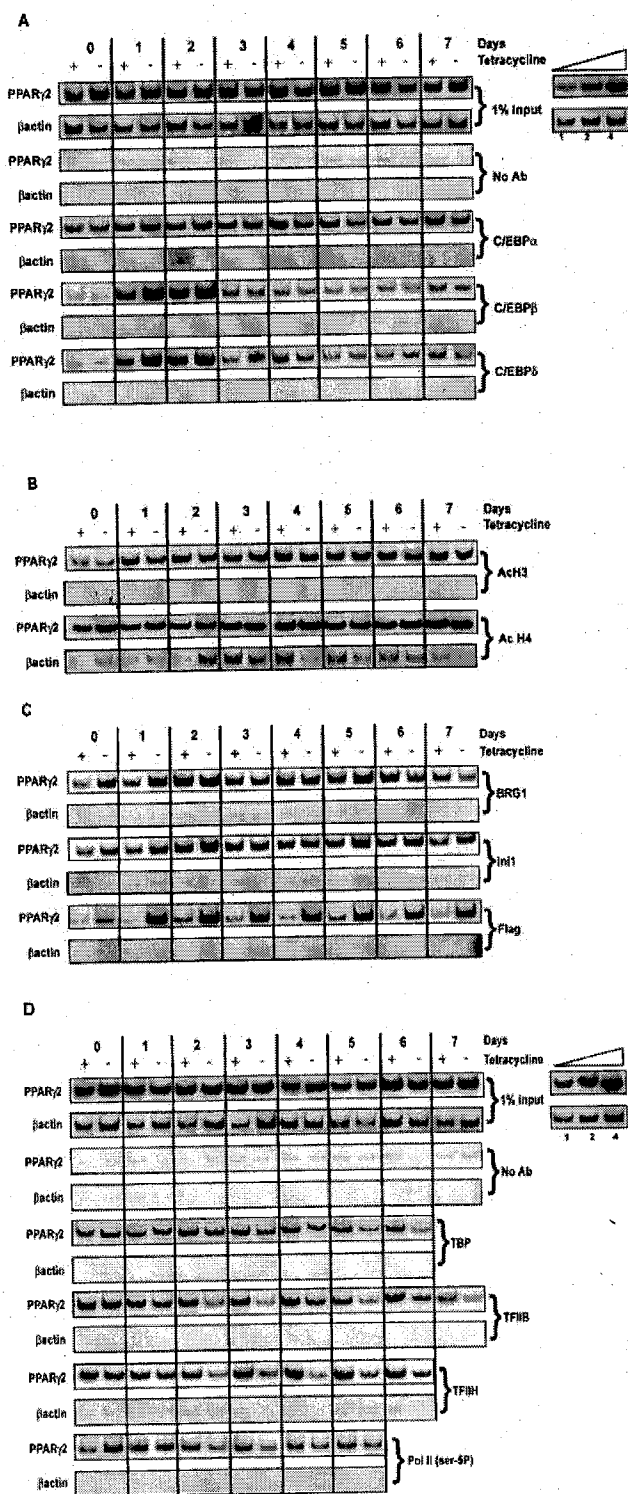


FIG. 5. ChIP analysis of interactions on the PPAR $\gamma$ 2 promoter as a function of time of differentiation. B22 cells were infected with a retroviral vector encoding C/EBP $\alpha$ , grown in the presence or absence of tetracycline, and differentiated. One percent of input for each experiment. Portions of the PPAR $\gamma$ 2 promoter or the  $\beta$ -actin 5' untranslated region and coding sequence were amplified from each sample. (A) Levels of interactions with C/EBP $\alpha$ , - $\beta$ , and - $\delta$ . (B) Levels of interactions with tetra-acetylated H4 and K9-, K14-diacetylated H3. (C) Levels of interactions with total Brg1, dominant-negative, FLAG-

GTFs, TBP, TFIIB, and TFIIF, indicated that each of these factors is present on the PPAR $\gamma$ 2 promoter on day 0, and occupancy in the presence of the dominant-negative SWI/SNF complex is essentially unaffected on days 0 and 1 (Fig. 5D). Thus, SWI/SNF function is not required to assemble these factors onto the promoter in the presence of ectopic C/EBP $\alpha$ . However, the presence of the C/EBP factors, Brg1-based SWI/SNF enzyme, and these GTFs on the promoter are not sufficient to initiate transcription at day 0. Maximal induction of PPAR $\gamma$ 2 transcription in the presence of functional SWI/SNF enzymes occurs on day 2 (Fig. 4). In contrast, starting on day 2 and continuing throughout the rest of the time course, the association of TBP, TFIIB, Pol II, and especially TFIIF is compromised in cells expressing the dominant-negative BRG1 (Fig. 5D). Western blot analysis of these GTFs and Pol II shows that the levels of these factors are not altered by differentiation or by expression of the dominant-negative BRG1 (data not shown). Thus, on or about day 2 of differentiation, SWI/SNF function is necessary to maintain the PIC on the PPAR $\gamma$ 2 promoter and to promote transcription.

**Examination of PPAR $\gamma$ 2 promoter accessibility and activation in differentiating 3T3-L1 preadipocytes.** Although forced differentiation of fibroblasts has been utilized for many years as a model system and has been essential in the identification and characterization of adipogenic regulatory proteins, we were concerned that the events leading to activation of the PPAR $\gamma$ 2 promoter under conditions where the C/EBP $\alpha$  activator was prematurely expressed might not precisely reflect the events that occurred during differentiation of a committed preadipocyte cell. We therefore examined PPAR $\gamma$ 2 activation in differentiating 3T3-L1 preadipocytes.

Initially, we examined changes in PPAR $\gamma$ 2 promoter structure as reflected by increases in nuclease accessibility. A schematic of the promoter is presented in Fig. 6A. Examination of two StuI sites, an EcoNI site, and a PstI site located between -100 and -1000 on the PPAR $\gamma$ 2 promoter revealed a dramatic increase in accessibility in this region on day 1 of the differentiation process (Figs. 6B to D), reflecting changes in promoter structure. A previous report (34) indicated that two DNase I hypersensitive sites exist on the PPAR $\gamma$ 2 promoter in undifferentiated 3T3-L1 cells (Fig. 6A). Our analysis indicated these DNase I hypersensitive sites were difficult to detect in undifferentiated, day 0 cells. However, by day 1, they were clearly visible, indicating that these hypersensitive sites are induced by differentiation (Fig. 6E). Together, the results clearly demonstrate a change in PPAR $\gamma$ 2 promoter structure on day 1 of 3T3-L1 differentiation, prior to induction of PPAR $\gamma$ 2 gene expression.

Northern analysis of PPAR $\gamma$  transcript levels revealed a robust induction of expression on day 2 of 3T3-L1 preadipocyte differentiation (Fig. 7A), in agreement with prior studies

tagged BRG1, and In1. (D) Levels of interactions with TBP, TFIIB, TFIIF p89, and Pol II phosphorylated on Ser-5 of the CTD. Inset: linearity controls for PCR amplifications. Each ChIP was repeated in two to five independent experiments. The data shown in parts A, B, and C derive from a single differentiation experiment. ChIPs in part D derive from a different differentiation experiment.

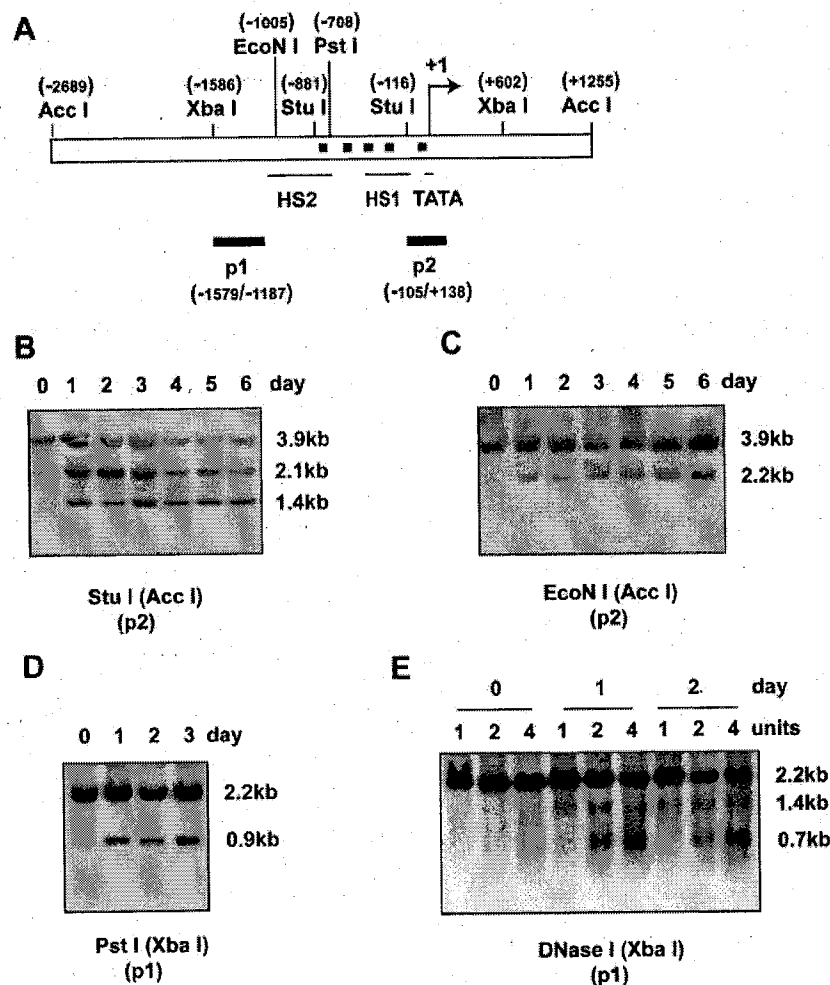


FIG. 6. Changes in PPAR $\gamma$ 2 promoter accessibility occur on day 1 of differentiation. 3T3-L1 preadipocytes were induced to differentiate, and nuclei were harvested at the indicated times for nuclease accessibility experiments. (A) Schematic of the PPAR $\gamma$ 2 promoter. The locations of relevant restriction enzyme sites are indicated, as are the previously reported DNase I hypersensitive sites (HS1 and HS2) (34). Black squares represent potential C/EBP binding sites. Black bars P1 and P2 indicate the fragments used as Southern blot probes. (B to D) Restriction enzyme accessibility of EcoNI, StuI, and PstI. The enzymes used for flanking digests are indicated in parentheses. (E) Accessibility of DNase I.

(45). Thus, induction of the PPAR $\gamma$ 2 is temporally distinct from changes in promoter accessibility.

**Binding of C/EBP factors, hyperacetylated histones, Pol II, and most GTFs precedes the onset of PPAR $\gamma$ 2 induction.** ChIP experiments to identify interactions of the C/EBP family of factors with the PPAR $\gamma$ 2 promoter showed an induction of C/EBP $\beta$  and C/EBP $\delta$  binding on days 1 and 2 of differentiation, respectively (Fig. 7B), largely consistent with the expression patterns of these C/EBPs in differentiating 3T3-L1 cells and with the ChIP results from the forced fibroblast system (Fig. 5A). Thus, interaction of C/EBP $\beta$  correlated temporally with changes in nuclease accessibility on the promoter. Over days 2 and 3, C/EBP $\beta$  and  $\delta$  disappeared from the promoter, concurrent with the appearance of C/EBP $\alpha$  (Fig. 7B). Thus, in differentiating 3T3-L1 cells, the C/EBP binding sites in the PPAR $\gamma$ 2 promoter undergo a transition of factor occupancy from C/EBP $\beta$  to C/EBP $\alpha$ .

As in the C/EBP $\alpha$  forced fibroblast differentiation experiments, ChIP analysis of tetra-acetylated H4 indicated that lev-

els were high prior to differentiation (Fig. 7C). In contrast to the forced fibroblasts, levels of diacetylated H3 were present on the PPAR $\gamma$ 2 promoter at day 0 but increased on day 3 (Fig. 7C). This increase clearly occurred after the onset of PPAR $\gamma$ 2 expression but correlated with the transition from binding of C/EBP $\beta$  and  $\delta$  to C/EBP $\alpha$ . Whether the change in diacetylated H3 levels is a cause or effect of the transition to C/EBP $\alpha$  binding has not been determined. Diacetylated H3 was also present at relatively high levels on the control  $\beta$ -actin sequences.

Examination of phosphorylated Pol II and associated GTFs by ChIP showed that Pol II, TBP, and TFIIB became associated with the promoter on day 1 of differentiation, prior to expression of PPAR $\gamma$ 2 (Fig. 7D). Collectively, these data agree with the results obtained with the fibroblast differentiation model and indicate that much of the transcriptional machinery is present on the promoter prior to the onset of gene activation.

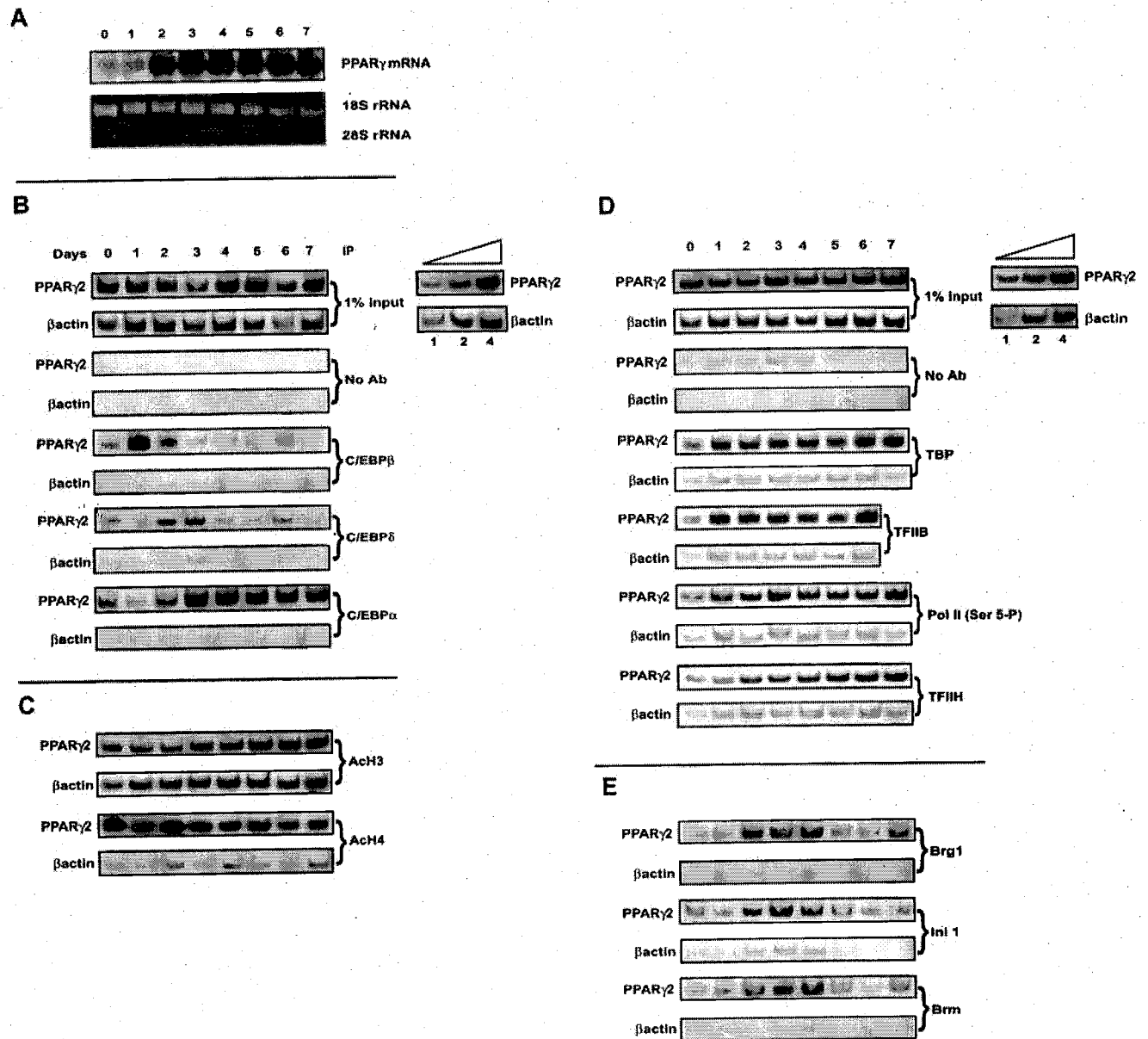


FIG. 7. (A) PPAR $\gamma$  expression levels in differentiating 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate. PPAR $\gamma$  expression levels were analyzed by Northern blotting. Ethidium bromide-stained 28S and 18S rRNA is shown as a control. (B to E) ChIP analysis of interactions on the PPAR $\gamma$ 2 promoter as a function of time of differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate and processed for ChIP assays at the indicated times. Portions of the PPAR $\gamma$ 2 promoter or the  $\beta$ -actin gene were amplified from each sample. (B) Levels of interactions with C/EBP $\alpha$ ,  $\beta$ , and  $\delta$ . (C) Levels of interactions with tetra-acetylated H4 and K9-, K14-diacetylated H3. (D) Levels of interactions with TBP, TFIIB, TFIH p89, and Pol II phosphorylated on Ser-5 of the CTD. (E) Levels of interactions with Brg1, Brm, and Ini1. Inset: linearity controls for PCR amplifications. Each ChIP was repeated in three to five independent experiments. The data shown in parts B, C, and E derive from a single differentiation experiment. ChIPs in part D derive from a different differentiation experiment.

**PPAR $\gamma$ 2 expression is coincident with the binding of TFIH and SWI/SNF components to the promoter.** In contrast to the other factors examined, the increase in association of TFIH did not occur until day 2 of 3T3-L1 differentiation (Fig. 7D). Similarly, association of the SWI/SNF components Brg1, Brm, and Ini1 occurred on day 2 (Fig. 7E), coincident with the onset of PPAR $\gamma$ 2 transcription. Unlike the case with Pol II and all of the GTFs examined, the interaction of SWI/SNF subunits was

transient, with only background levels present on the promoter after day 4. The Ini1 subunit is common to all SWI/SNF enzymes examined, but the Brg1 and Brm ATPases form distinct SWI/SNF enzymes, indicating that at least two distinct forms of the enzyme are present at the PPAR $\gamma$ 2 promoter at the onset of transcription. The data indicate that histone hyperacetylation, changes in promoter structure, C/EBP activator binding, and association of phosphorylated Pol II and multiple GTFs

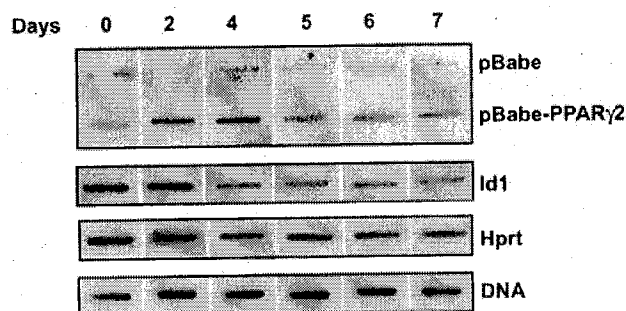


FIG. 8. The rate of PPAR $\gamma$  transcription decreases after day 4 of 3T3-L1 differentiation. Nuclear run-on assays were performed on nuclei isolated from differentiating 3T3-L1 cells on the days indicated. Run-on transcripts were hybridized to 3T3-L1 genomic DNA, linearized pBABE vector, linearized pBABE containing the PPAR $\gamma$ 2 cDNA, or PCR products corresponding to Id1 or Hprt cDNAs.

with the PPAR $\gamma$ 2 promoter occur over the first 24 h of adipocyte differentiation but that these events are not sufficient to initiate transcription. PPAR $\gamma$ 2 expression is facilitated by association of TFIID and SWI/SNF enzymes on day 2 of differentiation.

**A decrease in the rate of PPAR $\gamma$  transcription correlates with the dissociation of SWI/SNF enzyme components from the PPAR $\gamma$ 2 promoter.** The ChIP experiments shown in Fig. 7E clearly demonstrate that SWI/SNF components were no longer stably associated with the PPAR $\gamma$ 2 promoter after day 4 of differentiation, even though the GTFs and C/EBP $\alpha$  remained. Analysis of stable mRNA levels indicated that PPAR $\gamma$  mRNA was abundant on days 5 to 7 (Fig. 7A). Two possible explanations for these data exist. The promoter may achieve a stable structure that is permissive for continued transcription in the absence of SWI/SNF, or the transcription of PPAR $\gamma$ 2 decreases or stops after day 4 and the PPAR $\gamma$  message observed on days 5 to 7 represents stable mRNAs produced on day 4 or earlier.

To distinguish between these possibilities, we performed a time course of nuclear run-on experiments. Linearized plasmid DNA containing the PPAR $\gamma$ 2 cDNA or empty vector was immobilized on membranes and hybridized to radiolabeled run-on transcripts produced by nuclei isolated from differentiating 3T3-L1 cells on the days indicated. The data demonstrated induction of PPAR $\gamma$  transcription on day 2, continued transcription on day 4, but little transcription on days 5 to 7 (Fig. 8). Thus, the rate of PPAR $\gamma$  transcription decreased between days 4 and 5 and correlated with the loss of SWI/SNF enzyme components on the PPAR $\gamma$ 2 promoter (Fig. 7E). In contrast, the rate of Id1 transcription decreased as a function of differentiation (Fig. 8), in agreement with previously published results from the differentiation of human preadipocyte cells (26). We utilized nonspecific hybridization to plasmid sequences and hybridization to 3T3-L1 genomic DNA as controls, as has been described (10, 24, 41, 47), since we were unable to identify other genes that gave a constant rate of transcription over the differentiation time course. The rates for *Hprt*, *36B4*, and *Gapdh* all decreased over the 7-day time course (Fig. 8 and data not shown).

We note that the immobilized template contained the entire

PPAR $\gamma$ 2 cDNA; thus, PPAR $\gamma$ 1 transcription would also have been detected. We were unable to detect run-on signal when only the short, 121-bp portion of the cDNA unique to PPAR $\gamma$ 2 (52) was used for the hybridizations (data not shown). However, PPAR $\gamma$  mRNA at day 5 of 3T3-L1 differentiation and beyond is predominantly expressed from the PPAR $\gamma$ 2 promoter (38, 44). Thus, the data most likely reflect a decrease in the rate of PPAR $\gamma$ 2 or in the rates of both PPAR $\gamma$ 2 and PPAR $\gamma$ 1 transcription.

## DISCUSSION

PPAR $\gamma$  is involved in adipocyte differentiation, insulin sensitivity and diabetes, atherosclerosis, and the control of cell proliferation in some cancer cells (reviewed in references 5 and 37). Consequently, its function has been the subject of intense investigation. Relatively little, however, is known about the mechanisms controlling its expression. Here we utilized two different cellular models for adipocyte differentiation to temporally describe the molecular interactions that occur at the promoter of the inducible PPAR $\gamma$ 2 gene during adipocyte differentiation, with particular emphasis on the requirement for SWI/SNF chromatin-remodeling enzymes. Through use of a differentiation system driven by introduction of the adipogenic regulator, C/EBP $\alpha$ , we demonstrate a requirement for SWI/SNF enzymes in the activation of the PPAR $\gamma$  regulator as well as in the activation of adipogenic marker genes expressed later during differentiation. Moreover, these experiments revealed that this requirement for SWI/SNF enzymes was relatively late in the cascade of events leading to PPAR $\gamma$ 2 activation. Activator binding, Pol II and associated GTF interactions at the promoter, and histone H3 and H4 acetylation occurred prior to and independently of SWI/SNF function. Instead, the data revealed a role for SWI/SNF enzymes in the function of the PIC components at the promoter at the time of transcriptional activation.

Because of the inherent differences between forcing differentiation of fibroblasts into the adipocyte lineage and genuine preadipocyte differentiation, we analyzed differentiation of 3T3-L1 preadipocytes and confirmed both the general order of events that occur during PPAR $\gamma$ 2 activation and a role for SWI/SNF enzymes in facilitating PIC function. We expect that the differences exhibited by the two systems reflect the ability of the C/EBP $\alpha$  activator to recruit GTFs and SWI/SNF enzymes (32) prematurely at the initiation of the forced differentiation program. Despite the differences, it is important to note that the data from both systems are consistent with a need for SWI/SNF enzymes to promote the function of the PIC.

The order of events occurring in differentiating 3T3-L1 cells is diagrammed schematically in Fig. 9. Acetylation of H4 occurs before the onset of differentiation, followed by concurrent changes in promoter accessibility, binding of the C/EBP $\beta$  and  $\delta$  activators, and assembly of Pol II and most of the GTFs on day 1 of differentiation. Subsequently, on day 2, SWI/SNF enzymes and TFIID associate with the promoter, indicating that the SWI/SNF enzymes likely facilitate completion of the preinitiation complex, thereby permitting PPAR $\gamma$ 2 transcription to commence. On day 3, there is both an increase in the levels of H3 acetylation and a transition from binding of C/EBP $\beta$  and  $\delta$  to binding of C/EBP $\alpha$ . Which event, if either,



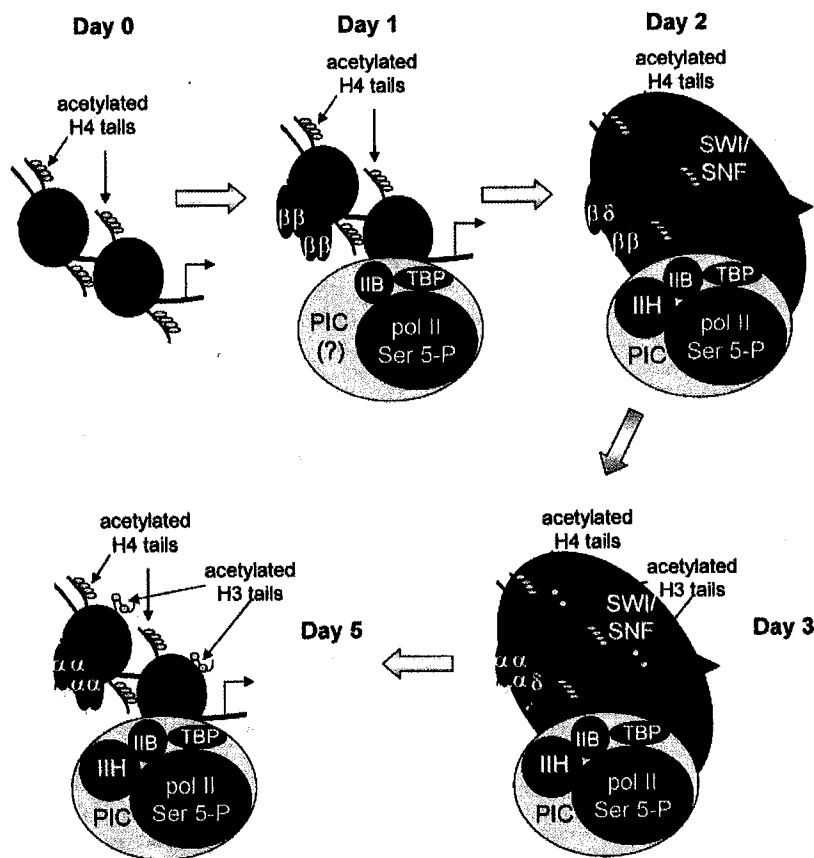


FIG. 9. Schematic model of the temporal changes in factor interactions at the PPAR $\gamma$ 2 promoter during 3T3-L1 preadipocyte differentiation. Nucleosome positions are presented for illustrative purposes only.

is causal remains to be determined. Following day 4, the SWI/SNF enzymes disappear from the promoter and the rate of PPAR $\gamma$  transcription drops, indicating that the presence of SWI/SNF is required for continued transcription.

The data from differentiating 3T3-L1 preadipocytes indicate that both BRG1 and BRM are present on the PPAR $\gamma$ 2 promoter, suggesting that both complexes are contributing to function. Alternatively, the two complexes could be redundant in function, and the presence of either SWI/SNF enzyme might be sufficient. Our studies using cell lines that inducibly express dominant-negative BRG1 or BRM also suggest that both ATPases are required for PPAR $\gamma$ 2 activation and adipocyte differentiation. However, because the BRG1 and BRM SWI/SNF complexes share multiple subunits, it is possible that expression of one mutant ATPase deleteriously affects both complexes by sequestering subunits from the other, endogenous ATPase. Thus, we cannot rigorously state at present whether BRG1, BRM, or both are required for PPAR $\gamma$ 2 activation and adipocyte differentiation.

One of the interesting results from our studies is the demonstration that the C/EBP binding sites undergo a transition during the time course of differentiation from binding C/EBP $\beta$  and  $\delta$  to binding C/EBP $\alpha$ . Although the kinetics of expression for these factors has long supported this idea, this is the first documentation that such a transition occurs at a promoter

expressed during adipogenesis. These results differ from previously published work that showed that C/EBP $\alpha$  and  $\delta$ , but not C/EBP $\beta$ , could bind to the C/EBP sites in the PPAR $\gamma$ 2 promoter in a gel shift study and could activate a transiently transfected PPAR $\gamma$ 2 reporter plasmid (16). The differences between the studies may be attributed to the likelihood that the chromatin structure at the genomic locus differs from that on a transfected template and undergoes changes during the differentiation process that affect factor interactions.

Previous reports documenting the potential of C/EBP $\alpha$  and  $\beta$  to physically interact with BRM in cells transfected with both the C/EBP isoform and BRM suggested that these factors may recruit SWI/SNF enzymes (19, 32). Our temporal analysis of factor interactions on the PPAR $\gamma$ 2 promoter during C/EBP $\alpha$ -driven differentiation strongly suggests that targeting of SWI/SNF by C/EBP $\alpha$  can occur. However, C/EBP $\alpha$  is prematurely present on the promoter in this differentiation system; the temporal differences in the appearance of the different C/EBP factors on the PPAR $\gamma$ 2 promoter in the differentiating 3T3-L1 adipocytes suggest that in a more natural differentiation context, C/EBP $\beta$  and  $\delta$  may target SWI/SNF enzymes, which then later recruit C/EBP $\alpha$ .

Changes in nuclease accessibility and the binding of C/EBP factors, Pol II, and many of the GTFs on day 1 prior to the appearance of SWI/SNF enzymes on the PPAR $\gamma$ 2 promoter



indicate that other factors must control the initial accessibility of these promoter sequences. Changes in H4 acetylation did not correlate with initial factor binding to the promoter. Changes in other histone modifications at the PPAR $\gamma$ 2 promoter have not been tested but potentially could mediate factor accessibility. Alternatively, a different ATP-dependent remodeling enzyme(s) could alter chromatin structure and promote activator binding prior to SWI/SNF function. This hypothesis is supported by in vitro studies showing that ISWI containing chromatin-remodeling enzymes facilitated stable interaction of RAR $\alpha$ :RXR on chromatin templates prior to SWI/SNF enzyme-mediated stimulation of transcription (15). Finally, transcriptional regulators present on the promoter prior to differentiation, such as GATA-2 and -3 (43) and KLF2 (4), might influence chromatin structure in a manner that promotes the transition to an actively transcribing gene.

Our analysis of GTF interactions on the PPAR $\gamma$ 2 promoter also revealed that serine 5-phosphorylated Pol II is present at the promoter before TFIIF. TFIIF contains a kinase activity that is capable of phosphorylating the Pol II CTD; however, the temporal order of factor appearance at the PPAR $\gamma$ 2 promoter suggests that either a different kinase is responsible for the CTD phosphorylation or TFIIF mediates CTD phosphorylation independently of promoter binding. Additionally, the presence of Pol II phosphorylated at serine 5 of the CTD raises the possibility that the polymerase may be transcriptionally engaged at day 1 and that the inclusion of SWI/SNF enzymes and TFIIF on day 2 promotes release of the polymerase and/or elongation. Multiple other genes are regulated at the level of transcriptional elongation, including *hsp70*, where elongation can be stimulated by SWI/SNF enzymes in vitro and in vivo (6, 9).

The concurrent entry of SWI/SNF enzymes and TFIIF onto the PPAR $\gamma$ 2 promoter in differentiating 3T3-L1 cells suggests that SWI/SNF facilitates the interaction of TFIIF with the rest of the preinitiation complex. Such a role for SWI/SNF enzymes has not previously been documented. However, the data presented here agree with and extend findings from temporal analyses of other mammalian promoters that show the following: (i) some form of histone hyperacetylation precedes association of SWI/SNF with the promoter, and (ii) SWI/SNF enzymes work late in the activation of many genes, typically after some, if not most, of the components driving transcription have associated with the promoter (1, 23, 25, 40). Thus, the data we present on PPAR $\gamma$ 2 activation during adipocyte differentiation support a general model where SWI/SNF enzymes function subsequent to activator binding by completing or stabilizing preinitiation complex formation and/or by promoting promoter clearance and elongation.

#### ACKNOWLEDGMENTS

We are grateful to B. Spiegelman and S. Sif for providing reagents and to P. Pekala, R. Kingston, C. Peterson, and members of the Imbalzano and Peterson labs for suggestions, advice, and discussion.

This work was supported by the UMMS Diabetes Endocrine Research Center, by a Scholar Award from the Leukemia and Lymphoma Society, and by an NIH grant to A.N.I.

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# APPENDIX B

# Temporal recruitment of CCAAT/enhancer-binding proteins to early and late adipogenic promoters *in vivo*

N Salma, H Xiao and A N Imbalzano

Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655, USA

(Requests for offprints should be addressed to A N Imbalzano; Email: anthony.imbalzano@umassmed.edu.)

## Abstract

The CCAAT/enhancer-binding protein (C/EBP) family of transcriptional regulators is critically important for the activation of adipogenic genes during differentiation. The C/EBP $\beta$  and  $\delta$  isoforms are rapidly induced upon adipocyte differentiation and are responsible for activating the adipogenic regulators C/EBP $\alpha$  and peroxisome proliferator activated receptor (PPAR) $\gamma$ 2, which together activate the majority of genes expressed in differentiating adipocytes. However, mitosis is required following the induction of adipogenesis, and the activation of C/EBP $\alpha$  and PPAR $\gamma$ 2 gene expression is delayed until cell division is underway. Previous studies have used electromobility shift assays to suggest that this delay is due, at least in part, to a delay between the induction of C/EBP $\beta$  protein levels and the acquisition of DNA binding capacity by C/EBP $\beta$ . Here we used *in vivo* chromatin immunoprecipitation analysis of the C/EBP $\alpha$ , PPAR $\gamma$ 2, resistin, adiponectin, and leptin promoters to examine the kinetics of C/EBP protein binding to adipogenic genes in differentiating cells. In contrast to prior studies, we determined that C/EBP $\beta$  and  $\delta$  were bound to endogenous regulatory sequences controlling the expression of these genes within 1–4 h of adipogenic induction. These results indicated that C/EBP $\beta$  and  $\delta$  bind not only to genes that are induced early in the adipogenic process but also to genes that are induced much later during differentiation, without a delay between induction of C/EBP protein levels and DNA binding by these proteins. We also showed that each of the genes examined undergoes a transition *in vivo* from early occupancy by C/EBP $\beta$  and  $\delta$  to occupancy by C/EBP $\alpha$  at times that correlate with the induction of C/EBP $\alpha$  protein levels, demonstrating the generality of the transition during adipogenesis and indicating that the binding of specific C/EBP isoforms does not correlate with timing of expression from each gene. We have concluded that C/EBP family members bind to adipogenic genes *in vivo* in a manner that follows the induction of C/EBP protein synthesis.

*Journal of Molecular Endocrinology* (2006) **36**, 139–151

## Introduction

The major transcription factor families involved as key regulators of adipocyte differentiation include the nuclear hormone receptor peroxisome proliferator activated receptor (PPAR) $\gamma$  and the CCAAT/enhancer-binding proteins (C/EBPs) (Darlington *et al.* 1998, Fajas *et al.* 1998, Lane *et al.* 1999, Morrison & Farmer 2000, Rangwala & Lazar 2000, Rosen *et al.* 2000, Camp *et al.* 2002, MacDougald & Mandrup 2002). The C/EBP family members belong to the basic leucine zipper (bZIP) class of transcription factors, and bind to specific DNA sequences as dimers with other C/EBPs (reviewed by Lekstrom-Himes & Xanthopoulos 1998, Ramji & Foka 2002). When adipocyte differentiation is induced in preadipocyte cell lines, C/EBP $\beta$  and  $\delta$  are rapidly and transiently induced (Cao *et al.* 1991, Yeh *et al.* 1995). These regulators act synergistically to modulate the expression of C/EBP $\alpha$  and PPAR $\gamma$ 2 via interaction with the C/EBP regulatory elements present in the proximal promoters of these genes (Christy *et al.* 1991, Zhu *et al.* 1995, Clarke *et al.* 1997, Tang *et al.* 1999). Subsequently, C/EBP $\alpha$  and PPAR $\gamma$ 2 play a prominent role regulating

the expression of adipocyte genes necessary for the development of functional, mature adipocytes (Lin & Lane 1994, Tontonoz *et al.* 1994, Fajas *et al.* 1998, Wu *et al.* 1999, Rosen *et al.* 2000).

The essential role of the C/EBP proteins in adipocyte differentiation has been established. Ectopic expression of C/EBP $\beta$  or C/EBP $\alpha$  is able to force non-adipogenic cell lines to differentiate into adipocytes (Freytag *et al.* 1994, Wu *et al.* 1995, Yeh *et al.* 1995). In contrast, expression of antisense C/EBP $\alpha$  RNA in preadipocyte cell lines prevents the differentiation program (Lin & Lane 1992). Additionally, analysis of promoter regions of adipogenic genes as well as studies of knockout mice have demonstrated the involvement of this family of transcription factors in regulating adipogenesis and other important physiological processes (reviewed by Cornelius *et al.* 1994, MacDougald & Lane 1995, Tanaka *et al.* 1997, Darlington *et al.* 1998, Gregoire *et al.* 1998, Lane *et al.* 1999, Rangwala & Lazar 2000, Ramji & Foka 2002).

Most regulatory sequences controlling the expression of adipocyte-specific genes contain at least one functional C/EBP binding site, from which transactivation

is mediated by members of the C/EBP family (reviewed by Hwang *et al.* 1997, Gregoire *et al.* 1998, Cowherd *et al.* 1999, Morrison & Farmer 2000, Rangwala & Lazar 2000). Work on the PPAR $\gamma$ 2 and C/EBP $\alpha$  promoters has focused on the role of C/EBP $\beta$  and  $\delta$  as primary inducers of the expression of key regulators. PPAR $\gamma$ 2 and C/EBP $\alpha$  are expressed by day 2 of the differentiation process, following one or two rounds of mitotic clonal expansion. The induction of C/EBP $\beta$  and  $\delta$  protein levels, however, occurs almost immediately after addition of differentiation inducers at the onset of differentiation (Cao *et al.* 1991, Yeh *et al.* 1995, Darlington *et al.* 1998, Tang & Lane 1999). Thus, even though both C/EBP $\beta$  and  $\delta$  are expressed at high levels at the beginning of the differentiation program, the target genes C/EBP $\alpha$  and PPAR $\gamma$ 2 are not expressed until nearly 2 days later (Lane *et al.* 1999, Rosen *et al.* 2000). Previous work using electrophoretic mobility shift assay (EMSA) analysis (Tang & Lane 1999) determined that the lag in C/EBP $\alpha$  expression is due to a delay in acquisition of C/EBP $\beta$  and  $\delta$  DNA binding activity, therefore pausing the transcriptional activation of the gene. The need for such a delay fits well with the numerous observations that C/EBP $\alpha$  is anti-mitotic in both preadipocytes as well other cell types (Umek *et al.* 1991, Lin *et al.* 1993, Timchenko *et al.* 1996, Wang *et al.* 2001).

The transcriptional activity of the C/EBP $\beta$  protein is regulated at several levels, including transcription, translation, association with other proteins, and post-translational modification, which includes the regulation of the phosphorylated state by multiple effectors. Multiple phosphorylation sites have been characterized on C/EBP $\beta$ , some of which result in attenuation or enhancement of DNA binding and transactivation activity (Mahoney *et al.* 1992, Trautwein *et al.* 1993, 1994, Park *et al.* 2004a, Tang *et al.* 2005 and references therein). At least some of these phosphorylation events occur almost simultaneously with induction of C/EBP $\beta$  levels, and it has been suggested that phosphorylation causes a conformational change in C/EBP $\beta$  that transforms it from a repressor to an activator (Kowenz-Leutz *et al.* 1994).

We previously examined the temporal interactions of C/EBP family members, modified histones, and subunits of the SWI/SNF family of ATP-dependent chromatin remodeling enzymes at the PPAR $\gamma$ 2 promoter as a function of adipocyte differentiation (Salma *et al.* 2004). In that study, we used chromatin immunoprecipitation (ChIP) assays to determine that C/EBP $\beta$  was bound at the PPAR $\gamma$ 2 promoter at 24 and 48 h following the initiation of the differentiation program in both differentiating 3T3-L1 cells and in fibroblasts forced to differentiation into adipocytes by ectopic expression of C/EBP $\alpha$  (Salma *et al.* 2004). Continuation of these ChIP studies at earlier time-points revealed that the C/EBP $\beta$

and  $\delta$  isoforms were found on the regulatory regions of numerous adipocyte-specific genes in differentiating 3T3-L1 cells within a few hours of the onset of differentiation. These genes included PPAR $\gamma$ 2, C/EBP $\alpha$ , and several genes expressed later in the differentiation process. Thus the binding of C/EBP $\beta$  and  $\delta$  to adipogenic genes in cells correlates with the kinetics of C/EBP $\beta$  and  $\delta$  expression. In addition, binding of C/EBP $\beta$  and  $\delta$  was replaced at each of the loci by the binding of C/EBP $\alpha$ . C/EBP $\alpha$  binding was noted on each promoter between 20 and 48 h post-differentiation, indicating that it was associating with promoters as soon as it was expressed and that binding did not strictly correlate with the time at which the locus became transcriptionally active. We have concluded that the binding of C/EBP transcription factors to regulatory sequences controlling the expression of adipogenic genes *in vivo* occurs rapidly and without significant delay following the induction of each isoform during adipogenic differentiation.

## Materials and methods

### Cell lines and differentiation methods

3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA), maintained in growth medium consisting of Dulbecco's minimum essential medium containing 10% calf serum, and induced to differentiate as described previously (Wu *et al.* 1995). Cells were collected at 0, 1, 2, 4, 8, 12, 16, 20, and 24 h and then at 24-h intervals for 7 days after addition of differentiation cocktail (Salma *et al.* 2004) for western blot, RT-PCR, and ChIP analysis. In experiments where 3T3-L1 cells over-expressed C/EBP $\beta$ , cells were infected with pBABE retrovirus containing C/EBP $\beta$  or empty vector as described previously (Tontonoz *et al.* 1994, Salma *et al.* 2004). The generation of the retrovirus has been described previously (Pear *et al.* 1993, Salma *et al.* 2004). Samples were collected at 0, 4, 24, 48, and 168 h in the presence or absence of differentiation cocktail for western blots, RT-PCR, and ChIP analysis.

### EMSAs

Nuclear extracts isolated from 3T3-L1 cells differentiated in the presence or absence of cocktail were prepared as described (Hasegawa *et al.* 1997). The binding reaction contained 6  $\mu$ g nuclear extract and 5 fmol  $^{32}$ P-labeled double-stranded oligonucleotide probe corresponding to the region from -343 to -306 bp from the mRNA start site in the mouse PPAR $\gamma$ 2 promoter. This region contains a functional C/EBP binding site (Zhu *et al.* 1995, Clarke *et al.* 1997). Binding reactions contained 10 mM Tris-HCl (pH 7.5), 5%

glycerol, 50 mM NaCl, 0.5 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, and 0.5 mg/ml Poly (dI-dC) in a volume of 10 µl. Reactions were incubated for 30 min at room temperature and separated electrophoretically on 4% non-denaturing polyacrylamide gels made with 0.5 × Tris-borate-EDTA buffer. Some reactions were preincubated for 10 min with 1 µl IgG or anti-C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-7962) prior to addition of the labeled oligonucleotides. The sequence of the oligonucleotide probe was: 5'-TAAAAAGCAATCAATATTGAACAA TCTCTGCTCTGGTAA-3'.

### RNA analysis

RNA isolation and analysis by northern blotting have been described previously (de la Serna *et al.* 2001b). Probes were derived from plasmids containing PPARγ (provided by B Spiegelman, Dana Farber Cancer Institute, Boston, MA, USA) and the ribosomal phosphoprotein 36B4 (obtained by RT-PCR) and labeled by random priming. Washed blots were exposed to a PhosphorImager (GE Healthcare Chalfont St. Giles, UK). For RT-PCR, total RNA (3 µg) was reverse transcribed with Moloney murine leukemia virus RT (Invitrogen). cDNA was amplified by PCR with QIAGEN HotStart Taq master mix in the presence of 2 µCi [<sup>32</sup>P]dATP. The sequences of the primers were as follows: 5'-CCG GCC GCC TTC AAC GAC-3' and 5'-CTC CTC GCG GGG CTC TTG TTT-3' for C/EBPα (288 bp product); 5'-GAA CTG AGT TGT GTC CTG CT-3' and 5'-TGC ACA CTG GCA GTG ACA-3' for resistin (340 bp product); 5'-GAT CAA TGA CAT TTC ACA CA-3' and 5'-GGA CGC CAT CCA GGC TCT CT-3' for leptin (281 bp product); 5'-CAG TGG ATC TGA CGA CAC CA-3' and 5'-CGA ATG GGT ACA TTG GGA AC-3' for adiponectin (433 bp product); and 5'-CTC CAA GCA GAT GCA GCA GA-3' and 5'-TCA ATG GTG CCT CTG GAG AT-3' for the ribosomal phosphoprotein 36B4 (351 bp). The PCR conditions for leptin and 36B4 were: 95 °C for 15 min, followed by 24 cycles of: 95 °C for 30 s; 62 °C for 40 s; 72 °C for 30 s, and a final round of extension for 5 min. The PCR conditions for adiponectin were the same, except that the number of cycles was 20. For resistin, the conditions were the same as for leptin except that the annealing temperature was 58 °C. For C/EBPα, the PCR conditions were: 95 °C for 15 min, followed by 25 cycles of: 95 °C for 50 s; 66 °C for 55 s; 72 °C for 50 s, and a final round of extension for 5 min.

### Protein extracts and Western analysis

Isolation of protein and western blotting have been described (de la Serna *et al.* 2001a). Antibodies utilized

included the following from Santa Cruz Biotechnology: C/EBPα (sc-61), C/EBPβ (sc-7962), C/EBPδ (sc-151), and cyclin A (sc-596). Phosphatidylinositol 3-kinase (PI-3K) antibody (06-496) was obtained from Upstate (Charlottesville, VA, USA).

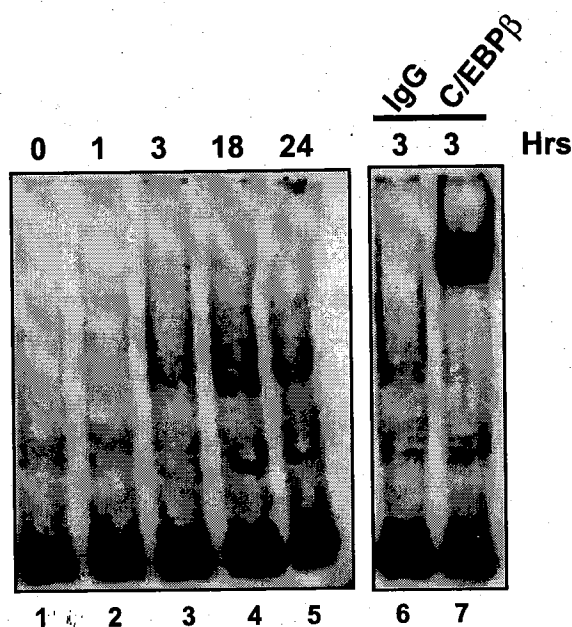
### ChIP

The ChIP procedure was adopted from the Upstate protocol and was performed as described by Salma *et al.* (2004). One-tenth of the immunoprecipitated DNA and 1% of the input DNA were analyzed by PCR. Antibodies used included Santa Cruz antibodies: C/EBPα (sc-61), C/EBPβ (sc-7962), and C/EBPδ (sc-151). PCRs were performed with QIAGEN Hot Start Taq master mix in the presence of 2 µCi [<sup>32</sup>P]dATP under the following conditions: a pre-heating at 94 °C for 15 min, followed by 24–30 cycles of 94 °C for 30 s, 62 °C for 40 s (except for PPARγ2 which was 49.5 °C), 72 °C for 30 s, followed by a 72 °C extension for 5 min. PCR products were resolved in 6–8% polyacrylamide-1 × Tris-borate-EDTA gels, dried, and exposed to a PhosphorImager. Primers used were the following: β-actin, 5'(+31) GCTTCTTTGCA GCTCCTTCGTTG-3' and 5'(+135) TTTGCACAT GCCGGAGCCGTTGT-3' (Rayman *et al.* 2002); PPARγ2 promoter, 5'(-413) TACGTTTATCGGT GTTTCAT-3' and 5'(-247) TCTCGCCAGTGA CCC-3'; upstream region of the PPARγ2 promoter, 5'(-1871) GGGCGTTAAAACACAATCCT-3' and 5'(-1707) TCTCTCCTCCTTCCCTTCC-3'; C/EBPα promoter, 5'(-315) TGACTTAGAGGCTTAAA GGA-3' and 5'(-32) CGGGGACCGCTTTTATAG AG-3'; resistin promoter, 5'(-177) CACCATGGTC CCTGGTGTTA-3' and 5'(+26) CTCAGTTCTGGG TATTAGCTC-3'; adiponectin promoter, 5'(-272) ATTGTCCTTACCCTTGCCCC-3' and 5'(-15); and leptin promoter, 5'(-323) GCCTTCTGTAGCC TCTTGCT-3' and 5'(-22) GCTCCATGCCCTG CCTGC-3'. Representative experiments from at least three independent experiments are shown.

### Results

#### *In vitro* binding of C/EBPβ at a C/EBP site in the PPARγ2 promoter occurs as early as 3 h post-differentiation

While investigating the role of C/EBP isoforms in the activation of adipogenic genes, we examined *in vitro* binding of C/EBPβ at a C/EBP regulatory element present in the PPARγ2 promoter by EMSA during a short time-course of 3T3-L1 preadipocytes induced to differentiate into adipocytes. We found that C/EBPβ present in nuclear extracts prepared from cells differentiated for 3, 18, and 24 h was able to bind to a



**Figure 1** *In vitro* binding of C/EBP $\beta$  to a C/EBP site in the PPAR $\gamma$ 2 promoter occurs as early as 3 h following the induction of 3T3-L1 adipocyte differentiation. EMSA was performed using nuclear extracts prepared from preadipocytes (day 0) or from differentiating preadipocytes at 1, 3, 18, and 24 h after the addition of the differentiation cocktail (lanes 1–5). The double-stranded,  $^{32}$ P end-labeled oligonucleotide probe encoded the C/EBP binding site between –343 and –306 relative to the start site of PPAR $\gamma$ 2 transcription. Supershift experiments were performed by adding purified IgG from pre-immune serum (lane 6) or antibodies against C/EBP $\beta$  (lane 7).

C/EBP site in the PPAR $\gamma$ 2 promoter (Fig. 1, lanes 3–5). Confirmation that the shifted band in the EMSA was C/EBP $\beta$  was demonstrated by the appearance of a supershifted band upon addition of C/EBP $\beta$  antibody to the reaction while addition of purified IgG had no effect (Fig. 1, lanes 6 and 7). The results revealed that the C/EBP $\beta$  regulator has the capacity to bind to DNA as early as 3 h following the induction of differentiation. In addition, we observed that the apparent level of C/EBP $\beta$  binding under the reaction conditions used did not increase between 3 and 24 h post-differentiation (Fig. 1, lanes 3–5).

These results diverged somewhat from data published previously where the *in vitro* binding activity of C/EBP $\beta$  was delayed until 12–16 h following the stimulation of adipogenic differentiation (Tang & Lane 1999). We note that the previous report demonstrated that C/EBP $\beta$  binding could be observed at 4 h post-differentiation but that binding was significantly induced at the 12–16 h time-points. This dissimilarity in results is possibly due to differences in reaction conditions, or perhaps to the use of different oligonucleotide probes. In the previous study (Tang & Lane 1999), EMSA was performed using a C/EBP site from the C/EBP $\alpha$  promoter, while our

experiment utilized a probe that contained a C/EBP binding site from the PPAR $\gamma$ 2 promoter. Nevertheless, the results presented in Fig. 1 raised a question about the timing of C/EBP $\beta$  binding to adipogenic regulatory sequences. To better address this issue, we decided to perform ChIP experiments at different times following the induction of adipocyte differentiation in 3T3-L1 cells in order to examine the binding of C/EBP isoforms *in vivo*.

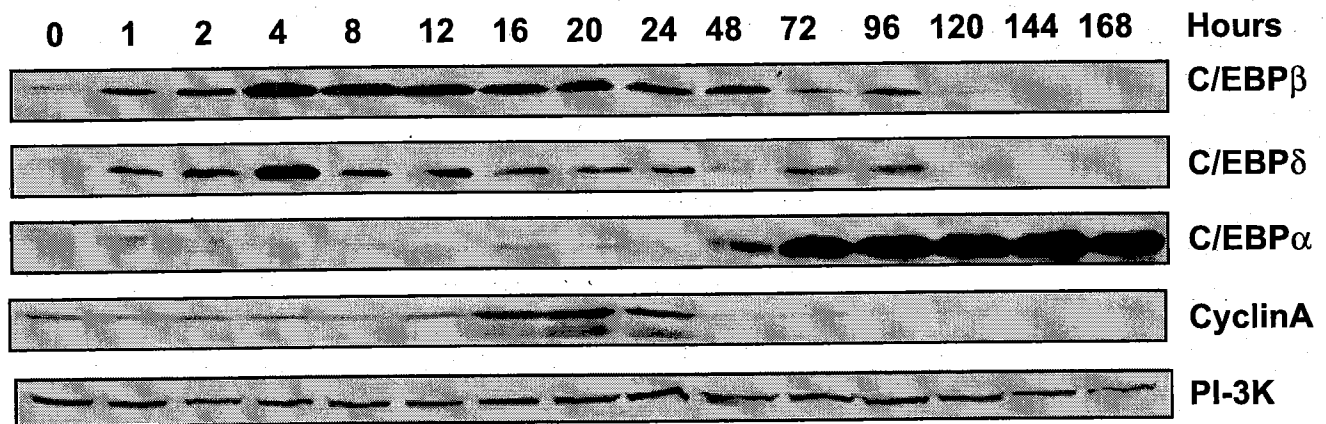
### Kinetics of C/EBP expression in differentiating 3T3-L1

Since the EMSA data presented in Fig. 1 indicated a potential difference from previously published studies, we first performed a series of control experiments to analyze the expression levels of C/EBP $\beta$ ,  $\delta$ , and  $\alpha$  during adipocyte differentiation of 3T3-L1 cells by western blot in order to eliminate the possibility that our results might be due to differences in the experimental handling of the differentiating 3T3-L1 cells. It has been well established that initiating differentiation of 3T3-L1 preadipocytes activates a cascade of gene expression events. Among the initial events are the rapid induction of C/EBP $\beta$  and  $\delta$ , which are stimulated by components of the differentiation cocktail, followed by the induction of C/EBP $\alpha$  on the second day of differentiation (Yeh *et al.* 1995, Wu *et al.* 1996). Western blot analyses corroborated that induced protein levels of C/EBP $\beta$  and  $\delta$  were detectable at 1 h, reached a maximum at 4 h, and were declining at 48 and 120 h respectively (Fig. 2). Expression of C/EBP $\alpha$  occurred later; significant induction began at about 48 h post-differentiation and expression was maintained throughout the time-course (Fig. 2).

To corroborate that the cells began the mitotic expansion phase, we performed a western blot with antibodies directed to cyclin A (Fig. 2). As was expected, cyclin A levels were increased between 16–24 h post-differentiation, indicating that the cells had entered the cell cycle prior to 16 h and had exited by approximately 48 h. All of the data presented in Fig. 2 indicate that the differentiation of the 3T3-L1 cells occurred as expected and confirm previously published results (Morrison & Farmer 1999, Tang *et al.* 2003a,b). Thus we have demonstrated the integrity of the 3T3-L1 cells and the differentiation protocol used for this and subsequent experiments.

### *In vivo* recruitment of C/EBP $\beta$ , $\delta$ , and $\alpha$ to the PPAR $\gamma$ 2 and C/EBP $\alpha$ promoters during adipogenesis

The results presented in Fig. 1 show that C/EBP $\beta$  was able to bind to C/EBP binding sites on the PPAR $\gamma$ 2 promoter at times that correlated with the induction of C/EBP $\beta$  levels. To determine whether binding occurs



**Figure 2** Western blot analysis of transcriptional and cell cycle regulators during differentiation of 3T3-L1 cells. Protein extracts were prepared from differentiating cells at the indicated times and the kinetics of protein expression for the indicated proteins were determined by western blot analysis. PI-3K was used as a loading control.

on endogenous adipocyte promoters at early times after the induction of adipocyte differentiation, we performed ChIP experiments and temporally analyzed binding, not only of C/EBP $\beta$ , but also of C/EBP $\delta$  and C/EBP $\alpha$ , to specific adipocyte promoters. First, we chose to evaluate binding at the C/EBP $\alpha$  and the PPAR $\gamma$ 2 promoters, since these two essential adipogenic regulators are expressed early during differentiation, on day 2 (Fig. 3A and B).

Regulation of the C/EBP $\alpha$  and the PPAR $\gamma$ 2 promoters by C/EBP family members has been characterized previously (Christy *et al.* 1991, Zhu *et al.* 1995, Clarke *et al.* 1997, Tang & Lane 1999, Tang *et al.* 1999, 2004, Elberg *et al.* 2000, Yang & Chow 2003). The C/EBP $\alpha$  proximal promoter contains a C/EBP regulatory element at  $-187$  relative to the transcriptional start site that mediates transactivation by C/EBPs. The PPAR $\gamma$ 2 promoter contains two previously characterized C/EBP recognition elements at  $-340$  bp and  $-327$  bp relative to the transcriptional start site in addition to other potential sites that diverge from the C/EBP consensus. As shown in Fig. 3A, recruitment of C/EBP $\beta$  as well as C/EBP $\delta$  at the C/EBP regulatory element on the PPAR $\gamma$ 2 promoter was induced in a manner consistent with the protein expression patterns of both proteins (Fig. 2), with binding of both proteins apparent 2 h after the onset of differentiation. Subsequently, C/EBP $\beta$  and C/EBP $\delta$  were replaced by C/EBP $\alpha$ , which initiated binding to the promoter at 48 h and was maintained throughout the time-course. These results confirmed the transition of binding from C/EBP $\beta$ / $\delta$  to C/EBP $\alpha$  at this promoter *in vivo* (Salma *et al.* 2004, Tang *et al.* 2004).

Analysis of the C/EBP $\alpha$  promoter revealed the same pattern found at the PPAR $\gamma$ 2 promoter, except that binding occurred even earlier following differentiation (Fig. 3B). C/EBP $\beta$  and C/EBP $\delta$  were bound as early as 1 h post-differentiation and remained present until 48 h

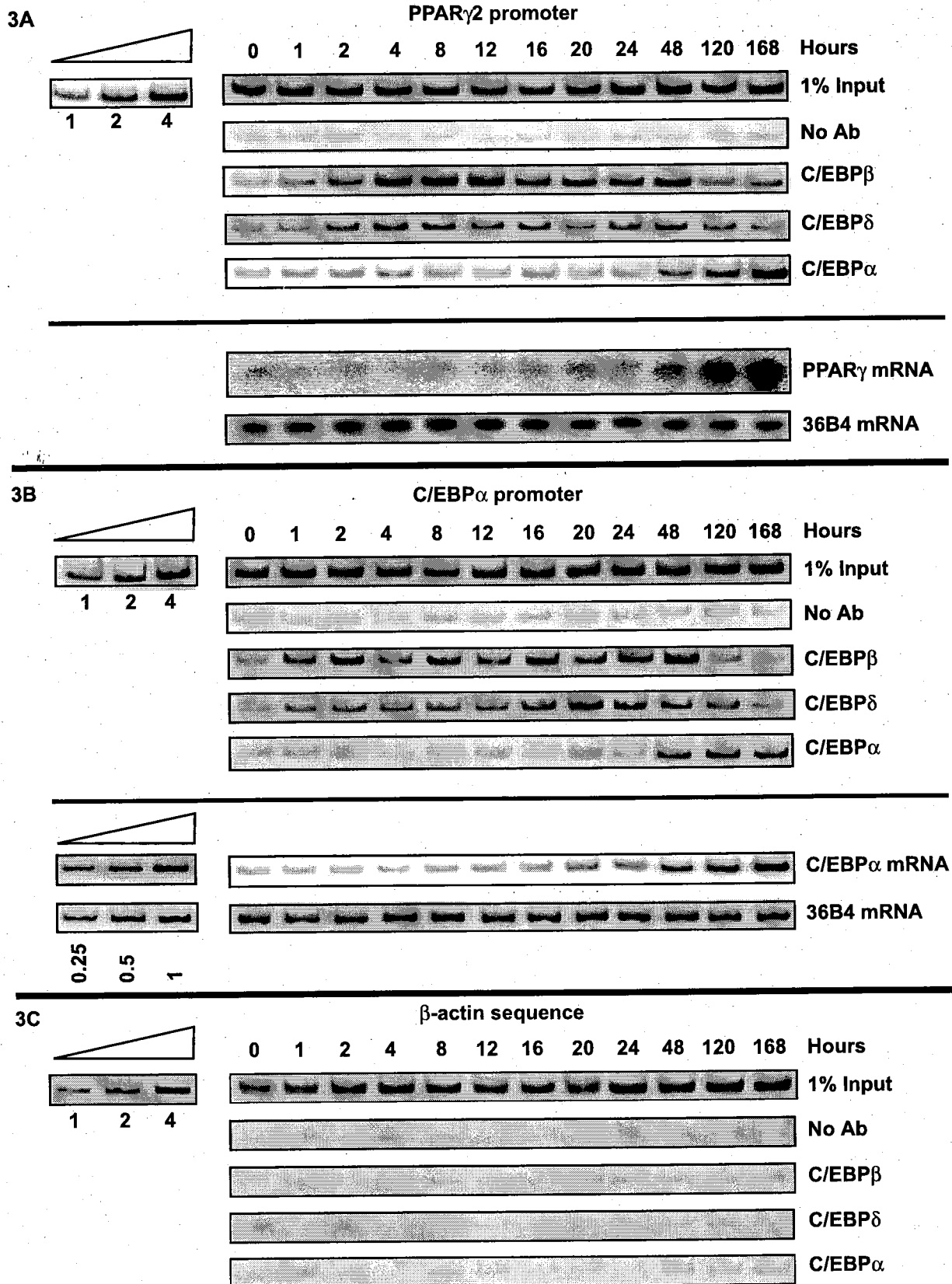
and 120 h respectively. Induction of C/EBP $\alpha$  binding to the C/EBP $\alpha$  promoter began at 48 h as reported previously (Tang *et al.* 2004). Therefore, the transition in binding of the C/EBP isoforms that was observed at the PPAR $\gamma$ 2 promoter also occurred at the C/EBP $\alpha$  promoter.

The appearance and disappearance of binding of the different C/EBP isoforms at the PPAR $\gamma$ 2 and C/EBP $\alpha$  promoters at different times post-differentiation indicate specificity of binding. No antibody controls provide further evidence for specific binding (Fig. 3A and B). As an additional control, we analyzed C/EBP factor interactions at the  $\beta$ -actin locus (Fig. 3C) and at sequences 1.8 kb upstream of the PPAR $\gamma$ 2 start site (data not shown). Neither  $\beta$ -actin sequences nor sequences upstream of the PPAR $\gamma$ 2 promoter were immunoprecipitated by any of the antibodies used in the ChIP procedure.

#### ***In vivo* recruitment of C/EBP $\beta$ , $\delta$ , and $\alpha$ to additional adipogenic gene promoters during adipogenesis**

We next examined if C/EBP $\beta$ ,  $\delta$ , and  $\alpha$  are recruited to the resistin, adiponectin, and leptin promoters. These adipocyte-secreted peptides, collectively referred to as adipocytokines, have generated considerable interest since they are important regulators of body mass and their misregulation may play a role in obesity (Miner 2004). The proximal promoters of these genes contain C/EBP binding sites that are necessary for expression. The resistin promoter contains a C/EBP site at  $-56$  relative to the transcriptional start site, adiponectin contains two identified C/EBP sites at  $-775$  and  $-264$ , and two potential binding sites at  $-117$  and  $-73$ , and leptin has three consensus C/EBP binding sites at nucleotides  $-55$ ,  $-211$ , and  $-292$ . The activity of these C/EBP sites has been confirmed by reporter assays





for each of these genes (de la Brousse *et al.* 1996, Hwang *et al.* 1996, Hartman *et al.* 2002, Park *et al.* 2004b).

Binding of C/EBP $\beta$  and  $\delta$  to the C/EBP site at the resistin proximal promoter was evident at 2–4 h post-differentiation and did not decline until after 48 h (Fig. 4A). A modest increase in binding of C/EBP $\alpha$  was observed from 20 to 48 h, and was further increased at 120 h (Fig. 4A). These results indicate that C/EBP $\beta$  and  $\delta$  bind early after differentiation to the resistin promoter and suggest that all three C/EBP isoforms play a role regulating this adipocyte gene. It was previously demonstrated that C/EBP $\alpha$  binds specifically to the C/EBP element on the resistin promoter that is essential for expression (Hartman *et al.* 2002); however, a transition in binding of these regulators has not been previously demonstrated.

The adiponectin promoter contains two C/EBP sites at –775 and –264 and two potential binding sites at –117 and –73; however, the C/EBP element at –264 and the potential C/EBP sites at –117 and –73 confer promoter activity as defined in transient promoter studies, EMSA, DNase I footprinting, and ChIP assays (Park *et al.* 2004b, Seo *et al.* 2004). Consequently, we performed ChIPs in the region of the proximal promoter that contains these C/EBP sites. The analysis of binding of C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\alpha$  to the adiponectin promoter showed a pattern nearly identical to that observed for the resistin promoter, despite the fact that adiponectin expression initiated later than resistin expression (Fig. 4B). C/EBP $\beta$  and  $\delta$  were bound to the promoter at 2 h and maintained until 48 h. Definitive binding of C/EBP $\alpha$  was present at 48 h, although a modest increase was noticed at 20–24 h.

Finally, we assessed the recruitment of the C/EBP members on the leptin promoter. The proximal promoter contains three consensus C/EBP binding sites. DNase I footprint analysis, reporter gene assays, and EMSA studies have demonstrated that one of these C/EBP sites, located at –53 relative to the transcriptional start site, is functional (de la Brousse *et al.* 1996, Hwang *et al.* 1996, Mason *et al.* 1998). However, because the C/EBP sites are near each other, we designed PCR primers to amplify a region of the proximal promoter containing all three sites. The recruitment of C/EBP $\beta$  occurred at 4 h and remained relatively constant until 48 h (Fig. 4C). In contrast, C/EBP $\delta$  was detectable from 12 h to 48 h. Binding of C/EBP $\alpha$  was observed on the

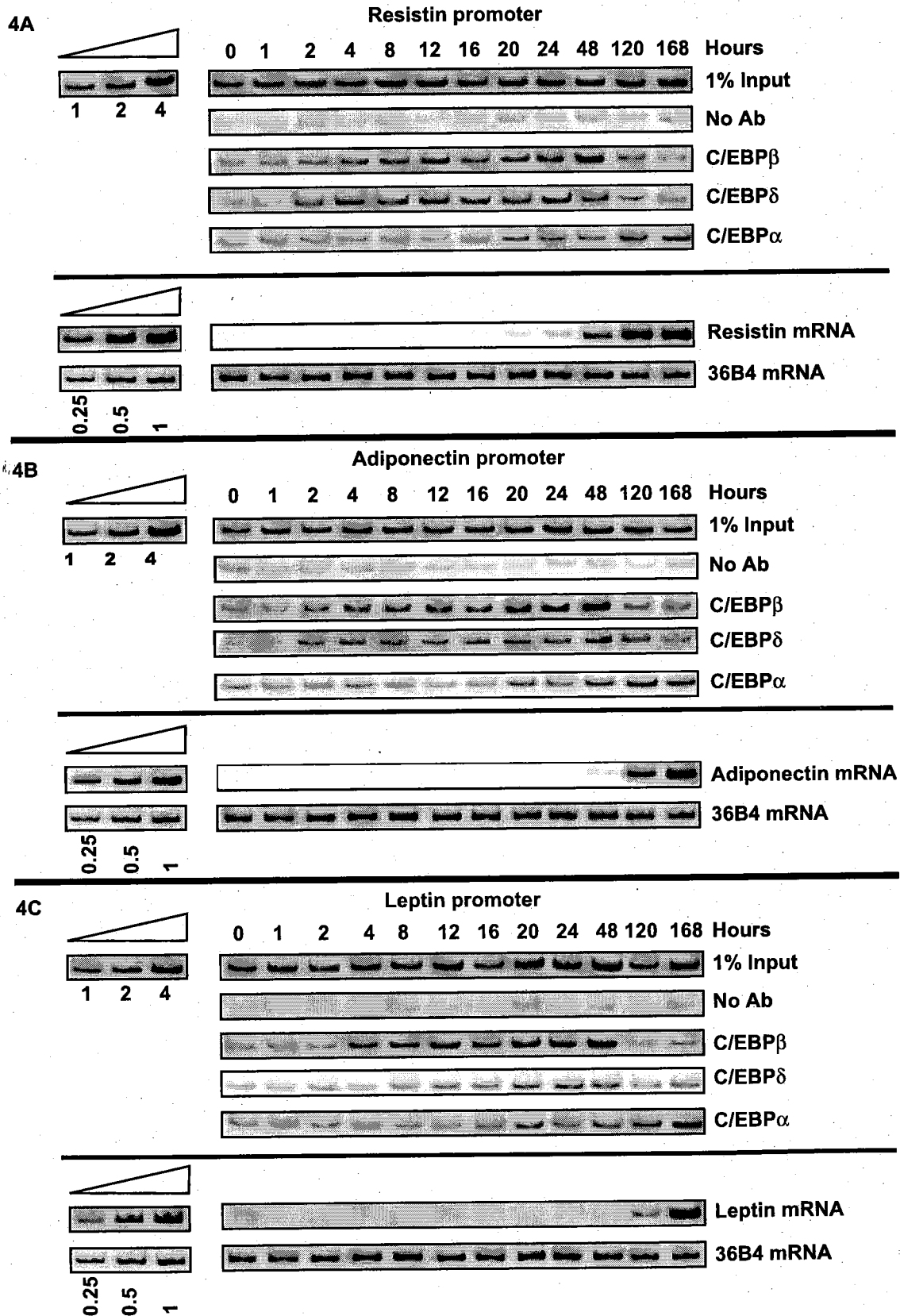
promoter at 20 h, but was not robust until 120 h post-differentiation, which coincides with the start of leptin mRNA accumulation. These results indicate that C/EBP $\beta$  and  $\delta$  bind quickly after the induction of differentiation to adipogenic promoters that are not expressed until much later in the differentiation process. Furthermore, the transition from C/EBP $\beta$  and  $\delta$  to C/EBP $\alpha$  binding on these promoters also occurred prior to gene expression, suggesting that C/EBP factor interactions with adipogenic promoters is independent of the time at which gene expression is initiated.

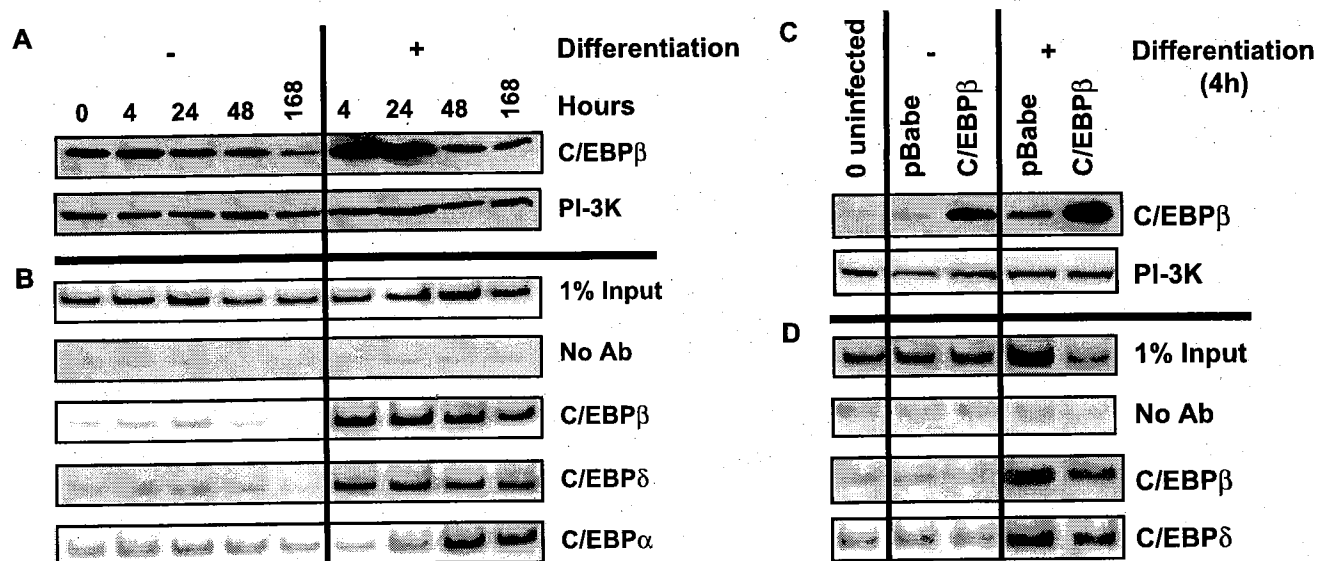
#### Overexpression of C/EBP $\beta$ is not sufficient to promote C/EBP protein binding to the PPAR $\gamma$ 2 promoter in differentiating 3T3-L1 cells

The detection of C/EBP $\beta$  on adipogenic promoters *in vivo* within a few hours of the onset of adipogenic stimulation caused us to evaluate whether overexpression of C/EBP $\beta$  in 3T3-L1 preadipocytes would be sufficient to induce binding of C/EBP $\beta$  to the regulatory sequences examined. 3T3-L1 preadipocytes were infected with a retroviral vector expressing C/EBP $\beta$  and were allowed to reach confluence, but no differentiation cocktail was added. Instead, cells were maintained in 10% calf serum as a confluent plate, and C/EBP binding was assessed at 0, 4, 24, 48, and 168 h. Despite the ectopic expression of C/EBP $\beta$  (Fig. 5A), no binding of C/EBP $\beta$ ,  $\delta$ , or  $\alpha$  was observed at the PPAR $\gamma$ 2 promoter, whereas control plates treated with differentiation cocktail showed C/EBP $\beta$  and  $\delta$  binding to the PPAR $\gamma$ 2 promoter within 4 h post-differentiation and C/EBP $\alpha$  binding by 48 h post-differentiation (Fig. 5B).

The data suggested that overexpression of C/EBP $\beta$  in cells is not sufficient to promote C/EBP $\beta$  binding to adipogenic promoters in the absence of differentiation cocktail. A potential caveat to this conclusion is that ectopic expression did not provide a high enough level of C/EBP $\beta$  protein to surpass a threshold level of C/EBP $\beta$  protein necessary to achieve binding. We note that the inoculum of pBABE-C/EBP $\beta$  retrovirus used in these experiments is the same as we have previously used to trans-differentiate fibroblast lines into adipocyte-like cells; thus the levels of C/EBP $\beta$  provided are sufficient to reprogram cells of a different lineage (Salma *et al.* 2004). However, to more directly address this concern, we compared the levels of C/EBP $\beta$  protein present in

**Figure 3** ChIP assays reveal that C/EBP $\beta$  binding to the PPAR $\gamma$ 2 and C/EBP $\alpha$  promoters occurs *in vivo* within 2 h of the induction of 3T3-L1 adipocyte differentiation. ChIP assays were performed at the indicated time-points using the indicated antibodies and amplified for the PPAR $\gamma$ 2 promoter (A, top panel), the C/EBP $\alpha$  promoter (B, top panel), or the  $\beta$ -actin 5' untranslated region and coding region (C); 1% of each input is shown. A twofold titration (number of  $\mu$ l indicated) of input from the 0 h sample is shown on the left to demonstrate linearity of the PCR reactions. (A, bottom panel) Duplicate samples were used to prepare RNA and a Northern blot showing the levels of PPAR $\gamma$  and 36B4 mRNA at each time-point is shown. (B, bottom panel) C/EBP $\alpha$  and 36B4 mRNA levels were determined by RT-PCR. A twofold titration (number of  $\mu$ l indicated) of input reverse transcribed RNA from the day 7 sample is shown on the left to demonstrate linearity of the PCR reactions. Ab, antibody.





**Figure 5** Overexpression of C/EBP $\beta$  in undifferentiated 3T3-L1 preadipocytes is not sufficient to induce the binding of C/EBP factors at the PPAR $\gamma$ 2 promoter. Subconfluent 3T3-L1 preadipocytes were infected with either pBabe retrovirus or pBabe retrovirus encoding C/EBP $\beta$ . For differentiation, cells were placed in fresh media lacking or containing the differentiation cocktail, and samples were harvested at the indicated times for (A and C) Western blot analysis or (B and D) ChIP analysis. Ab, antibody.

uninfected cells and in cells infected with pBabe-C/EBP $\beta$  or with the pBabe empty retrovirus that were differentiated in the presence or absence of differentiation cocktail for 4 h. A western blot (Fig. 5C) demonstrates that cells infected with the C/EBP $\beta$  virus contained greater levels of C/EBP $\beta$  protein in both the presence and absence of differentiation cocktail (compare lane 2 with lane 3 and lane 5 with lane 6), as expected. We also observed that C/EBP $\beta$  levels in vector-infected cells that were differentiated in the presence of cocktail were lower than C/EBP $\beta$  levels in the pBabe-C/EBP $\beta$ -infected cells that were differentiated in the absence of cocktail (compare lanes 4 and 3). The levels of C/EBP $\beta$  protein in pBabe-infected cells treated with differentiation cocktail were sufficient to permit C/EBP $\beta$  binding to the PPAR $\gamma$ 2 promoter, whereas higher levels of C/EBP $\beta$  in the C/EBP $\beta$ -infected cells differentiated in the absence of cocktail were not (Fig. 5D, compare lanes 4 and 3). The results exclude the possibility that insufficient levels of C/EBP $\beta$  were present in the cells not treated with differentiation cocktail. The C/EBP $\beta$  protein undergoes a number of post-translational modifications that are associated with

the induction of adipogenesis; it is likely that such modifications are induced by addition of the differentiation cocktail and are necessary to promote rapid binding to adipogenic gene regulatory sequences *in vivo*. Thus, simple overexpression of C/EBP $\beta$  is not sufficient to induce C/EBP $\beta$  binding in 3T3-L1 preadipocytes.

## Discussion

The C/EBP family of transcription factors is widely expressed and is a key regulator of a variety of target genes important in physiological events, including energy metabolism, inflammation, hematopoiesis, cellular proliferation, and differentiation (Darlington *et al.* 1998, Lekstrom-Himes & Xanthopoulos 1998, Rosen *et al.* 2000, Ramji & Foka 2002, Kovacs *et al.* 2003). Of note is the essential role C/EBP family members play during adipogenesis (reviewed by Darlington *et al.* 1998, Lane *et al.* 1999). Almost immediately upon induction of adipogenesis, the C/EBP family members  $\beta$  and  $\delta$  are induced in a manner dependent on several signal transduction cascades that result in phosphorylation of

**Figure 4** ChIP assays show that C/EBP $\beta$  binding to the adipocytokine promoters occurs *in vivo* within 4 h of the induction of 3T3-L1 adipocyte differentiation. ChIP assays were performed at the indicated time-points using the indicated antibodies and amplified for the resistin promoter (A, top panel), the adiponectin promoter (B, top panel), or the leptin promoter (C, bottom panel); 1% of each input is shown. A twofold titration (number of  $\mu$ l indicated) of input from the day 0 sample is shown on the left to demonstrate linearity of the PCRs. Duplicate samples were used to prepare RNA at each time-point and mRNA levels for resistin (A, bottom panel), adiponectin (B, bottom panel), and leptin (C, bottom panel) as determined by RT-PCR are shown. 36B4 mRNA levels are shown as a control. A twofold titration (number of  $\mu$ l indicated) of input reverse transcribed RNA from the day 7 sample is shown on the left to demonstrate linearity of the PCRs. Ab., antibody.

these proteins (Park *et al.* 2004a, Bezy *et al.* 2005, Tang *et al.* 2005 and references therein). However, the activation of the early adipogenic regulators, PPAR $\gamma$ 2 and C/EBP $\alpha$ , which are dependent on C/EBP $\beta$  and  $\delta$ , does not occur until day 2 of the differentiation process. During the first 2 days, cells undergo one or two rounds of mitosis, a process termed mitotic clonal expansion (Bernlohr *et al.* 1985, Cornelius *et al.* 1994, MacDougald & Lane 1995). Thus, the transcriptional activation potential of the C/EBP $\beta$  and  $\delta$  proteins are masked or repressed until clonal expansion commences.

Over the past several years data have accumulated that suggest that the binding capacity of C/EBP $\beta$  for its cognate binding site is delayed 12–20 h post-induction of adipocyte differentiation (Lane *et al.* 1999, Tang & Lane 1999, Tang *et al.* 2003b, 2005). This observation fits well with the need to delay expression of C/EBP $\alpha$ , which has anti-mitotic properties (Umek *et al.* 1991, Lin *et al.* 1993, Timchenko *et al.* 1996, Wang *et al.* 2001), until clonal expansion occurs. Moreover, the kinetics of DNA binding activation fit well with the kinetics of other events that occur at this time, including the appearance of phosphorylated Rb and the localization of C/EBP $\beta$  to pericentric heterochromatin, which contains numerous C/EBP binding sites in the satellite DNA sequences (Tang & Lane 1999). How this change in sub-nuclear distribution relates to gene expression has not been well established, but the observation raises the possibility that localization of proteins to specific nuclear compartments contributes to the complexity of adipocyte gene regulation.

In the course of examining the activation of the PPAR $\gamma$ 2 promoter during adipogenesis, we noted that *in vitro* binding of C/EBP $\beta$  to a C/EBP binding site in the PPAR $\gamma$ 2 promoter did not appear to change significantly between 3 and 24 h post-differentiation (Fig. 1). Given the wide range of variable conditions that can affect protein binding in a gel shift assay, we initially did not view this as contradictory to the existing models explaining the delay in activation of C/EBP $\alpha$  and PPAR $\gamma$ 2 gene expression. However, ChIP assays, which specifically detect *in vivo* protein:DNA interactions at endogenous loci, clearly demonstrated that C/EBP $\beta$  and C/EBP $\delta$  were capable of binding to both the C/EBP $\alpha$  and the PPAR $\gamma$ 2 promoter at very early times post-differentiation. Moreover, C/EBP $\beta$  and  $\delta$  could also bind at early times to adipocyte specific promoters that do not begin to transcribe until much later in the differentiation process. Our results do not alter the original conclusion that mechanisms exist during the time of mitotic clonal expansion to delay activation of C/EBP $\alpha$  and PPAR $\gamma$ 2 gene expression and the target genes that they subsequently activate. Instead, they indicate that the rate-limiting step is not the interaction of the C/EBP $\beta$  protein with binding sites at the endogenous target gene promoters.

Numerous possibilities to restrict the transcriptional activating properties of C/EBP $\beta$  exist even if the protein is DNA bound. The exact isoform of C/EBP $\beta$  that is bound could influence the transcriptional potential. Interaction between C/EBP $\beta$  and repressor proteins (Ron & Habener 1992, Tang *et al.* 1999) would not necessarily be restricted to solution interactions; repression could occur via interactions at promoter sequences as shown previously (Mo *et al.* 2004). DNA-bound C/EBP $\beta$  could be and likely is still subject to post-translational modifications, including phosphorylation, acetylation, and sumoylation (Kim *et al.* 2002, Eaton & Sealy 2003, Xu *et al.* 2003, Park *et al.* 2004a, Tang *et al.* 2005), that may modulate transcriptional capacity. C/EBP $\beta$ -bound loci could remain transcriptionally silent because other activators, RNA polymerase II (pol II)- or pol II-associated general transcription factors have not been synthesized, are spatially restricted, have not yet undergone the appropriate post-translational modification, or cannot bind in the absence of specific chromatin modifications or alterations. In support of this last possibility, Wiper-Bergeron *et al.* (2003) showed that histone deacetylase1 (HDAC1) could affect the acetylation status of the C/EBP $\alpha$  promoter in a manner regulated by glucocorticoids. Finally, C/EBP $\beta$ -bound loci may change position within the nucleus, becoming associated or disassociated with specific sub-nuclear structures such as pericentric heterochromatin or splicing domains. None of these possibilities are mutually exclusive, and it is likely that multiple mechanisms are acting co-operatively to control the timing of expression for each individual target gene.

We also note that, in other cell types, C/EBP $\beta$ -mediated activation of endogenous target genes can occur without induction of C/EBP $\beta$  levels or induction of post-translational modifications. Lipopolysaccharide-mediated induction of C/EBP $\beta$  target genes in B cell-derived lines lacking C/EBP proteins could be accomplished by constitutive expression of the bZIP domain of C/EBP $\beta$  (Hu *et al.* 2000). More recently, C/EBP $\beta$  binding to and subsequent activation of target genes in lipopolysaccharide-stimulated macrophages were shown to occur prior to induction of C/EBP $\beta$  protein levels and in the absence of induced C/EBP $\beta$  phosphorylation or nuclear translocation (Bradley *et al.* 2003). Thus, different mechanisms likely control C/EBP $\beta$  function in different cell types.

The *in vivo* binding capacity of C/EBP $\beta$  and  $\delta$  to adipogenic genes as early as 1 h post-differentiation seems likely to be modulated by components of the differentiation cocktail. ChIP assays performed with 3T3-L1 over-expressing C/EBP $\beta$  in the absence of differentiation inducers showed that this regulator is not recruited to the PPAR $\gamma$ 2 (Fig. 5). This result shows that the physical presence of C/EBP $\beta$  alone is insufficient to

be recruited to specific promoters. Further studies will be required to determine the nature of modifications required for both C/EBP $\beta$  and  $\delta$  to rapidly bind to adipogenic regulatory sequences following differentiation signaling as well as the relative importance of C/EBP $\beta$  homodimers and C/EBP $\beta$  and  $\delta$  heterodimers.

Limited data on the binding of C/EBP $\beta$  and  $\delta$  to gene promoters other than C/EBP $\alpha$  and PPAR $\gamma$ 2 exist. In differentiating 3T3-L1 cells, C/EBP $\beta$  binds to the aP2 promoter between 24–72 h post-differentiation (Tang *et al.* 2004), and a recent report shows both C/EBP $\beta$  and  $\delta$  on the adiponectin promoter in mouse adipose tissue (Park *et al.* 2004b). These data raise the question of why C/EBP $\beta$  and  $\delta$  are bound to such promoters when evidence strongly suggests that the genes are not activated until later times when C/EBP $\alpha$  has replaced C/EBP $\beta$  and  $\delta$  at these loci. We speculate that the presence of C/EBP $\beta$  may be part of the process by which C/EBP $\alpha$  is recruited to adipogenic promoters. Alternatively, or perhaps, additionally, all of the adipogenic loci undergo structural changes at the onset of differentiation, and the binding of C/EBP $\beta$  to these loci serves as a mark for subsequent gene activation.

The data have also clearly demonstrated that the C/EBP binding sites on each of the genes undergo a transition in binding from C/EBP $\beta$  and  $\delta$  to C/EBP $\alpha$  *in vivo*. We and others have previously demonstrated that this occurred on the PPAR $\gamma$ 2 promoter during adipogenesis (Salma *et al.* 2004, Tang *et al.* 2004). From the data presented here, we predict that this transition occurs on all adipocyte genes containing C/EBP binding sites with similar kinetics, indicating the importance of the transition and a role for C/EBP $\beta$  and  $\delta$  in both early and late events of the differentiation program. We note that the binding of C/EBP $\beta$  and  $\delta$  correlated with the induction of overall cellular levels of C/EBP $\beta$  and  $\delta$ . Similarly, C/EBP $\beta$  and  $\delta$  were replaced on each promoter between 24 and 48 h post-differentiation, the time at which C/EBP $\alpha$  protein levels begin to be induced. Thus the binding of the specific C/EBP proteins to adipocyte specific genes *in vivo* does not correlate with the time of target gene expression but instead occurs rapidly after the induction of C/EBP protein levels.

## Acknowledgements

We are grateful to Y Ohkawa and the members of our laboratory for advice and discussion. This work was supported by a Scholar Award from the Leukemia and Lymphoma Society and by grants from the NIH and the University of Massachusetts Medical School Diabetes Endocrine Research Center to A N I. N S was supported in part by a Zelda Haidak Memorial Scholar Fellowship. The authors declare that there is no conflict of interest

that would prejudice the impartiality of this scientific work.

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Received 7 October 2005

Accepted 17 October 2005

Made available online as an Accepted Preprint 9 November 2005