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DISSERTATION

REGULATION OF ADIPOCYTE FATTY ACID BINDING PROTEIN (a-FABP) GENE EXPRESSION DURING THE EARLY EVENTS OF ADIPOCYTE DIFFERENTIATION

Submitted by
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In partial fulfillment of the requirements
for the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Summer 1999

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COLORADO STATE UNIVERSITY

April 02, 1999

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY PHILIPPE THUILLIER ENTITLED REGULATION OF ADIPOCYTE FATTY ACID BINDING PROTEIN (a-FABP) GENE EXPRESSION DURING THE EARLY EVENTS OF ADIPOCYTE DIFFERENTIATION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT of DISSERTATION

REGULATION OF ADIPOCYTE FATTY ACID BINDING PROTEIN (a-FABP) GENE EXPRESSION DURING THE EARLY EVENTS OF ADIPOCYTE DIFFERENTIATION

The conversion of preadipocytes to adipocytes is stimulated by the insulin growth factor 1 (IGF-1) and appears to be one of the consequences of insulin resistance seen in patient suffering from obesity and non insulin dependent diabetes milletus (NIDDM). The study of the adipocyte specific gene a-FABP has yielded important information regarding the molecular mechanisms regulating adipocyte differentiation. One of the most significant contribution was the discovery of the adipogenic transcription factor mPPARγ2. Original studies suggested that an increase in mPPARγ2 expression was sufficient to trigger the adipogenic program, and this event was amplified by ligand activation. The work presented here tested the timely expression of mPPARγ2 and of RXRα, its binding partner, during the early event of fat cell terminal differentiation. Our work suggest a novel regulatory mechanism for the control of expression of genes that are target for mPPARγ2 and more precisely for aFABP. An increase in mPPARγ2 was detectable but an increase in RXRα protein expression appeared to be the key regulatory event for the activation of the heterodimer. During the process of studying these events we also characterized a novel 3T3-L1 derivative cell line that contained a defective signaling system which did not permit ligand activation of mPPARγ2 and terminal differentiation.

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Acknowledgment

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DEDICATION

This dissertation is dedicated with love to my wonderdul, loving, patient and supportive wife Catriona , to my loving mother, Claudine Marouani, to my inspiring brother Henri-Georges, and to my parents in-law, Sonia Buist and Neil Buist for their encouragement and never ending support. Thank you all for believing in me.

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Introduction

Non insulin dependent diabetes milletus (NIDDM) is a disorder characterized by insulin resistance in muscle and adipose tissue and an inability of insulin to shut down hepatic gluconeogenesis (1,2). Antecedent to the development of NIDDM there appears to be the development of a malfunction in the insulin signal transducing mechanisms which operate to impair glucose utilization (3). Such an impairment may reflect the suppression of genes coding for proteins essential to glucose metabolism. Thus, a defect in nuclear signals that operate to govern the expression of genes involved in glucose metabolism may explain why observable symptoms of NIDDM are preceded by anomalies in the intracellular metabolism of glucose (1, 4, 5).

With the quest to understand the physiological and molecular events leading to NIDDM, the conversion of preadipocytes to adipocytes has been studied by many groups over the last ten years (6-10). Although some key breakthroughs were yielded recently, the molecular events regulating adipocyte differentiation are still unclear. Adipocyte differentiation is stimulated by the insulin growth factor 1 (IGF-1) or by insulin (6,7). The study of adipocyte differentiation has widely focused on the regulation of the adipocyte-fatty acid binding protein (a-FABP) expression because it is one of the specific early event markers of adipocyte terminal differentiation (8, 9). We have discovered that the a-FABP gene is a target for insulin during the early events of adipocyte differentiation (7, 10). In addition, pioglitazone (piog), a novel anti-diabetic agent was discovered to increase the insulin/IGF-1 action on adipocyte differentiation, which can be monitored at the molecular level by an increase in

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a-FABP mRNA expression several fold within the first 12 hours following treatment (5, 10, 11). This suggested a transcriptional control of a-FABP expression by piog and insulin and the existence of a specific target to insulin and piog in the 5' flanking region of the a-FABP gene. An adipose differentiation specific enhancer was characterized in the 5' flanking region of the a-FABP gene (9, 12) and the factor binding to this element, termed adipose response factor 6 (ARF6), consisted of the murine peroxisome proliferator activated receptor gamma 2 (mPPARγ2) and of the retinoid X receptor alpha (RXRα) (13). Although a multitude of information has been generated regarding PPARγ2 expression during the late stages of terminal fat cell differentiation, the mechanisms regulating the expression and activity of ARF6 during the first 24 hours of adipocyte differentiation have not been completely elucidated. In particular it is not clear whether an increase in PPARγ2 expression alone is responsible for an increase in a-FABP expression during fat cell terminal differentiation, or if synthesis of other factors, such as RXRα, is necessary. In addition, it is not known whether piog increase in a-FABP expression is due to a direct effect on PPARγ2 expression or on its cellular localization.

Thus, this project will focus on determining the relative levels of ARF6 during the activation of a-FABP expression, in particular during the first 24 hours following differentiation induction. Reportedly, mPPARγ2 appears to be the limiting factor in the formation of ARF6 complex (13). Hence we will examine some of the possible mechanisms regulating its levels of expression and its localization in the cells including transcription, translation and cellular

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localization. Finally we will test whether piog plays any role in the regulation of these events. Therefore, the specific aims that this project will address are:

1- Characterize the temporal pattern of expression for mPPARγ2 during the 0 to 24 hour period following piog and insulin induction of differentiation. This will require the development of anti-mPPARγ2 antibodies.

2- Characterize the temporal relationship between mPPARγ2 expression, its cellular localization, and the initiation of a-FABP transcription.

3- Determine the impact of piog on mPPARγ2 expression and cellular localization during the first 24 hours following induction of differentiation.

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Chapter I

Background

I.A. Adipose tissue and the differentiation program

I.A.1. *Adipose Tissue*

During the past two decades, growth and development of white adipose tissue (WAT) and brown adipose tissue (BAT) has been extensively studied in various mammals including humans. Despite a wealth of data, key information is lacking regarding the origin of fat cells and is minimal regarding adipose tissue development during embryogenesis and early life (14). However, the relationships between WAT and BAT and the molecular mechanisms leading to adipose differentiation are now better understood. Quite recently the importance of adipose tissue as an endocrine, autocrine, and intracrine organ has been established. Adipocytes represent between one third and two thirds of the total number of cells in adipose tissue. The remaining cells are various blood cells, endothelial cells, pericytes, adipose precursor cells of varying degrees of differentiation and, most likely, fibroblasts (Figure I.1)(15). Over the last decade, convincing evidence has accumulated concerning the existence of very small fat cells in addition to mature adipocytes (16, 17, 18). The relationships between a definition of cell types based upon ultrastructural studies and one based upon biological studies are summarized in figure I.1. Most of the cell population of adipose precursor cells has been previously defined in developing and adult rodents as "interstitial cells" (15, 19, 20-22) or

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Figure I.1 Relationships between morphological types in vivo and stages of cell differentiation in vitro.

The adipocyte fraction corresponds to adipocytes and some very small fat cells. The stromal-vascular fraction corresponds to a mixture of the other cell types. See text for more details. *Adapted from Ailhaud et al 1992, (14)*

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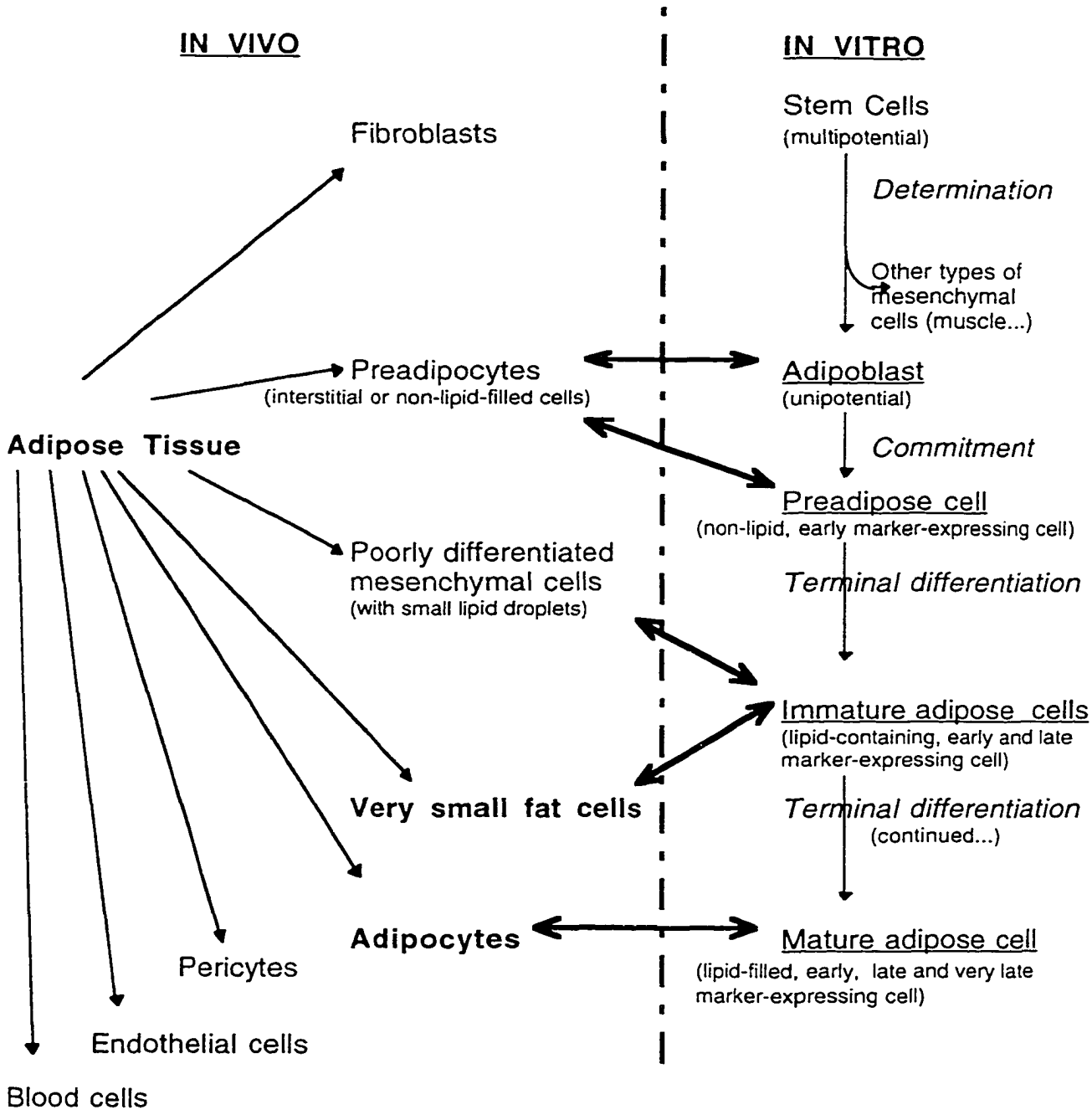

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"non lipid-filled mesenchymal cells" (23) or "other mesenchymal cells", which includes both "undifferentiated" and "poorly differentiated" mesenchymal cells (24). From a biological perspective, it is generally accepted that adipose precursor cells arise from multipotent stem cells, the origin of which is still unclear. From an embryological perspective, the process of determination from multipotent stem cells leads to the formation of unipotent adipoblasts (25). Commitment of adipoblasts leads to the formation of preadipocytes, defined as cells that have expressed early, but not yet late, markers and that have not yet accumulated triacylglycerol stores. Thus by definition, the population of adipoblasts and preadipocytes should correspond to interstitial cells or non-lipid-filled cells (15). Although adipoblasts are formed during embryonic development, it is unclear whether some remain postnatally or whether only preadipocytes are present. Clearly, biochemical and molecular markers are needed to carry out decisive studies of the development of adipose tissue.

I.A.2. *Proliferation and differentiation of white adipose tissue.*

I.A.2.1. Embryonic development

White adipose tissue cannot be detected macroscopically during embryonic life or at birth in rodents (rat, mouse), however, it is present at birth in the pig, rabbit, guinea pig and human. The development of white adipose tissue has been studied in pig fetuses (26, 27). Approximately at the beginning of the last third of the gestation period, large and small arterioles can be detected in the vicinity of small developing fat cell clusters that are surrounded by extensive stroma. Cell cluster size increases steadily with fetal age, but the number of clusters does not change significantly before birth. The importance of tissue vascularization is underscored by the fact that fat cell density appears to be positively associated with capillary density and that the largest fat cell clusters

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are located near the entry points of large blood vessels. The embryonic development of human adipose tissue takes place earlier than that of the pig, i.e. at the beginning of the second third of the gestation period in various sites (buccal, neck, shoulder, gluteal, perirenal) (28, 29, 30). Adipose tissue appears and develops in these areas where it remains after birth. Initially, the aggregation of a dense mass of mesenchymal cells (mesenchymal lobules with no lipid accumulation) is associated with the organization of a vascular structure. Primitive fat cell clusters are then formed, composed of densely packed fat cells adjacent to capillaries. In the last stage, growth of adipose tissue is mainly due to an increase in size of fat cells clusters surrounded by mesenchyme, which condenses rapidly and then thickens to form septa among the clusters. During both pig and human embryogenesis, angiogenesis appears to be tightly coordinated with the formation of fat cell clusters in time and space. Therefore, the characterization of specific growth factors able to trigger and modulate the development of capillaries and fat cell clusters represents an important issue. A wide variety of ubiquitous angiogenesis factors have been described. TGF-β and PGE_2 are synthesized and secreted by adipocytes. Monobutyrin (1-butyrylglycerol) has been described as a novel angiogenesis factor that stimulates motility (31) and that appears to be fat-specific (32). Conversely, microvascular endothelial cells from human omental adipose tissue (most likely mesothelial cells) in culture, secrete more insulin-like growth factor-I (IGF-I) and IGF-binding protein(s) than do epithelial cell types (33). Clearly, IGF-I mRNA and secreted IGF-I in the human fetus are preferentially localized in fibroblasts and other cells of mesenchymal origin (34). It is now generally accepted that IGF-I acts as an endocrine hormone via the blood, and locally as a paracrine/autocrine factor. In addition, growth hormone (GH) controls its synthesis both in liver cells and in peripheral cells of

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mesodermal origin (35, 36). Taken together, these observations agree with in vitro data and suggest that the requirement for IGF-I, necessary for the proper development of adipose tissue, is met during embryogenesis.

I.A.2.2. Postnatal development

Methods of determining fat cell number and size have been used to study the postnatal development of WAT, particularly in rodents. Unfortunately, they are not sufficiently accurate to detect modest changes in cell number and, in any event, they only count lipid-filled cells. In other words, preadipocytes (Figure I.1), i.e. cells that have expressed early phenotypes but do not yet contain triacylglycerol droplets, are not counted. This technique excludes a priori a true estimate of the developmental potential of the various adipose deposits. The expression of S-100 proteins, a protein identified in various mammalian tissues, helped solve this problem. During the development of rat epididymal fat tissue, non lipid-filled cells (preadipocytes) stain positively for these proteins while endothelial cells, pericytes, and fibroblasts do not stain (37). Numerous studies using ^{3}H-thymidine have been performed in order to distinguish between cell proliferation and the lipid-filling process. One study combined pulse-labeling with ^{3}H-thymidine followed by autoradiography and histochemical visualization of a-naphtyl acetate hydrolase activity, i.e. most likely lipoprotein lipase (LPL) and/or monoglyceride lipase activity (38). With this approach, Pilgrim (38) was able to distinguish between lipid-free mesenchymal cells (presumably adipoblasts), lipid-free, esterase-positive cells (presumably preadipocytes), and lipid-filled, esterase-positive cells (adipocytes). The proliferative activity is highest in preadipocytes, which suggests that committed cells are able to proliferate, whereas fully differentiated cells lose the capacity to divide. Using a similar approach in mice, Cook &

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Kozak (39) have observed the highest labeling index in cells negative for glycerol-3-phosphate dehydrogenase (GPDH) activity, a late marker of adipose cell differentiation. A dramatic decrease of the labeling index precedes the rise of GPDH activity, which is detected subsequently in all triacylglycerol containing cells. Since the labeling index reflects cell division and growth, it appears that early marker-expressing cells undergo mitoses before terminal differentiation takes place.

The ability of adult rodents and humans to increase the number of adipocytes has been long known (40) and depends on the localization of the adipose depot, the nature of the diet, and the environmental conditions to which the animals were exposed. Some controversy exists, however, as to whether the formation of new fat cells takes place during refeeding after a prolonged period of food deprivation. Initial reports indicated that such a treatment caused loss and recovery of endothelial and non-lipid filled mesenchymal cells only and that no loss or gain of fat cells occurred (22). More recently, a similar study has shown that poorly differentiated mesenchymal cells can replicate (41). Some loss of adipocytes appears possible under severe pathological conditions. A reduction in the number of fat cells from parametrial and retroperitoneal sites has been reported in diabetic rats (15) but the turnover of adipocytes is clearly a slow process. Proliferation of mature fat cells has remained a controversial issue. Sugihara and coworkers have reported (42) that approximately 2% of cultured adipocytes from very young rats can undergo mitoses in culture. Possibly, this phenomenon is related to the rearrangement of the extracellular matrix in vitro following collagenase treatment of the abdominal subcutaneous adipose tissue; this phenomenon might not take place in vivo.

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I.A.2.3. Late Development

The hyperplastic development of adipose tissue in aging animals fed a high-carbohydrate or high-fat diet has been thoroughly studied. Sprague-Dawley rats are able to increase the number of fat cells in most of their adipose depots (retroperitoneal, perirenal) in response to a high-carbohydrate or a high-fat diet (40). The life-long potential to make new fat cells has been clearly illustrated in rodents. For instance, the perirenal fat depots of very old mice from both sexes contain large amounts of early markers of adipocyte differentiation, i.e. acyl CoA oxidase (ACO) mRNAs, LPL mRNAs and IGF-I mRNAs, indicating that preadipocytes at stages 2 and 3 (figure 2) are indeed present in these depots (25). A similar conclusion can be directly drawn in humans, since a significant proportion of stromal-vascular cells from subcutaneous fat tissue of elderly men and women is able to differentiate in vitro into adipose cells (43) . This finding indicates that adipose precursor cells, which could give rise to new fat cells, are indeed present in those individuals. This observation can also explain the acquisition of new fat cells, which is known to take place at the adult stage in normal subjects and obese patients.

I.A.3. *Adipocyte differentiation, the 3T3-L1 model.*

The process of determination from multipotential stem cells to unipotential adipoblasts (Figure I.1) cannot be easily studied. However a determination-like process can be induced by treatment of mouse 3T3 cells (44) with insulin (45). In most cases these treated mesenchymal cells were able to differentiate into adipocytes. Adipose precursor cells or adipoblasts have been cloned from this mixed population of cells from mouse embryo (3T3-L1, 3T3-F442A) and from adult mice (Ob17) (18, 25, 33, 44- 47). The process of

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Figure I. 2. Multiple stages of adipose cell differentiation. The abbreviations are LPL, lipoprotein lipase; FA, fatty acid; ACO, acyl CoA oxidase, ACS, acetyl-CoA synthase; FATP, fatty acid transporter protein; C/EBP, CAAT/ enhancer binding protein; PPAR, peroxisome proliferator activated receptor; IGF-1, insulin-like growth factor 1; GLUT4, insulin sensitive glucose transporter 4; ME, malic enzyme; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthetase; GPDH, glycerol-3-phosphate dehydrogenase; a-FABP, adipocyte fatty acid binding protein; HSL, hormone sensitive lipase; GPAT, glycerophosphate acyltransferase; LPAT, lysophosphatidate acyltransferase; DGAT, diglyceride acyltransferase; ACBP, acyl CoA binding protein; PEPCK, phosphoenol-pyruvate carboxykinase; β2-AR, β2-adrenoreceptor; β3-AR, β3-adrenoreceptor; α2-AR, α2-adrenoreceptor. *Adapted from Ailhaud et al 1992, (14).*

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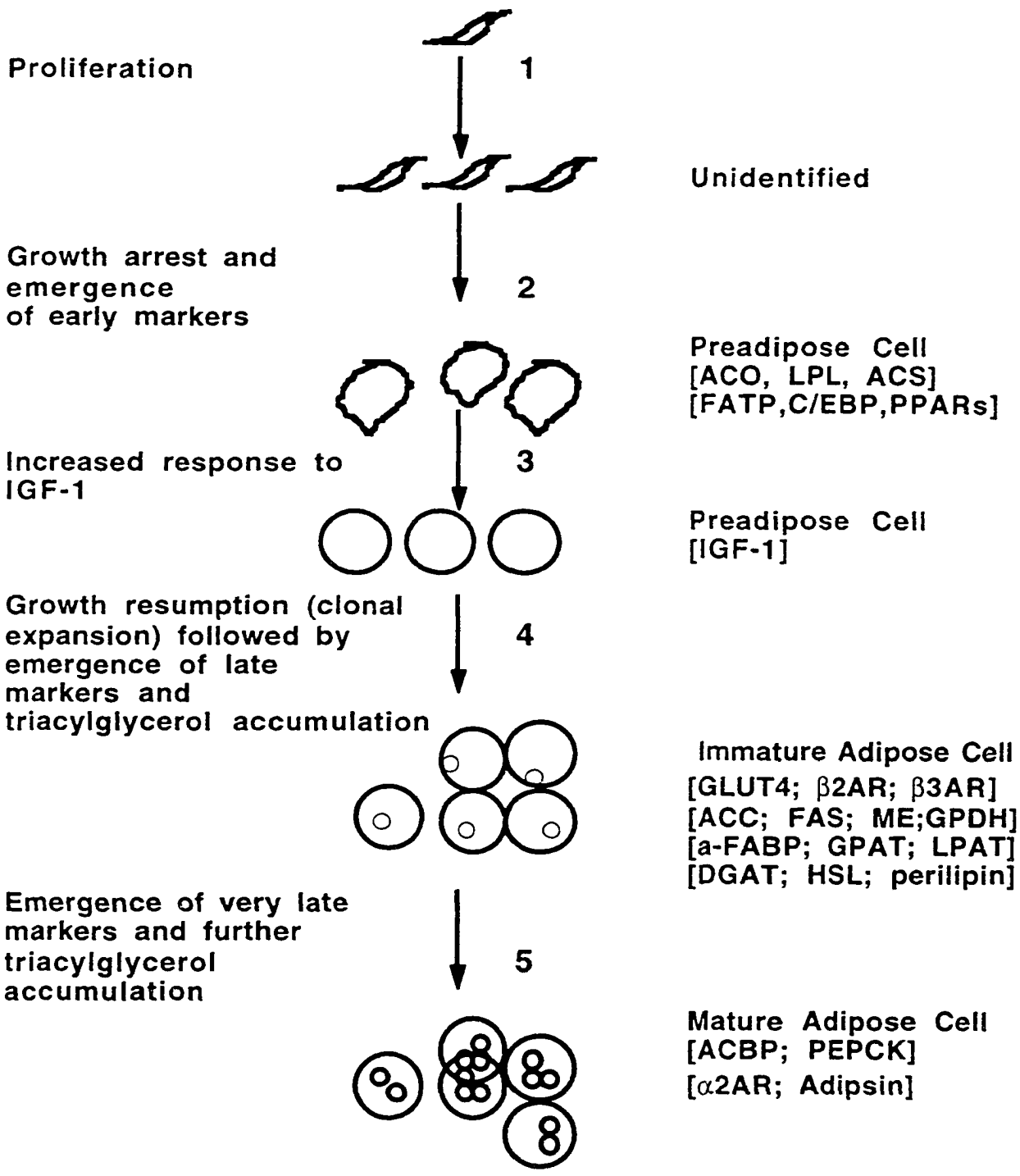

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adipose cell differentiation, which can be analyzed in vitro, corresponds to the phenotypic changes: adipoblast -----> preadipose cell (usually termed preadipocyte) -----> immature adipose cell -----> mature adipose cell (Figure I.1 and I.2).

I.A.3.1. Sequential events

The 3T3-L1 mouse cell line is a clonal derivative of Swiss mouse embryo fibroblasts (46, 47) that undergoes adipose conversion under appropriate experimental conditions (48). The conversion of 3T3-L1 cells from fibroblasts to adipocytes represents a spontaneous and heritable phenotype and can be facilitated by treating the cells with various hormones, drugs or nutrients such as insulin or IGF-1 (6, 7, 49). Once the cells are committed to differentiate and begin to accumulate fat droplets in the cytosol, the process is irreversible and the adipocytes represent a population of terminally differentiated end-cells. The 3T3-L1 cell line has been widely used as a model to study adipose differentiation in mammals (6-8, 12-14, 43-57) . Morphologically, after reaching confluence, fibroblast-like 3T3-L1 cells become round, enlarge, and accumulate triacylglycerol droplets in their cytoplasm. Once differentiated, mature adipose cells show most, if not all, biochemical characteristics and hormonal responses of adipocytes. The main events, based upon numerous studies by various investigators are summarized in Figure I.2. Growth arrest at the G1/S stage of the cell, rather than contact among arrested cells, is necessary to trigger the process of cell commitment (51). This commitment is associated with the emergence of potential regulatory genes such as ACO, LPL mRNAs and LPL activity (52), as well as the emergence of a selective uptake of long-chain fatty acids (53). The regulation of expression of these early genes takes place primarily at a transcriptional level and appears to be independent of various hormones which, in contrast, are required for terminal differentiation

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(52, 54). The expression of late and very late genes is associated with limited growth resumption of these committed early marker-expressing cells. DNA synthesis of preadipose cells precedes terminal differentiation. At least one cell doubling has been consistently observed, and this process of clonal amplification of committed cells (defined as postconfluent mitoses) is limited both in magnitude and duration. Cell division of preadipose cells appears to be essential for terminal differentiation (defined by the emergence of GPDH activity), providing that cells are exposed to the appropriate hormonal milieu (51, 55). The process of terminal differentiation is characterized by the induction of late and very late markers (Figure I.2 steps 4 and 5). The emergence of some late markers, is primarily due to increased gene transcription (56, 57). As represented in figure I.2, the enzymatic machinery required for lipogenesis and triacylglycerol synthesis is then turned on and is responsible for lipid accumulation. Clearly, in order for the cell to differentiate from a fibroblast to an adipocyte, a specific and ordered sequence of events must take place in the cell. This program requires inactivation of certain genes and expression of new genes to produce new proteins necessary and specific to the function of the adipocyte. Some of these newly expressed genes are molecular markers specific to adipose differentiation, which are needed for a better understanding of adipose tissue development. One such gene is the gene coding for the adipocyte fatty acid binding protein (a-FABP).

I.B. Regulation of a-FABP expression and key regulatory factors of adipocyte differentiation.

I.B.1. *The adipocyte fatty acid binding protein.*

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a-FABP was isolated (58) and structurally characterized as a low molecular mass cytosolic protein of 14.6 kd, and as the adipocyte specific member of a multigene family represented by different isoforms in different tissues (59). Cytosolic a-FABP is not only thought to play a role in the trafficking process of fatty acids, but also in the regulation of the free fatty acid concentration which can be stored as triglycerides. This protects the cell from the detergent action of fatty acids (60) but more importantly, it allows the adipose tissue to act as an energy storage bank for the animal. When glucose supplies become low, the adipose cells hydrolyze triglycerides and release the fatty acids and glycerol for utilization elsewhere. With respect to fatty acid metabolism, the increase in uptake of fatty acid is concomitant with the induction of LPL. Fatty acid entry then becomes sufficient to activate the transcription of a-FABP. This process is very important for the energy metabolism of higher animals and is under the control of numerous hormones such as insulin, epinephrine and glucagon (14, 61). An important phenomenon for the functioning of a-FABP may be its phosphorylation at tyrosine 20 in response to hormone stimuli. a-FABP is a substrate for the insulin receptor kinase (62), and fatty acid binding activates phophorylation, but phosphorylation blocks ligand binding without a dramatic effect on overall protein organization (63, 64). This suggests that an insulin-regulated phosphorylation/dephosphorylation cycle may be utilized for directionally orienting lipid flux in adipocytes. One of the current hypotheses is that the insulin-stimulated phosphorylation of the a-FABP-fatty acid complex at the plasma membrane may serve to target the complex to the microsomal membrane. The turnover of phosphorylated a-FABP may however also be an intermediate step in the insulin signaling pathway (63). Gene expression of a-FABP is mediated by insulin and IGF-1 (6, 55,56, 65) and by other "factors" or hormones yet to be determined (7, 8, 10, 57, 61). The

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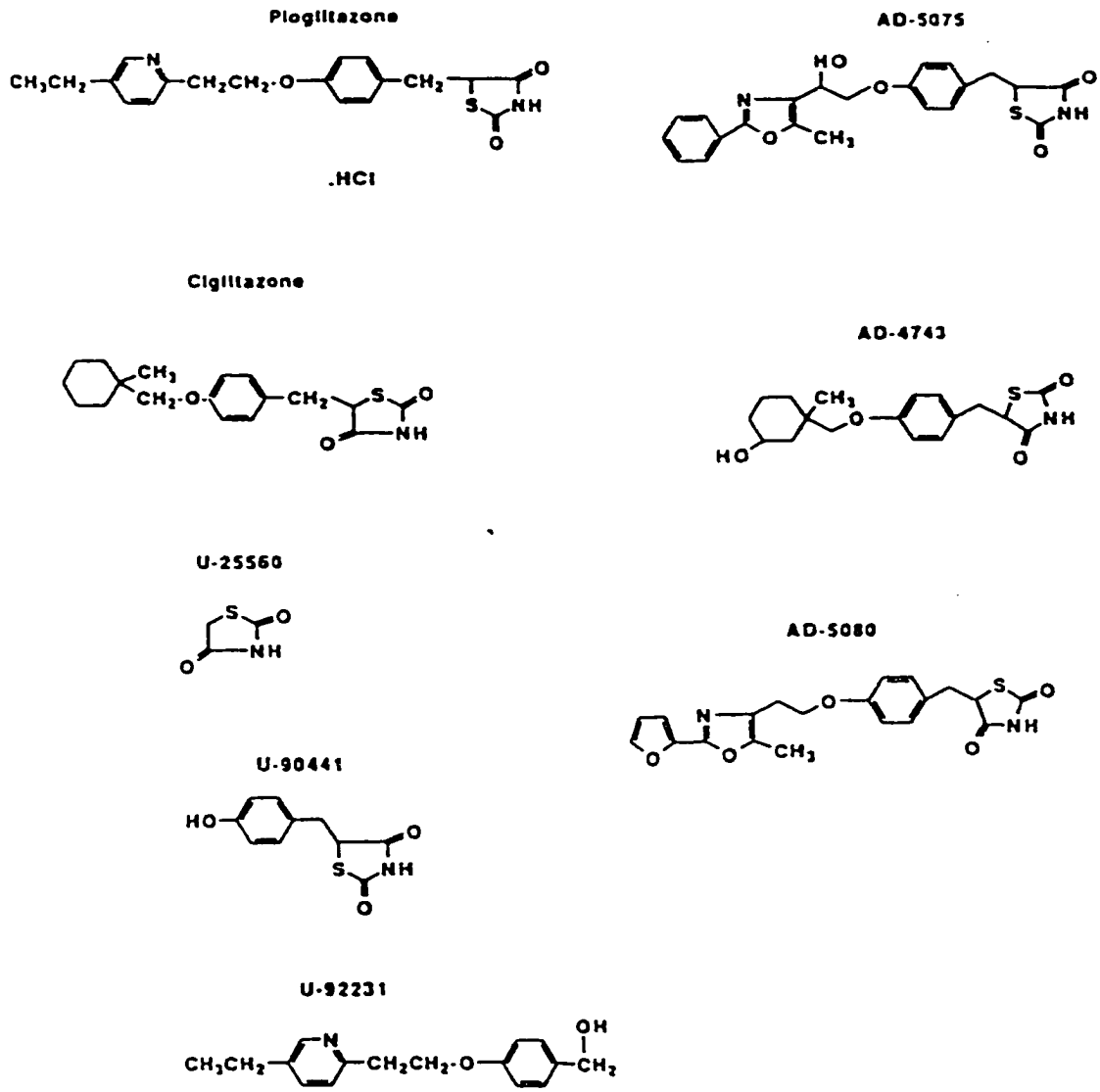

Figure I.3. Structures of pioglitazone and related analogs of the thiazolidinedione family. *Adapted from Kletzien et al 1992 (7)*

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positive effect that insulin and IGF-1 exert on adipocyte differentiation appears to be amplified by an anti-diabetic drug, pioglitazone (piog) that belongs to a family of compounds termed thiazolidinediones (7, 10) (Figure I.3). One of piog effects was to increase a-FABP mRNA levels during adipocyte differentiation. An increase in mRNA in the cell could be the result of an increase in stability of the mRNA or an increase in transcription of the gene. Previous studies showed indeed, that during differentiation of 3T3-L1 preadipocytes, a-FABP mRNA levels increased 20-fold and that transcription rates were increased 10-fold (8, 65). Increase in gene transcription reflects an increase in activity of the transcriptional machinery. Such variations result from specific interactions between trans-acting factors and cis-elements on the gene which have the ability to act at a distance to stimulate transcription. The regulation of a-FABP expression was shown not only to be transcriptional during differentiation but also to result from stabilization and transcriptional activation after differentiation (61). The characterization of the 5'-flanking region of a-FABP is ongoing and has led to the identification of several cis elements which contribute to the regulation of a-FABP expression.

I.B.2. *The 5'-flanking region of the a-FABP gene.*

The 5'-flanking region of the a-FABP gene contains several cis-acting elements that are important determinants of a-FABP gene transcription (Figure I.4): an AP1 site at -120 bp. (64); a C/EBP site at -140 bp. (66); and an adipose specific enhancer between -5.4 to -4.9 kb (9); plus several consensus motifs for glucocorticoid elements (64), and a non traditional cAMP cis-element (67). While the AP1 and the C/EBP binding sites are important for high expression of a-FABP gene in differentiating preadipocytes, the elements responsible for adipocyte specific expression of a-FABP appear to reside in the region of -5.4 to

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-4.9 kb of the 5'-flanking region of the a-FABP gene (9). Initially, an enhancer region mapping between -5.4 to -4.9 kb of the a-FABP gene was found to be sufficient to yield adipocyte specific expression of the chloramphenicol acetyl transferase (CAT) activity in transgenic mice. Studies by Spiegelman et al. have shown the existence of two adipose specific enhancers in this region of -5.4 -4.9 kb of the a-FABP gene (Figure I.4) (12). These two enhancers, termed adipose response element 6 and 7 (ARE6 and ARE7), are imperfect direct repeats (DR) of the sequence AGGTCA, with one base space similar to the DR1 type hormone response element (HRE) that is a recognition sequence for receptors of the steroid hormone receptor family.

I.B.3. *PPARs are adipogenic factors.*

One of the factors binding to ARE6 was recently cloned (13) and identified as PPARγ2, a member of the peroxisome proliferator activated receptor (PPAR) family, a sub-family of the steroid hormone receptors super family which includes the receptors for retinoids (RAR), thyroid hormones (TR), vitamin D, glucocorticoids (GR), estrogen (ER) and the receptors COUP and RXRα (68). PPARs are ligand-activated transcription factors modulating the expression of a variety of target genes including genes encoding adipose fatty acid-binding protein (13), phosphoenolpyruvate carboxykinase (PEPCK)(69), lipoprotein lipase (LPL) (70), stearoyl-CoA desaturase 1 (SCD1)(71), malic enzyme (ME)(72), uncoupling protein (UCP) (73), UCP-2 (74) and apolipoprotein A (ApoA) (75). PPARs are zinc finger proteins that bind to their cognate response element as heterodimers with RXRs (76, 77). Because receptors belonging to this class bind DNA as dimers in which both partners contact the DNA, two copies of the core motif are necessary to constitute a

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Figure 1.4: Representation of the 5' flanking region of the a-FABP gene.

Shown schematically are the localization of identified response elements in the basal and distal promoter region of the a-FABP gene. Distance is expressed in kilobases (Kb). The different factors and elements represented are: the cAMP response element (CRE), the CAAT enhancer binding protein (C/EBP), the glucocorticoid response element (GRE), the activator protein 1 (AP1), and the adipose response elements 6 and 7 (ARE6 and ARE7 respectively). The start site of transcription is represented by an arrow. The CAAT box and the TATA box are also indicated. Pioglitazone and IGF/1-Insulin are indicated in the area of the gene where they are believed to exert their action.

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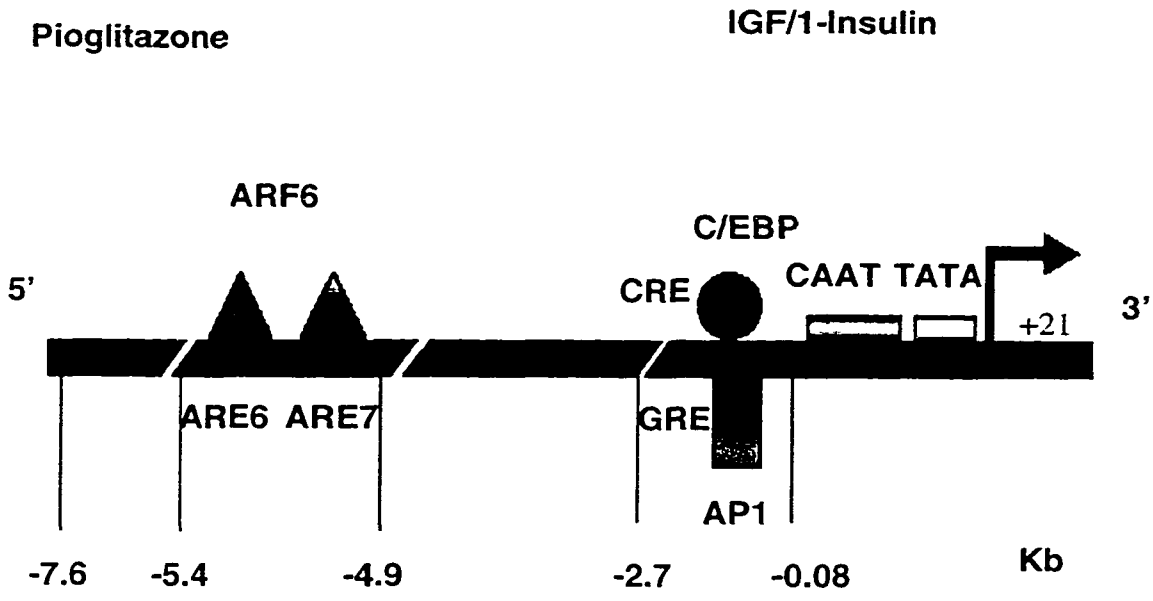

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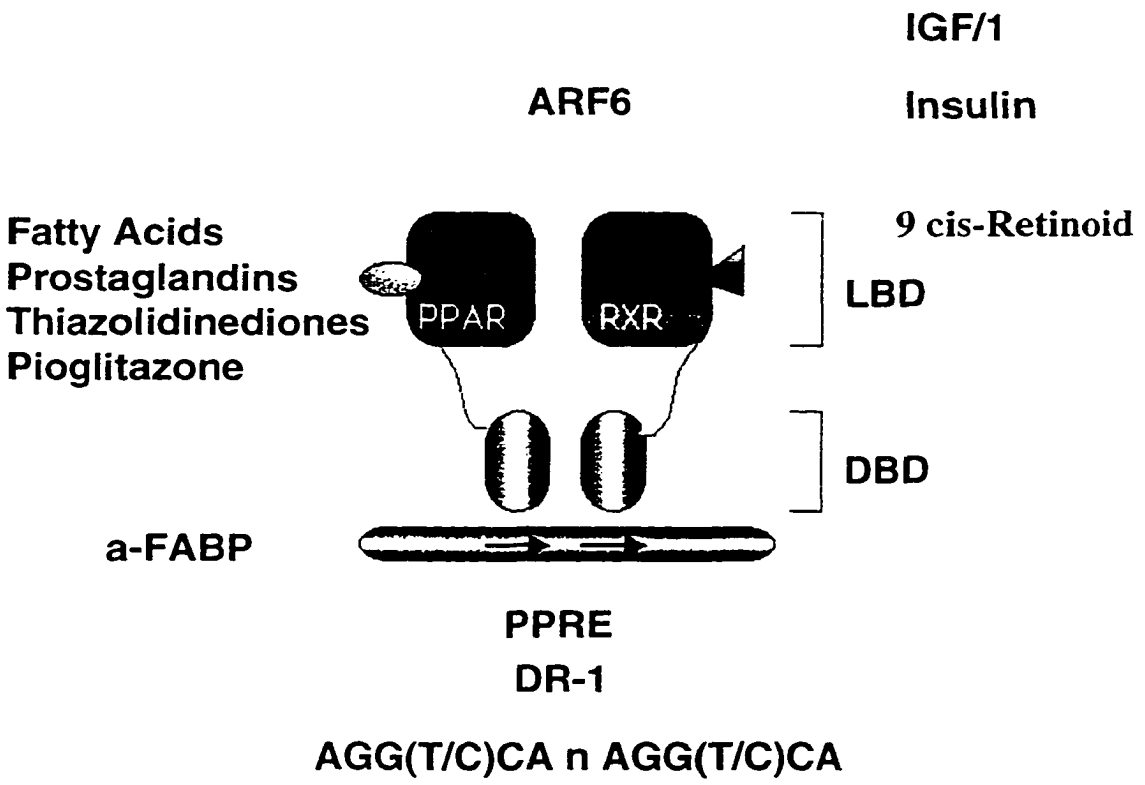

Figure I.5. PPAR and RXR heterodimerize on the DR1 type HRE (PPRE). LBD, ligand binding domain; DBD, DNA binding domain, *adapted from Tontonoz et al. (166) and Forman et al. (178)*

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functional HRE (Figure I.5). The recognition of a specific DNA sequence and the dimerization of the receptors are determined by two amino acids sequences on the receptors termed P-box and D-box. As determined by mutagenesis studies, the P box sequence CEGCKG (Figure I.6) determines DNA recognition specificity to the consensus HRE half-site AGGTCA. Classification of nuclear hormone receptors according to three decisive amino acids within the P-box (Figure I.6 in boldface) revealed that there are two principal groups of receptors: the GR-like group whose members recognize a consensus AGAACA half site HRE, and all the other receptors which bind to a consensus AGGTCA half-site HRE. Interestingly, the latter group shows a certain limited variability in the P-box sequence. The first of the three decisive positions appears to require an acidic amino acid (Glu or Asp), the second amino acid can be a Gly, or Ser, and the third a Gly, Ser or Ala. The D-box, located between the first pair of Cys residues in the second zinc finger, contains only three amino acids in PPARs but five amino acids in all other receptors. It is involved in the dimerization of receptors and the determination of response element half-site spacing. The relative orientation (e.g. direct repeat, palindromic, or inverted palindromic configuration) and spacing of the two motifs dictates which particular set of receptors bind to a given HRE. PPARs contain highly conserved DNA-binding domains but more divergent ligand-binding domains (Figure I.7) (78). Thus, although they bind to the same DNA consensus direct repeat sequences spaced by one base (DR-1) in vitro, the activation profiles of these receptors are pharmacologically distinct (77, 79). Three PPAR isoforms have been identified that define a subfamily of nuclear hormone receptors (Figure I.7). PPARα , the first member of the PPAR family to be identified, was cloned in 1990 as an orphan receptor activated by agents that induce peroxisome proliferation,

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Figure I.6. Partial classification of nuclear hormone receptors according to their P-box sequence.

Shown schematically are the conserved structures of nuclear hormone receptors with the A-F domains and a more detailed view of the DNA binding domain (C) with its characteristic two zinc fingers. The P-box situated in the first zinc finger contains the second pair of cysteines and determines the specificity of response element recognition. The D-box is involved in the dimerization of receptors and half-site spacing of the response element. The nuclear hormones receptors indicated are the receptors for: glucocorticoid (GR), progesterone (PR), androgen (AR), thyroid hormone (TR), estrogen (ER), retinoic acid (RAR), retinoic X (RXR), vitamin D (VDR), peroxisome proliferator-activated (PPAR), chicken ovalbumin upstream promoter (COUP), COUP-TF-II (ARP-1) and COUPg (EAR2). *Adapted from Mangelsdorf et al. (68)*

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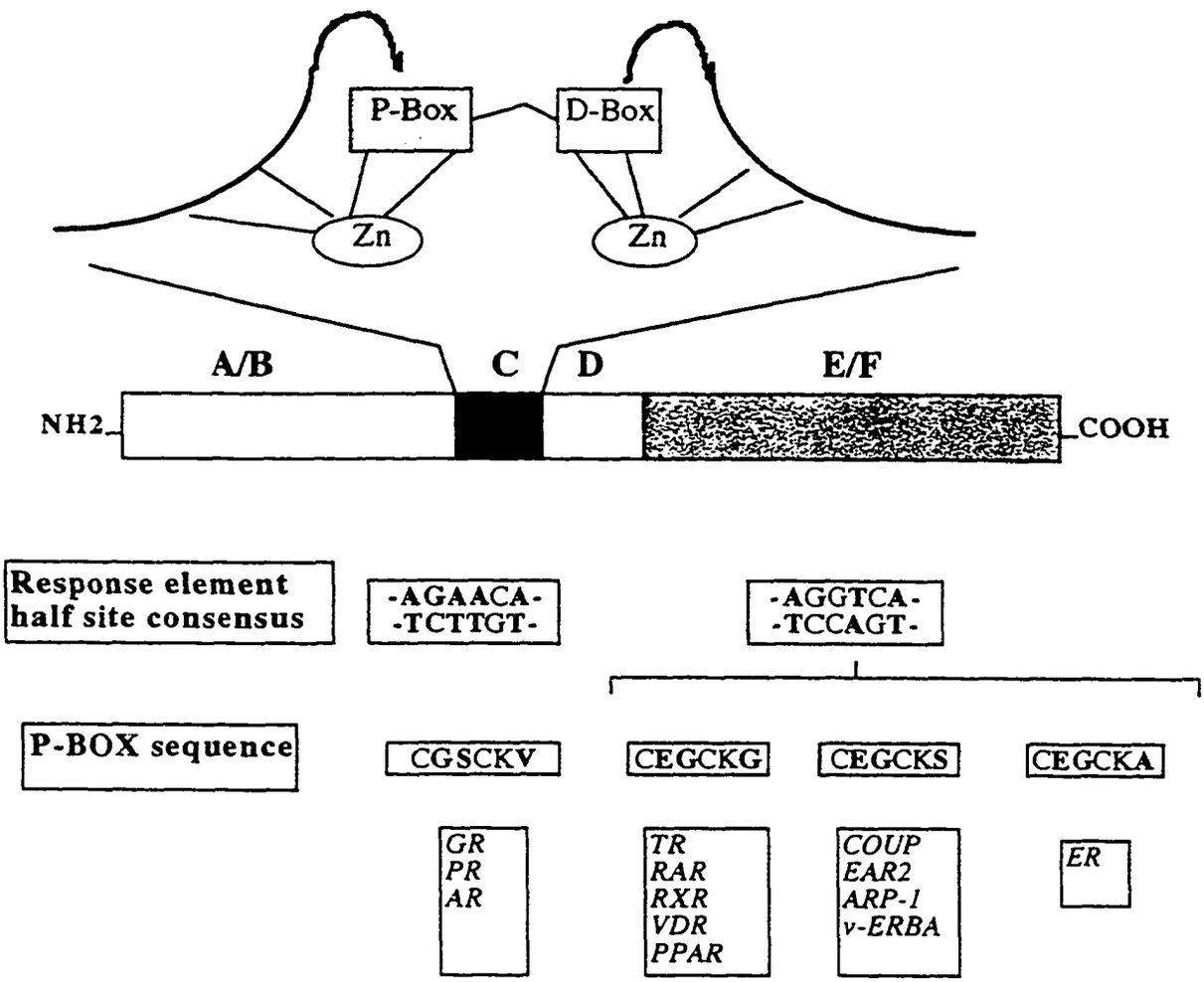

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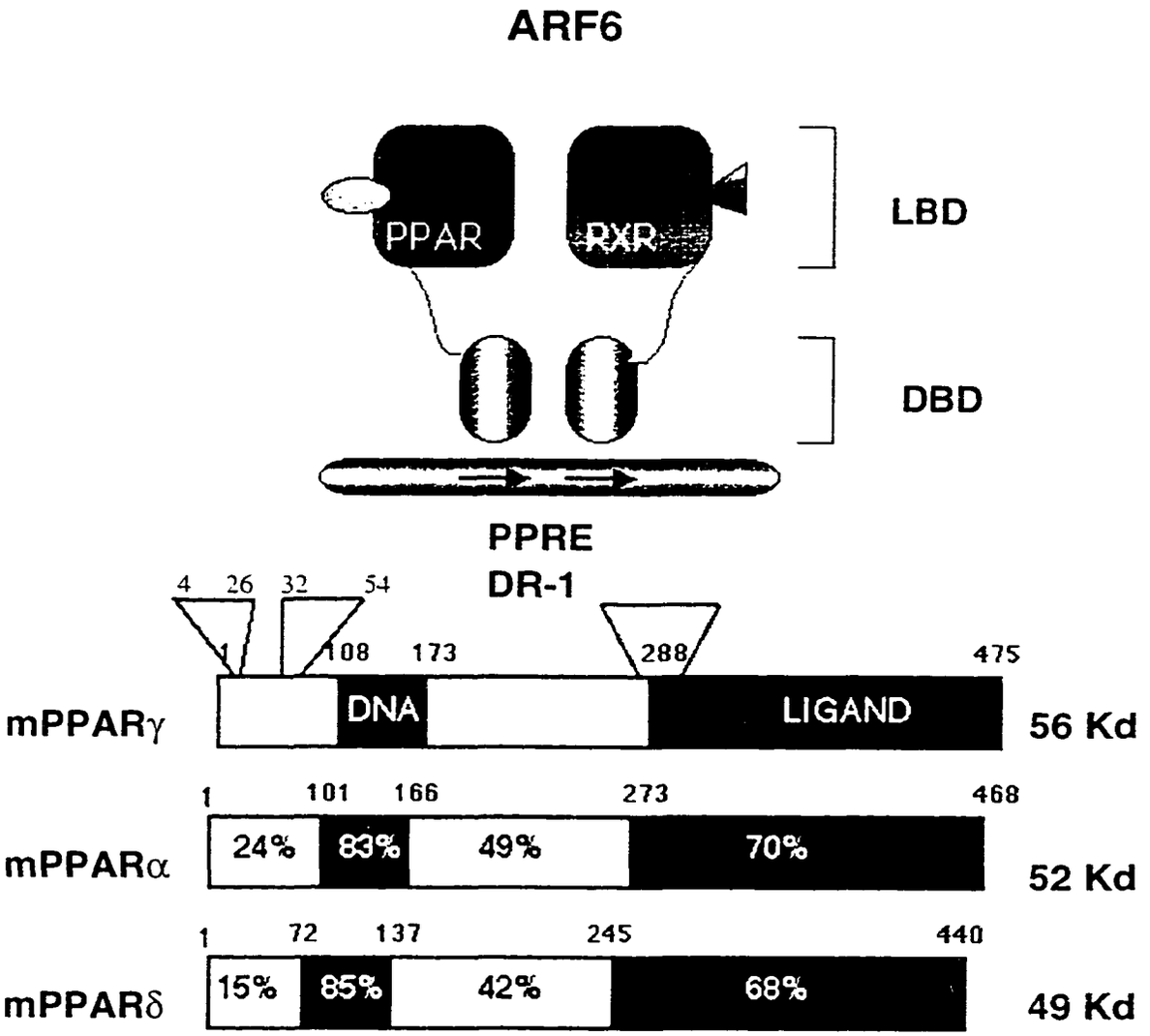

Figure I.7: Relative homology between the three murine PPAR isoforms. LBD, ligand binding domain; DBD, DNA binding domain; PPRE, PPAR response element; DR-1, direct repeat 1; ARF6, adipose response factor 6. *Adapted from Kliewer et al. (78) and Forman et al. (178).*

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primarily in liver (80, 100). PPARα, expressed mainly in liver, heart, kidney and adipose tissue is thought to regulate transcription of many genes involved in the β-oxidation of fatty acids (81) and have recently been associated with cancer (82). PPARδ also referred as NUC1 (84), PPARβ (85) and FAAR (86), is expressed in nearly all tissues (78, 84, 86), suggesting that this isoform may play a general housekeeping role. PPARγ is the most adipose-specific of the PPARs. It is expressed at low levels in kidney, spleen, and adrenal glands but is most abundant in adipose tissue (13, 78, 87). Its adipogenic activity is markedly enhanced by the presence of insulin in the well established 3T3-L1 and 3T3-F442A preadipocyte cell lines (88). Two isoforms of PPARγ, PPARγ1 and PPARγ2, are generated by alternative splicing and alternate translation initiation (13, 89, 90). Although PPARγ2 appears to be more adipose-specific than PPARγ1 (91), functional differences between the two isoforms have not been detected. The recent identification of the human PPARγ promoter (92, 93), will be helpful in determining the regulation of expression of these two isoforms. Because PPARs activate many genes that are involved in lipid metabolism, including both catabolism and anabolism, understanding the impact that they have on fatty acid metabolism has been crucial in gaining insight into their role in lipid homeostasis and their effect on adipocyte differentiation.

I.C Role of PPARs in lipid metabolism.

I.C.1 *Fatty acid metabolism and lipid homeostasis.*

Liver is a pivotal organ in determining the metabolic fate of fatty acids, which can be either esterified into triglycerides or oxidized. The esterification of fatty acids leads to the secretion into the blood stream of excess triglycerides as

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VLDL, which are mainly taken up by adipose tissue for triglyceride storage. When plasma fatty acids levels are high, the oxidative pathway leads to the production and secretion of ketone bodies that serve as fuel for brain, muscle and kidney. Hence, by regulating the relative rates of fatty acid uptake, their esterification into triglycerides and their oxidation, the liver is able to regulate the levels of the three circulating fat fuels: non-esterified fatty acids, triglycerides and ketone bodies.

Many PPAR target genes have been identified, and their functions suggest that PPARα plays an important role in fatty acid oxidation in liver. Typically, upon ingestion, fatty acids travel through the gut where medium chain fatty acids (MCFA, containing 8-12 carbons and saturated), are absorbed more quickly than long chain fatty acids (LCFA, containing 14 or more carbon atoms and up to one or more double bonds). MCFA are then transported directly to the liver as non-esterified fatty acids carried by serum albumin via the portal circulation while LCFA are preferentially incorporated into VLDL or chylomicrons as long chain triglycerides and transported via lymph. Once transported to the liver, triglycerides are hydrolyzed extracellularly into glycerol and fatty acids by lipoprotein lipase (LPL) (94). The released free fatty acids are either oxidized to generate ATP in muscle, stored in adipose tissue, or secreted in milk by the mammary gland. In contrast to LPL, apo C-III appears to antagonize plasma triglyceride metabolism and inhibits triglyceride hydrolysis by LPL and hepatic lipase. Hence triglyceride levels are regulated by both LPL and apo C-III levels but with opposite effects. Cellular uptake of long-chain fatty acids is facilitated by fatty acid transporters such as the fatty acid transporter protein (FATP) (95) and the fatty acid translocase (FAT) (96). Once imported into hepatocytes, FFA may follow various catabolic pathways including β-oxidation, ω-oxidation, and peroxisomal oxidation. While FATP facilitates the

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passage of FFA across the plasma membrane, the acyl-CoA synthase (ACS)-dependent esterification of these FFA to acyl-CoA derivatives prevents efflux of the incorporated fatty acids from the cell, rendering uptake unidirectional (95). This activates the FFA for utilization in catabolic (β-oxidation) or anabolic pathways (conversion into more complex cellular lipids). The majority of lipids are catabolized by mitochondrial β-oxidation. Acyl-CoA synthase (ACS), esterify FFA to their acyl-CoA derivatives. Acyl-CoA may then either be esterified or alternatively, may enter the pathway of β-oxidation. Long (C14 to C20) and medium chain (C8 to C12) fatty acids can be oxidized either in peroxisomes or mitochondria. The flux of fatty acids into mitochondria is controlled by a carnitine dependent facilitated transport system. The acyl-CoA derivatives are transformed into acylcarnitine by carnitine palmityl transferase (CPT) I, cross the mitochondrial membrane and are regenerated as acyl-CoA derivatives by CPT II. These mitochondrial acyl-CoA derivatives undergo oxidation with production of acetyl-CoA which can then be further oxidized through the Krebs cycle. High levels of acetyl-CoA in the mitochondria are conducive to ketone body formation via 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, a key enzyme in ketogenesis. HMG-CoA synthase catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to generate HMG-CoA which is eventually converted to ketone bodies. An alternate route for cytosolic fat utilization is peroxisomal oxidation. In liver, very long-chain fatty acyl-CoA (C>20) is β-oxidized in peroxisomes. In addition, at high concentrations of FA in the perfused liver, it is estimated that 25% of oxidation occurs in the peroxisome (97). Unlike mitochondrial β-oxidation, peroxisomal β-oxidation produces H_2O_2 and is not coupled to a respiratory chain. However, induction of peroxisomal and mitochondrial β-oxidation is coordinated (94) and mitochondrial β-oxidation always exceeds and precedes β-oxidation occurring in the peroxisome.

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Peroxisomal β-oxidation involves three enzymes, acyl-CoA oxidase (ACO), enoyl-CoA hydratase 3-hydroxyacyl-CoA dehydrogenase (also termed multi-functional enzyme), and 3 ketoacyl-CoA thiolase. These three enzymes act sequentially to catabolize fat by an energetically inefficient pathway that may contribute to enhanced thermogenesis. ω-oxidation originated in the hepatocyte microsome, produces water soluble dicarboxylic acid which can be excreted in the urine. ω-hydroxy and dicarboxylic acids formed by FA ω-oxidation in the liver may then be further metabolized in the liver, accounting for acetate release from peroxisomes (97). Acetyl-CoA derived from FA and not directed toward ketone body formation or oxidation may be resynthesized into longer chain FA and esterified by activation of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). As discussed below, the expression and activity of many of the enzymes described above involved in regulating lipid homeostasis are regulated by PPARs through a PPRE or by peroxisome proliferators.

I.C.2. *Role of peroxisome proliferators and PPAR in fatty acid metabolism.*

Prior to the discovery of PPARs, peroxisome proliferation had been observed and characterized as a cellular response to a variety of chemical compounds and to certain pathophysiological conditions involving extreme changes in cellular morphology and enzymatic activity (98). This phenomenon mostly observed in liver and kidney may lead to hepatocarcinogenesis under certain conditions (99). Peroxisomes are small subcellular organelles involved in cellular metabolism that contain more than 50 enzymatic activities ranging from anabolic to catabolic pathways (100) . These activities include: β-oxidation of long chain fatty acids and derivatives, fatty acid elongation, hydrolysis and

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conversion of acyl-CoAs to acylcarnitines, the biosynthesis of ether glycerolipids, cholesterol and dolichols, the catabolism of polyamines, purines and amino acids, the conversion of glyoxylate, the metabolism of glucose via the hexose monophosphate pathway and the metabolism of reactive oxygen species (98, 101). Peroxisome proliferation is activated by a wide range of agents both natural and synthetic. Fibric acid derivatives (clofibrate, fenofibrate, bezafibrate, ciprofibrate, beclofibrate and etofibrate) reduce plasma triglycerides and cholesterol in hyperlipidemic patients (102) and result in liver peroxisome proliferation when administered to rats (103). A range of xenobiotics compounds such as phtalate, plasticizers, herbicides, leukotriene antagonists, acetylsalicylic acid, thio-substituted fatty acids and others, also induce peroxisome proliferation in rodents which are more susceptible than other species (98). Interestingly, although their only common structural property is an acid function, most of these agents also lower lipid levels. Similar effects caused by xenobiotics proliferators have been observed with natural factors such as high fat diets. These include diets enriched in partially hydrogenated fish oils which are poorly oxidized via the mitochondrial β-oxidation pathway and have a hypotriglyceridemic action (104). Peroxisome proliferation is also observed after increased dietary fat intakes during starvation (105) and during diabetes mellitus (106), suggesting that the peroxisome proliferation response can occur under certain pathophysiological conditions. Hormonal imbalances like hyperthyroidism (107) and treatment with synthetic glucocorticoids, such as dexamethasone, have also been reported to induce or enhance the enzymatic capacity of peroxisomes (108). Finally, certain vitamin derivatives, such as retinoic acid or vitamin E deficiency also induce peroxisomal enzymes (109).

Historically, peroxisome proliferation induction has been understood as an adaptive response to intracellular accumulation or overload by fatty acids,

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natural or xenobiotics compounds. Since mitochondria is the major site for fatty acid oxidation, an inhibition or overload of the mitochondrial pathway is sufficient to trigger peroxisome proliferation induction (110). Peroxisome β-oxidation stimulation can be viewed as a response that cells developed to metabolize fatty acids or analogs that are not oxidizable by the mitochondria. Hence peroxisome proliferating agents greatly affect hepatic lipid metabolism as a result of mitochondrial fatty acid oxidation inhibition (111). Consequently, long-chain dicarboxylic acids that are poorly oxidized in the mitochondria, accumulate and degrade in the peroxisomes which results in the induction of peroxisomal β-oxidation enzymes (112). Although alterations in lipid metabolism are always associated with peroxisome proliferation, the fact that some peroxisome proliferators do not inhibit mitochondrial β-oxidation or have the ability to modulate specific gene transcription rapidly, together with the description of a peroxisome proliferator-binding protein in rat liver (113), led to the proposition of a receptor-mediated model to explain peroxisome proliferation. Such a receptor was first isolated from mouse liver by Isseman and Green (80) and is now known as PPARα.

The discovery of PPARs urged scientists to define its role in directing peroxisome proliferator responsiveness to certain target genes. The best candidate genes were those involved in the peroxisomal β-oxidation pathway which is responsible for the metabolism of long-chain fatty acids and sequentially involves the enzymes ACO, the multi-functional enzyme, and 3 ketoacyl-CoA thiolase. The activities of these enzymes are stimulated in response to peroxisome proliferators (114). These changes correspond to an increase in the transcription rates of their genes (115) which contribute to decrease the lipid concentration in the serum. Analysis of the promoter sequences from the ACO gene and the multifunctional gene led to the

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identification of PPREs that conferred transcriptional activation by PPAR (116, 117). However , despite its transcriptional response to peroxisome proliferators, no PPRE has been identified in the ketoacyl-CoA thiolase gene, suggesting that the response of this gene to peroxisome proliferators could occur through a PPRE independent mechanism. Such a model is supported by the recent observation that the activation of the prolactin gene by PPARα is DNA binding-independent. Apparently PPARα activation of prolactin expression does not involve its dimerization with RXR on a PPRE but seems, rather, to exert its action by protein-protein interaction with other transcription factors and coactivators (118). The importance of PPARα in regulating enzymes involved in β-oxidation was clearly demonstrated by disruption of the ligand-binding domain of mouse PPARα by homologous recombination (119). In these transgenic animals, unlike in the wild type animals, peroxisome proliferators exposure (clofibrate and Wy-14,643) did not induce several enzymes of the β-oxidation and microsomal ω oxidation pathways. This implies that PPARα is involved in regulating a battery of lipid-metabolizing enzymes to balance perturbations in cellular lipid metabolism. In addition, the mice lacking mPPARα did accumulate fat droplets upon peroxisome proliferator treatment, which is indicative of an imbalance between the esterification and oxidative pathways, which may further be the result of lack of regulation of β-oxidation and ketogenesis. Following the discovery of PPRE in the ACO gene, a multitude of genes responsive to peroxisome proliferators and involved in lipid metabolism were discovered to harbor a functional PPRE in their promoter.

Peroxisome proliferation is accompanied by an increase in microsomal enzymes, in particular those belonging to the family of cytochrome P-450 IV enzymes (referred to as P-452 and CYP4A) (120). The enhanced activity of cytochrome P-450 IV enzymes results from a transcriptional induction possibly

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through a PPRE. These enzymes catalyze the ω-hydroxylation of a selection of substrates including fatty acids and prostaglandins. Induction of ω-hydroxylation precedes the induction of peroxisomal β-oxidation, suggesting that P-450 IV induction is an early and obligatory step in the induction of peroxisome proliferation (121). However, other reports suggest that P-450 IV might be necessary but not sufficient to ensure a peroxisome proliferative response (122).

Ketogenesis and mitochondrial β-oxidation are also enhanced by peroxisome proliferators. PPAR controls the induction of HMG-CoA synthase, a key mitochondrial enzyme in ketone body genesis, via a PPRE located in the proximal region of its gene (123). Interestingly, PPARα activates HMG-CoA synthase by physical interaction and stimulation of its translocation to the nucleus and transcriptional activation via the cognate PPRE of the HMG-CoA synthase gene (124). PPAR also mediates the transcriptional induction of the mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD) gene through a nuclear receptor regulatory element 1 (NRRE-1) different than the typical DR-1 (125). MCAD is one of the four different chain-length-specific enzymes that catalyze the initial reaction in the mitochondrial fatty acid (FA) β-oxidation cycle. Substrates for MCAD include medium chain length (C_6-C_{12}) acyl-CoA thioesters derived from: medium-chain FAs that enter mitochondria by diffusion, products of mitochondrial β-oxidation of saturated and unsaturated long-chain FAs, and products of peroxisomal β-oxidation of long-chain and very long-chain FAs. Because these diverse pathways of FA oxidation converge at this point, MCAD catalyzes a pivotal step in cellular FA metabolism. Peroxisome proliferators have also been shown to activate several cytosolic proteins such as palmitoyl-CoA hydrolase (126), malic enzyme (127), the acyl-CoA-binding protein (ACBP) (128), the liver fatty acid binding protein (L-FABP) (129) and a-FABP (13).

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Beside their action on intracellular lipid metabolism, PPARs are also important regulators of extracellular lipid metabolism. Apo A-I and apo-A-II are the major protein constituents of HDL, the protagonists of reverse cholesterol transport, a pathway that protects against coronary artery disease (130). Transgenic animal studies suggest a less protective role of apo A-II relative to apo A-I against the development of coronary heart disease (131). In rodents, fibrates and fish oils down regulate apo A-I and apo A-II gene expression, while in man, fibrates increase plasma levels of HDL and apo A-I and apo A-II. This opposite effect between the two species is attributed to an overall negative effect of fibrates and free fatty acids on the minimal apo A-I promoter, which is independent of PPAR (132). In humans but not in rats, this negative effect of fibrates and FFAs on apo A-I transcription is counteracted by PPAR activation of the apo A-I gene via a PPRE localized in the promoter of the human but not the rat apo A-I gene.

PPARs play an important role in the metabolism of triglyceride-rich lipoproteins. Lipoprotein lipase (LPL) whose gene is stimulated by PPARs (133), hydrolyzes the triglyceride moiety of chylomicrons and VLDL particles. LPL deficiency results in severe hypertriglyceridemia and chylomicronemia (133), illustrating its importance in lipid homeostasis. In vivo data indicate that fibrates and fatty acids induce LPL mRNA in rat liver (134) and adipose tissue (135), and this effect is regulated transcriptionally via a PPRE in vitro (136). In addition, fibrates down-regulate the expression of apo C-III (137). This regulation in circulating apo C-III levels in response to fibrates should enhance the LPL-mediated lipoprotein lipolysis and the clearance of VLDL particles. Consequently, the dual action of fibrates on the expression of LPL and apo C-III provides a potential mechanism by which these drugs reduce plasma triglycerides and improve postprandial hypertriglyceridemia. Fibrates exert their

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hypolipidemic action by enhanced catabolism of triglyceride-rich lipoproteins and a concomitant increase in hepatic uptake of the release FFA (138). Both FATP and ACS are regulated by PPARα and PPARγ activators (139) in a variety of tissues and cells. In addition, ACS is regulated by PPARα at the transcriptional level (140). Interestingly, CPTI is strongly induced by peroxisome proliferators and fatty acids (94). Thus the gene encoding CPTI probably represents another PPARα target gene and is consistent with the observation that peroxisome proliferator-induced ACS activity generates acyl-CoA esters which are predominantly used for β-oxidation (141). Due to the dramatic increase in peroxisomal and mitochondrial β-oxidation pathways after treatment with peroxisome proliferators, fewer acyl-CoA esters are available for triglyceride synthesis. A reduction in acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) activity (142-144) will also decrease de novo fatty acid synthesis, further diminishing the intracellular levels of FFA available for triglyceride synthesis. Although polyunsaturated fatty acids suppress both ACC and FAS expression, recent studies by Ren et al. (145) on S14 regulation suggest that PUFA regulation of these genes is not directly mediated by PPARα. In addition to their effect on β-oxidation and triglyceride synthesis, peroxisome proliferators decrease VLDL and apo B production (146-147). As a consequence, a reduced secretion of VLDL particles, together with an enhanced catabolism of triglyceride-rich particles, most likely account for the hypolipidemic effect of fibrates. The concerted PPAR-mediated regulation of several genes involved in the metabolism of triglyceride-rich lipoproteins leads to the following: increased hydrolysis of triglycerides, stimulation of cellular fatty acid uptake and conversion to acyl-CoA derivatives, stimulation of β-oxidation and reduction in fatty acid and triglyceride synthesis and VLDL production. All

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these effects seem to act together to explain the hypolipidemic effect of fibrates and fatty acids in humans and rodents.

The minute dissection of PPAR action in lipid metabolism through the characterization of its target genes, has produced clues on the natural physiological environment in which PPARα would stimulate fatty acid catabolism. At low plasma levels of fatty acids, the esterification pathway competes effectively with that of oxidation (148). In contrast, fasting or stress situations stimulate adipose lipolysis, which leads to an increase of the plasma levels of non-esterified fatty acids. In turn, the rates of fatty acid uptake, fatty acid β-oxidation and ketogenesis are strongly activated in the liver. Thus fasting and stress represent physiological conditions in which fatty acids can activate PPARα and stimulate the oxidative pathway. Fasting is also associated with an increased rate of gluconeogenesis, a pathway regulated by PEPCK which contains a functional PPRE (149) and is therefore expected to be stimulated by PPAR.

I.C.3. *Impact of PPAR on adipocyte metabolism*

As described previously, white adipose tissue is composed of adipocytes which play a central role in lipid homeostasis and the maintenance of energy balance in vertebrates. The energy is stored in the form of triglycerides during periods of nutritional abundance and is released in the form of free fatty acids at times of nutritional deprivation. Excess of white adipose tissue leads to obesity and its absence is associated with lipodystrophic syndromes. In contrast to the development of brown adipose tissue, which mainly takes place before birth, white adipose development and differentiation is a continuous process throughout life (150). Adipocyte differentiation from adipoblasts (Fig. I.1 and I.2) is triggered under the interdependent action of two groups of transcription

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factors, PPARs and C/EBPs (107, 111). As depicted in figure I.2, during adipocyte differentiation several specialized proteins are induced, and most of them are involved in lipid storage and metabolism. Good examples of such proteins are a-FABP, PEPCK, ACS and LPL in whose genes PPRE were recently identified, suggesting the involvement of PPAR in their induction. The increase in lipolytic activity resulting from the induction of LPL by PPARγ (135, 136), will result in increased delivery of fatty acids to the adipocytes, a phenomenon further enhanced by the PPAR-mediated induction of FATP and ACS expression. As discussed below, fatty acid-derived prostaglandin derivatives, whose delivery to the cell is increased by FATP, bind to and activate PPAR (128). Thus, fatty acids will provide the necessary build-up for triglyceride accumulation, ultimately enhancing adipocyte differentiation. The increased delivery of fatty acids will result in triglyceride synthesis and accumulation in the cell , a characteristic of the adipocyte phenotype. Hence it appears that fatty acids and their analogues induce the expression of adipocyte specific genes and enhance adipocyte conversion (103, 111, 127, 122), creating a positive regulatory feedback loop involving continued PPAR activation of the LPL, FATP, ACS and other genes, and aimed at promoting and sustaining the mature adipocyte phenotype.

While PPARβ function remains unknown, PPARα and PPARγ appear to regulate both sides of lipid homeostasis, i.e. fatty acid oxidation and triglyceride storage respectively. Specifically, PPARα stimulates fatty acid catabolism in several tissues known for their high rates of fatty acid oxidation such as liver, heart, kidney and brown adipose tissue. In contrast, PPARγ stimulates triglyceride accumulation in adipocyte and the storage of fatty acids. The distinct physiological actions of these two types of PPAR is reflected by their differential expression. However, because each PPAR subtype appears to be

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activated by specific ligands, their differential expression could reflect an evolution of the cell in response to the specific response elicited by this ligand.

I.D. Activation and regulation of differentiation.

I.D.1. *Multiple PPARs activation*

Since all of the PPARs (α, δ, and γ) can bind to the same DNA sequences, and all are expressed in at least some fat depots, it is interesting to know whether α and δ play any role in adipogenesis. Unlike PPARγ, PPARα or δ are not expressed preferentially in fat, however, it has been suggested that both of these receptors could play a direct or indirect role in adipogenesis (151, 152). In a direct comparison of the adipogenic potential of PPARs expressed in 3T3 cells, it was shown that PPARα can induce some adipogenesis when stimulated by its strongest activators, while PPARδ could not stimulate detectable fat cell formation (153). Although ectopic expression of PPARδ fails to induce adipogenesis in NIH 3T3 cells, this receptor appears to up-regulate expression of a subset of adipose-specific genes in these and other cell types (152, 154, 155). The fact that PPAR activators stimulate adipogenesis of 3T3-L1 preadipocytes (156) as well as myoblasts (88, 153), which expresses PPARδ constitutively (but not detectable levels of other PPARs), suggests that PPARδ may have the capacity to initiate adipocyte differentiation in at least some cell culture models. While the role of each PPAR isoform in adipogenesis has not been clearly defined, ectopic expression of PPARs in NIH 3T3 cells revealed that PPARγ was by far the most adipogenic. These findings are consistent with PPARγ adipose-specific expression and its participation in the activation of adipose differentiation (157)

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I.D.2. *The C/EBPs*

PPARγ is not the only transcription factor that is significantly elevated in adipocyte differentiation. C/EBPα, β, and δ are all markedly induced during differentiation in culture, eventhough they are not expressed in an adipose-selective fashion in vivo. C/EBPα is increased relatively late in the process of adipogenesis but ectopic expression of high levels of this factor alone can induce the differentiation of many fibroblastic cell lines (159, 160). Precocious expression of C/EBPα can accelerate the endogenous differentiation program of preadipocytes while antisense mRNA blocks the differentiation markedly (161). The important role of C/EBPα in adipose development was shown in a knockout mouse. This genetic disruption caused a major reduction in the lipid content of the fat tissue, however, fat cell differentiation apparently still occurs (162). C/EBPα, like PPARγ, directly binds to many fat cell-specific genes (161). Unlike PPARγ, which tends to bind to distal enhancer regions, C/EBPα tends to bind at sites clustered in proximal promoter regions. A dramatically cooperative or synergistic relationship between PPARγ and C/EBPα is observed when both of these factors are expressed in the same cells. Under conditions lacking a PPARγ activator, where neither factor alone promoted significant adipogenesis in fibroblasts, co-expression gave abundant differentiation (163). This cooperation is even more obvious when a cell with little adipogenic potential, such as a determined myoblast, is challenged with these factors. Neither C/EBPα or PPARγ (with activator) alone could give rise to fat cell differentiation from G8 myoblasts, but co-expression caused the differentiation of a least 70% of these cells into adipocytes (88). Thus, while either PPARγ and C/EBPα can promote adipogenesis under favorable experimental conditions, such as in a very susceptible precursor cell or with the application of high levels of adipogenic hormones or PPARγ ligands, it is likely that these factors cooperate in many, if not most physiological circumstances.

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A very interesting role of C/EBPβ in adipogenesis has recently emerged from the labs of McKnight and Farmer. Ectopic expression of this factor also promotes adipogenesis in fibroblasts (164). Notably, expression of C/EBPβ, which is normally induced very early in adipogenesis, increases the expression of PPARγ (165). The differentiation of cells expressing C/EBPβ ectopically is dependent on the addition of PPAR activator, suggesting that an important role of C/EBPβ in adipogenesis may be to turn on PPARγ expression. C/EBPδ has also been implicated in the regulation of PPARγ; co-expression of both C/EBPβ and δ is necessary to fully induce PPARγ to levels seen in adipocyte (166). The promoter for PPARγ2, the isoform expressed most selectively in fat, contains two C/EBP binding sites (89), suggesting a direct regulatory circuit.

I.D.3. *RXRs are PPARs binding partners*

Like the other members of the TR/RAR class, PPARs heterodimerize with RXRs (77, 167, 168). Indeed, PPARs strictly depend on RXRs as DNA binding partners because they do not function as homodimers or monomers. The first PPAR response element (PPRE) characterized was found in the promoter of the acyl-CoA oxidase (ACO) gene and was defined as a direct repeat of two core recognition motifs spaced by one nucleotide (Figure I.8) or DR1 (169, 170). Systematic in vitro binding studies, using repeat elements (DR) with various spacers (from 0 to 5 base pairs; DR-1 to DR-5), revealed that PPAR-RXR heterodimers preferentially bind to DR-1 elements (168, 171). This binding specificity allows PPARs to discriminate between PPREs (DR-1) and other direct repeat response elements such as the binding sites recognized by VDR (DR-3), TR (DR-4), and RAR (DR-5). DR-1 elements are also recognized by RAR-RXR (172) heterodimers and by RXR, HNF-4 and COUP-TF homodimers (173). Hence, competition for binding to a DR-1 element provides one mechanism for

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interactions between the signaling pathways mediated by this group of receptors. For example, HNF-4 and COUP-TF homodimers have been thought to compete with PPAR signaling by displacing PPAR-RXR from its binding site (174). However, subtle sequence differences can also confer preference to a given receptor complex. Indeed, the core motifs of natural PPREs often exhibit considerable divergence from the idealized consensus AGGTCA sequence (Figure I.8). Because RXR homodimers bind poorly to non consensus DR-1 elements, the combination of divergent core motifs with consensus 5'-flanking sequences results in preferential binding of PPAR-RXR to natural PPREs (171, 175).

I.D.4. *ARE6 and ARE7 are targets for PPAR/RXR.*

As mentioned earlier, the ARE6 and ARE7 recognition sequences found in the 5'-flanking region of the a-FABP gene can be interpreted as imperfect versions of a DR-1. A comparison between DR-1, ARE6 and ARE7 sequences is shown in figure I.8. The ability of PPAR-RXR to bind ARE6 or ARE7 was demonstrated when recombinant mPPARγ2 was found to dimerize with recombinant RXRα and to bind to the ARE6 and ARE7 consensus in vitro (13, 68). Because RXRα is expressed in many different tissues and PPARγ2 appears to be a true adipogenic factor, many groups have been focusing their efforts in understanding the role of PPARγ2 in vivo during adipose conversion. Clearly, the composition of the factors forming ARF6 (PPARγ-RXR) in vivo is being unraveled. However, the mechanisms regulating ARF6 activation of a-FABP expression (i.e. regulation of expression of ARF6, post-translational modification of ARF6, activation of ARF6 binding to ARE6) are still not clear.

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Gene		PPRE	
ACO 1	GGGGACC	AGGACA A AGGTCA	CGT
ACO2	GAGAGCA	AGGTAG A AGGTCA	AGA
L-FABP	CAAATAT	AGGCCA T AGGTCA	GTG
Apo-AII	TCTACCA	GGGTAA A GGTTGA	AGG
HMG-CoAS	AAAAACT	GGGCCA A AGGTCT	CAG
ME	GCATTCT	GGGTCA A AGTTGA	TCC
PEPCK1	ACTCCCA	CGGCCA A AGGTCA	TGA
PEPCK2	CACAACT	GGGATA A AGGTCT	CGC
ACS	GTCTTTC	AGGGCA T CAGTCA	CAT
MCAD	TCTCTCC	GGGTAA A GGTGAA	GGC
HBV	GGCAACG	GGGTAA A GGTTCA	GGT
ARE6	TCTCTCT	GGGTGA A ATGTGC	ATT
ARE7	TCTTACT	GGATCA G AGTTCA	CTA
Consensus	NNNAACT	AGGNCA A AGGTCA	NGN
DR1		AGGTCA A AGGTCA	

Figure I.8: Conservation between peroxisome proliferator response element (PPREs).

The DR1 motif is highly conserved among PPREs from the following genes: Acyl CoA Oxidase (ACO), liver cytosolic fatty acid binding protein (L-FABP), apolipoprotein A-II (Apo-AII). HMG-CoA synthase (HMG-CoAS), malic enzyme (ME), phosphoenolpyruvate carboxykinase (PEPCK), acyl-coenzyme A synthase (ACS), mitochondrial medium-chain acyl-CoA dehydrogenase (MCAD), hepatitis B virus enhancer 1 element (HBV), adipose response element 6 and 7 (ARE6 and ARE7). A consensus between all these genes is also indicated. Underlined sequences correspond to DR-1 or DR-1 like sequences for ARE6 and ARE7. A consensus between all these genes is also indicated. *Adapted from Refs (167-175)*

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I.D.5. *Ligand activation of PPARγ*

The nuclear receptors are built to receive chemical signals that trigger their activity. Since at least one of PPARγ's activities: to promote adipogenesis, is both very dramatic and of considerable physiological importance, there has been an immediate surge of activity to identify ligands, both natural and synthetic. Recent studies from several investigators have enabled the discovery of high affinity ligands for PPARγ2 (79, 80, 81, 151, 152, 158, 176). The identification of a natural ligand for PPARγ was preceded by research showing that many polyunsaturated fatty acids and eicosanoid derivatives, such as ETYA and WY14,643, were activators of PPARs (177). Using transcriptional activation of a PPRE through a reporter gene as an assay, it was found that prostaglandins of the J2 series had high activity (178, 179). Two groups found that 15-deoxy-$\Delta^{12,14}$ PGJ2 was a potent and rapid activator and could bind directly to PPARγ2 with a Ki of ~2 μM (154, 155). As expected for a direct agonist, 15-deoxy-$\Delta^{12,14}$PGJ2 was very effective in promoting adipogenesis when added to cells that express PPARγ. The better studied prostaglandin hormones are known to act via cell surface receptors. However, the notion that these lipids can permeate cell membranes and enter the nucleus is feasible. It is not known whether 15-deoxy-$\Delta^{12,14}$ PGJ2 is generated in cells undergoing adipogenesis at levels that would suggest that it could function as a true endogenous ligand for PPARγ. Recently, Lazar's group suggested that prostaglandins F2α and PGJ had opposite effects on adipogenesis through PPARγ2 and that this regulation involved activation of MAP kinase (180). This is supported by two recent reports that suggest that PPARγ2 exists as two separate forms, a phosphorylated inactive form and a dephosphorylated active form. Thus it appears that MAP kinase might play an important role in keeping PPARγ2 phosphorylated, indicating that the activation of PPARγ2 could be

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regulated by an inhibitory action of phosphatase on MAP kinase (180, 181). In addition, the outcome of MAP kinase activation could be to stimulate the synthesis of a new protein or to modify an existing protein needed for activation of ARF6 stimulation of a-FABP expression. Such candidate proteins could be the docking heat shock protein 90 (hsp 90), a type of protein whose synthesis increases upon heat stress, a feature presumably important for the survival of cells and organisms (182). Hsps have been shown to be involved in the translocation of certain members of the nuclear hormone superfamily, such as the estrogen receptor (ER) or the glucocorticoid receptor (GR), both members of the steroid receptor family (183). In the nuclear translocation model, the GR pool is located mainly in the cytosol of the cell as a heteromeric complex associated with a variety of hsps (184, 185), and is translocated to the nucleus upon activation by hormone. These hsps, under heat shock or chemical stress, were able to delocalize the unliganded GR to become tightly bound within the nucleus (185). Clearly, regulation of translocation of the ER from the cytosol to the nucleus allows regulation of ER action in the nucieus by post translational regulation in the cytosol (183). Because, PPARs belong to the same family of receptors as ER and GR, the possibility that ARF6 binding to ARE6 could result from a similar translocational activation of PPARγ2 cannot be ignored. However, it has not yet been explored. In addition, PPARγ was recently found to interact with hsp90 (186), giving more support to this model. Hence, if PPARγ2 were activated according to this model, then one would expect its early detection in the cytosol of the cell prior to its appearance in the nucleus, and would expect that activation by hormones or ligands would trigger translocation. As a consequence a-FABP transcription would be activated. One of the ligands with the highest affinity for PPARγ was recently identified as the synthetic drug pioglitazone (piog) (158, 178, 187)

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I.D.6. *Pioglitazone, an anti-diabetic drug and an accelerator of adipocyte differentiation.*

Piog belongs to the thiazolidinedione family (11, 108) (Figure I.3) and was originally discovered for its ability to improve hyperinsulinemia and to decrease blood glucose in non-insulin dependent diabetes milletus (NIDDM) rodents (5, 109). NIDDM is a disorder characterized by insulin resistance in muscle and adipose tissue and an inability of insulin to shut down hepatic gluconeogenesis (190, 191). The effects of piog on the symptoms of NIDDM were correlated with the ability of piog to increase the expression of genes instrumental to glucose metabolism and transport, i.e. the lipoprotein lipase gene (LPL) and the glucose transporter 4 (GLUT 4) gene respectively in adipose tissue (7). The effect of piog on adipose glucose metabolism is dependent upon insulin and is closely tied to an accelerated conversion of preadipocytes to adipocytes (7, 192). Piog accelerates adipocyte differentiation (7), presumably by mimicking some naturally existing factor that works synergistically with insulin during adipocyte differentiation.

As discussed previously, the study of adipocyte differentiation has been facilitated by the availability of preadipocyte precursor cell lines such as the 3T3-L1. Studies using this commercial cell line and others have yielded a remarkable amount of information regarding the series of molecular events taking place during adipocyte terminal differentiation. The induction of 3T3-L1 differentiation is achieved by treatment of the cells with a series of cocktails in a timely fashion, as illustrated in our differentiation protocol schematized in Figure I.9. Preadipocytes that have a fibroblast like morphology are grown in serum rich medium containing 10% of fetal calf serum. When they reach 70% of confluence, these cells that continue proliferating are still termed true

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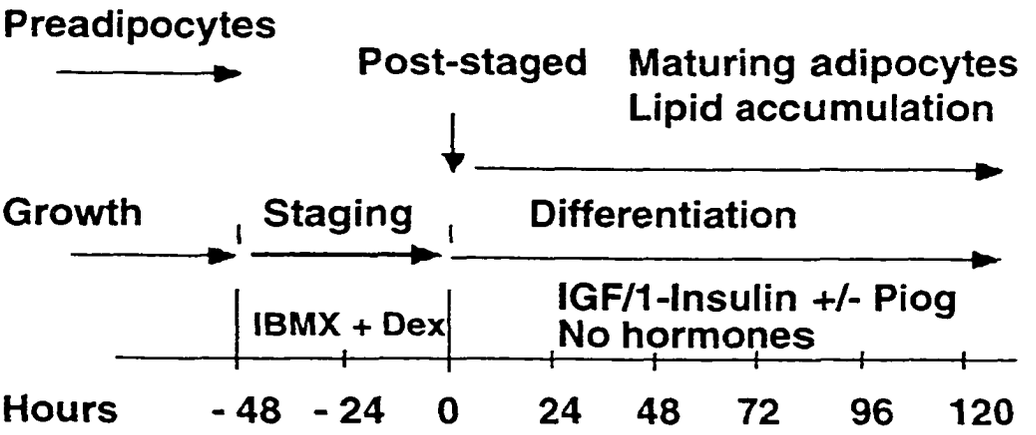

Figure I.9: Differentiation protocol for 3T3-L1

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preadipocytes as they have not yet started their final mitosis. At this point the cells are staged to respond to the differentiation stimulus by the addition of the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), and of the glucocorticoid analog, dexamethasone (Dex), to the medium. After 48 hours of this staging protocol, the cells are denominated post-staged or time 0h and start expressing early markers of differentiation. The medium is then changed to one containing 5% new born calf serum with the addition of desired hormonal treatment such as insulin, IGF-1, piog or with a combination of piog with either insulin or IGF-1. Adipocyte maturation then occurs with lipid accumulation and the expression of late markers. A representation of 3T3-L1 preadipocytes before induction of differentiation is shown in figure I.10A. The typical fibroblast-like and spindle-shaped morphology of the cells, as well as cell divisions, are very noticeable. Once treated with hormones the cells start to differentiate, accumulate lipids and express late markers of differentiation. Shown in figure I.10B after 3 days of treatment with insulin and piog, cells lost their long spindle-like arms and became more circular. In addition, lipids which have been stained with oil-red O can be seen starting to fill-up the cytoplasm. While the presence of Dex in the medium conditions the cells to respond to the differentiation stimuli, it is not sufficient alone to sustain and carry the terminal fat cell differentiation program without the presence of IGF-1 or insulin. Indeed, cells treated with IBMX and Dex in a medium deprived of growth factors were unable to fully carry-out adipocyte differentiation and expression of adipose specific markers. Hence, considerable work has been done to understand the targets and mechanisms of action of IGF-1 in adipocyte differentiation. In addition, a-FABP gene expression is increased by piog in vivo and in vitro (10). Piog stimulation of a-FABP mRNA levels is specific since other markers of adipocyte differentiation (LPL, Glut4) are not stimulated by piog alone. After 4

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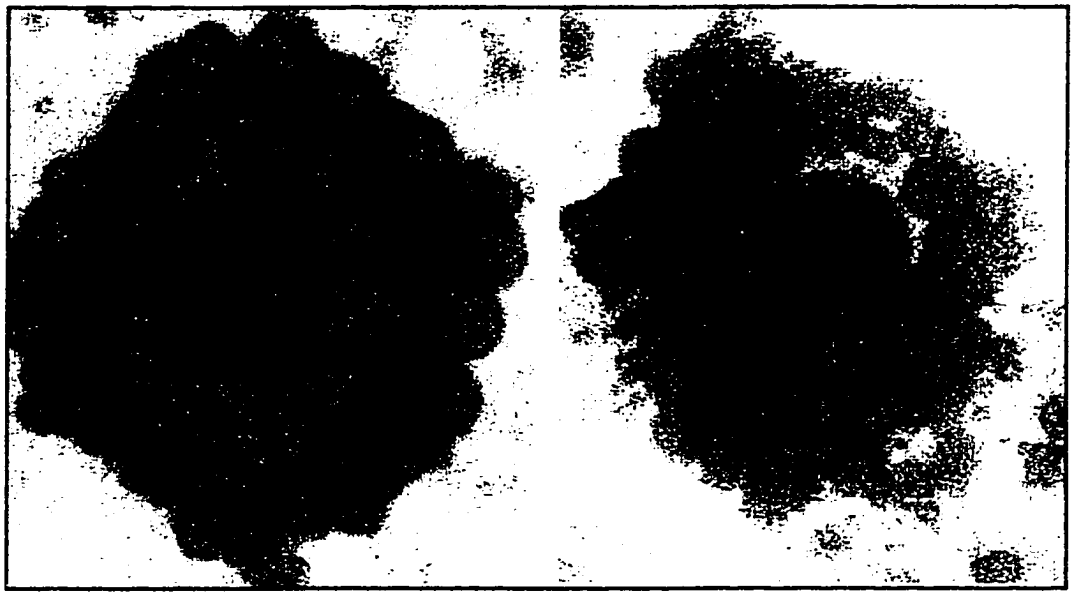
A

3T3-L1 Preadipocytes

B



Differentiating Adipocytes

Figure I.10: 3T3-L1 cells at different stages of differentiation, (A) preadipocytes (left: magnification 8x10X, right: magnification 8x20X). (B) differentiating adipocytes after treatment with insulin and pioglitazone for 3 days (magnification 8x20X). Nuclei were stained with hematoxillin, and lipids with oil red O.

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hours of treatment with piog and insulin, the relative abundance of a-FABP mRNA in cell culture starts to increase in comparaison to cultures treated with insulin alone and is up by 10-fold after 24 hours (10). Increase in the abundance of a-FABP mRNA primarily reflect increase in the rate of gene transcription (8). Thus one might expect that the piog response region resides in the 5'-flanking region of the a-FABP gene. One of the key steps in the search for the mechanisms and the factors responsible for terminal fat cell differentiation was the discovery of an adipose specific enhancer in the adipose specific gene a-FABP.

I-D.7. Localization of the adipose specific enhancer

Original studies by Clarke et al. led to the observation that the 5' flanking region of the a-FABP gene contained an adipose differentiation response element (7), which led to the investigation of the exact localization of this element. CAT activity in 3T3-L1 cells, stably transformed with a construct of the -7.2 kb of the 5'-flanking DNA of the a-FABP gene linked to the CAT gene (-7.2 kb pFABP/CAT), was increased 10 to 20 fold by the addition of piog (10). In contrast, 3T3-L1 cells stably transformed with the basal a-FABP promoter (-80 pFABP/CAT) displayed little or no basal or inducible CAT activity. Clearly, CAT expression from the -7.2 kb pFABP/CAT was induced by insulin, and piog amplified this response 10 to 20 fold. The piog response element was narrowed down to the region between -5.4 kb and -4.9 kb. This fragment, linked to a SV40 promoter/CAT gene, was able to increase CAT activity by 5 fold when cells were treated with piog and insulin (193). Analysis of the DNA sequence from the -5400 to -4900 enhancer (9) indicated the presence of multiple possible DNA/protein interaction sites within the -5400 to -5200 bp region of the a-FABP gene. However, DNA sequence analysis was not sufficient to

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determine whether these sites were functional, and more importantly, if any of these sites corresponded to the adipose differentiation specific response element (ARE). To address this question we originally hypothesized that an ARE was located within the -5400 to -4900 enhancer and that an adipose differentiation specific response factor (ARF) interacted with this ARE. We further hypothesized, that pioglitazone increased the ARE/ARF interaction, in agreement with its role as an inducer of a-FABP expression and an activator of terminal fat cell differentiation. To determine the location of the ARE within the -5400 to -4900 bp enhancer we developed a DNAse I footprinting assay and a nuclear protein extraction protocol. The hypothesis that ARF is adipose differentiation specific suggests that nuclear extract from differentiated adipocytes should yield a different footprint pattern than extracts from non differentiated cells. These differences in footprints would reflect the ability of ARF to bind to ARE. Binding to ARE could result in stimulation of transcription of a-FABP. Therefore, nuclear extracts from differentiated 3T3-L1 cells and from preadipocytes, were prepared and tested for their ability to footprint the -5400 to -4900 bp DNA fragment. This fragment was divided into two smaller fragments corresponding to -5400 to -5200 and -5200 to -4900 of the 5'-flanking region of the a-FABP gene. Furthermore, several PCR generated fragments from the -5400 to -4900 region of the a-FABP gene were cloned into the -100-a-FABP-CAT and termed PT(1-9)-CAT. The availability of these subcloned PCR fragments was essential for the determination of the functional localization of the putative adipose differentiation response element. When each of the PT-CAT clones was transiently transfected into differentiating 3T3-L1, treated in the presence or the absence of piog, no difference in CAT activity was found. Not only did additional experiments support these results, but the original -5400 to -4900-a-FABP-CAT had also lost its responsivity to differentiation when

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stimulated with piog. Furthermore, results from footprinting assay using the -5400 to -5200 or the -5200 to -4900 bp of the 5'-flanking region of the a-FABP gene, indicated no difference in DNA/protein interactions between nuclear extracts from preadipocytes or from differentiated adipocytes. These results denoted a sudden inability of these cells to respond to differentiation stimuli and suggested that the 3T3-L1 cells had lost their ability to express markers of differentiation. Northern analysis suggested that not only did the cells not express a-FABP, but also they had lost their response to insulin and piog. Morphological observations of the cells revealed that they had lost their ability to respond to differentiation stimuli and did not accumulate lipids. Further analysis and characterization of these cells confirmed that they were a derivative clone from the 3T3-L1 cells, that we termed RB-1, and which lacked the ability to differentiate into adipocytes and express specific markers of adipocyte differentiation. Results from these observations led to the publication of manuscript number II (Chapter III). While we were optimizing the footprinting procedure and recharacterizing our cell lines, Graves et al. (12) determined two target sequences for ARF, designated as ARE6 and ARE7, and localized at -5387-5375 and -5290-5274 of the a-FABP gene. ARF was then further characterized as a heterodimer of RXRα and of the adipose differentiation specific PPARγ2 (13). Soon after, Kletzien et al. demonstrated that the piog response element corresponded to ARE6 (193).

After the ARE6 binding factor ARF6 was cloned and shown to be PPARγ, several groups found that thiazolidinediones could activate PPARs (161, 181, 194). In particular, piog elicited a strong activation of PPARγ, with relatively little activity of PPARα and PPARδ. The potency of piog matches its anti-diabetic action, suggesting that PPARγ is the functional receptor in this action. However, the sequence of events leading to the activation of PPARγ by piog or by its

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possible natural ligand 15-deoxy-$\Delta^{12,14}$PGJ2, remains unclear. In particular, it has not been determined whether the ligand action requires changes in the levels of PPARγ2 or if this activation involves recruitment or activation of other factors. As discussed previously, PPARγ2 mRNA is found in high abundance in adipose tissue and its concentration reportedly increases as the preadipocyte undergoes terminal differentiation (12). However, it is not known whether the abundance of PPARγ2 protein is the limiting determinant for terminal fat cell differentiation or if translocation plays a role in PPARγ2 activation of fat cell terminal differentiation. Thus, we have hypothesized that an increase in PPARγ2 protein is required for the conversion of preadipocytes to mature fat cells, and that activating ligands of PPARγ2, such as piog, accelerate fat cell differentiation by additionally increasing the abundance of PPARγ2 protein.

I-D.8 *Analysis of PPARγ2 mRNA levels suggest that its regulation is post-transcriptional*

Because PPARγ2 was identified as an adipose differentiation specific gene, we postulated that it would not be detected in preadipocytes and that its expression would increase during the early stages of fat cell terminal differentiation. Total RNA was isolated from preadipocytes at different time points during the 48 hours of the staging protocol, as well as following 48 hours of induction of differentiation with or without pioglitazone. As shown in figure II.3 (Chapter II), PPARγ2 mRNA levels were high in preadipocytes and did not change significantly during differentiation. In addition, pioglitazone did not enhance the expression of PPARγ2 during that period. In contrast, a-FABP mRNA was undetected in preadipocytes and was expressed in a differentiation dependent manner and piog amplified a-FABP mRNA levels by 4 fold at 4h and up to 20 fold at 48h. It is now clear that PPARγ2 activation during fat cell

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differentiation involves its dimerization with RXRα and subsequently the dimer binds to the ARE. However, while PPARγ2 mRNA levels appear to be constant during the early stage of adipocyte terminal differentiation, the relative abundance of PPARγ2 protein during this period is unknown. Hence, we hypothesized that PPARγ2 regulation was post-transcriptional and that increases in a-FABP expression reflected an increase in PPARγ2 protein levels in the nucleus. Furthermore, we hypothesized that piog in its role of adipocyte differentiation accelerator, further stimulated the elevation of PPARγ2 protein levels in the nucleus.

I.E. Significance

NIDDM represents 75% of all diabetes cases in the United States. It is a disorder in which target tissues become unresponsive to insulin where insulin is unable to shut down hepatic gluconeogenesis, resulting in hyperglycemia. The loss of carbohydrate homeostatic control in these individuals is often accompanied by defective lipid metabolism and is highly associated with an excess of body fat, which can lead to further complications such as hypertension and coronary disease. Body fat content and capacity for glucose metabolism are directly related to the events regulating fat cell number and size. Stimulating differentiation of stem cells into mature adipocytes contributes to decreasing fatty acid concentration per cell. The 3T3-L1 cells, in the presence of the appropriate signals, undergo differentiation from fibroblastic adipoblasts to mature adipocytes and provide an excellent in vitro system for studying hormonal regulation and signaling in adipose tissue. In addition, the a-FABP gene is a unique model gene to study some of the mechanisms regulating adipocyte differentiation because of its specificity of expression in adipocytes, and its regulation of expression mediated by insulin and IGF-1 during the early

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events of fat cell differentiation (4, 6). Piog accelerates adipocyte differentiation and this effect is linked in time to an increase in a-FABP expression in vitro and in vivo (6, 9, 10). Hence the study of a-FABP expression, mediated by insulin and piog, is a good model system to further understand some of the key events that regulate the early events of adipocyte differentiation. Identification of the events that regulate a-FABP expression in differentiating adipocytes will greatly help to elucidate some of the mechanisms that control adipocyte differentiation and will further facilitate the development of a model to understand the regulation of cell differentiation in the complications of obesity and NIDDM.

I.F. Project objectives

A specific aim of this project is to determine the temporal relationship between expression and cellular localization of ARF6 (PPARγ2/RXRα) and a-FABP expression during fat cell differentiation. This will involve studying compartmental localization of both PPARγ2 and RXRα during adipogenesis. An additional goal is to elucidate the influence that piog exerts on mPPARγ2 or ARF6 cellular localization to determine if the insulin sensitizing action of piog involves an interaction at the level of mPPARγ2 expression and/or translocation. Therefore, the specific aims that this project will address are to:

1- Characterize the temporal pattern of expression for mPPARγ2 during the 0 to 24 hour period following piog and insulin induction of differentiation. This will require the development of anti-mPPARγ2 antibodies.

2- Characterize the temporal relationship between mPPARγ2 expression, its cellular localization, and the initiation of a-FABP transcription.

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3- Determine the impact of piog on mPPARγ2 expression and cellular localization during the first 24 hours following induction of differentiation.

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Chapter II

The following chapter has been accepted for publication in the Journal of Lipid Research: Vol 39 Issue 12 2329-2338 (1998) Certain figures that did not appear in the original publication, and mentioned in the text have been added in appendix, other figures and their legends have been modified according to the graduate committee recommendations.

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Cytosolic and nuclear distribution of PPARγ2 in differentiating 3T3-L1 preadipocytes.

II.1 ABSTRACT

In light of the pivotal role that PPARγ2 plays in the expression of fat specific genes (e.g. a-FABP), we have examined the hypothesis that a rise in PPARγ2 protein is required for the expression of a-FABP, and that the acceleration of fat cell differentiation by the thiazolidinedione agent, pioglitazone (PIOG), reflects an increase in the abundance of PPARγ2 mRNA and protein. Western analyses surprisingly revealed that undifferentiated 3T3-L1 fibroblasts contained significant levels of PPARγ2 protein; that the amount of total cellular PPARγ2 only increased 2-fold during differentiation; and that the levels of PPARγ2 protein and mRNA were not increased by PIOG even though fat cell differentiation was accelerated by PIOG as revealed by a 20-fold increase in a-FABP expression. Cell fractionation studies revealed that PPARγ2 was evenly distributed between the cytosolic and nuclear compartments in both undifferentiated and differentiating 3T3-L1 cells. Immunocytochemical studies with a PPARγ2-specific antibody indicated that PPARγ2 was diffusely distributed throughout the cytosol of undifferentiated 3T3-L1 cells, but as the differentiation progressed, the PPARγ2 became focused around the developing lipid droplets. In contrast to PPARγ2, undifferentiated 3T3-L1 cells contained no measurable quantities of RXRα, but once fat cell differentiation was initiated by treatment with IBMX and dexamethasone, the cellular content of RXRα increased several fold. The rise in RXRα content paralleled the induction of a-FABP, but the expression of RXRα was not enhanced by PIOG. Although the amount of PPARγ2 and RXRα was unaffected by PIOG, gel shift assays revealed that PIOG stimulated PPARγ2/RXRα binding to the adipose response element of a-FABP

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by 5-fold in less than 12h. Apparently, RXRα rather than PPARγ2 is the pivotal trans-factor essential for the initiation of terminal fat cell differentiation. However, the high cytosolic content of PPARγ2 and its association with the lipid droplet of differentiating 3T3-L1 cells suggests PPARγ2 may posses a cytosolic function in the developing fat cell.

II.2 INTRODUCTION

Adipocytes first appear late in fetal development in preparation for postnatal life when a substantial energy reserve is needed to survive periods of fasting. However, excessive development of adipose tissue affects over 30% of all adults in the United States and represents a significant risk factor for non-insulin dependent diabetes mellitus (NIDDM), coronary artery disease, and hypertension. Considerable progress has been made during the past few years in our understanding of the molecular control of adipogenesis and adipocyte-specific gene expression. Using the adipocyte-specific fatty acid binding protein (a-FABP) gene as a model, several cis-acting elements and trans-acting factors have been identified which act cooperatively to trigger the terminal differentiation program of adipocytes (1-5). Particular attention has focused on the fat specific region of the a-FABP gene which is located from -5400 to -4900 bp. This region of the gene contains the adipose response elements (AREs) which instill adipose tissue specific expression of a-FABP. The AREs are similar to the direct repeat (DR-1) response elements that bind members of the peroxisome proliferator-activated receptor (PPAR) family of transcription factors (5-14). The identification of the ARE (DR-1) element led to the cloning and identification of a new member of the PPAR family, PPARγ2, which is found in high abundance in adipose tissue and appears to be

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responsible for regulating the expression of adipose specific genes such as a-FABP (5, 15).

The induction of fat specific genes such as a-FABP by PPARγ2 requires that PPARγ2 bind to the ARE (DR-1) sequence as a heterodimer complex with the retinoid X receptor alpha (RXRα) (5,6). Moreover, interaction of the PPARγ2 with the ARE (DR-1) appears to be dependent upon its ability to bind a collection of ligand activators including eicosanoids (11-20), fatty acids, and insulin-sensitizing drugs such as pioglitazone (21-24). Although these observations demonstrate the importance of PPARγ2 in fat cell differentiation, it is unknown if the abundance of PPARγ2 protein is the limiting determinant for terminal fat cell differentiation. Thus, we have hypothesized that an increase in PPARγ2 protein is required for the conversion of preadipocytes to mature fat cells, and that activating ligands of PPARγ2 such as the thiazolidinedione, pioglitazone, accelerate fat cell differentiation by increasing the abundance of PPARγ2 protein. In fulfilling our objective we have made two interesting discoveries: (a) nearly 50% of the total cellular content of PPARγ2 is located in the cytosol compartment of both preadipocytes and mature fat cells; and (b) early events of fat cell differentiation appear to be dependent upon the induction of RXRα expression rather than upon the expression of PPARγ2.

II.3 METHODS

Cell culture. 3T3-L1 preadipocytes (ATCC #F8979) were grown in Dulbecco's modified Eagle's medium (Gibco BRL) that contained 5% fetal calf serum. When the cells were 70% confluent they were staged to differentiate by changing the medium to one containing 5% newborn calf serum plus 10 μM IBMX and 1 μM dexamethasone. The IBMX and dexamethasone were removed after 48h (designated as time 0h), and the medium was changed to one

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containing either no hormones, 1 μM insulin, or insulin plus 10 μM pioglitazone.

Anti- PPARγ2 antibody preparation. To address our hypothesis that an increase in PPARγ2 is a crucial step in terminal fat cell differentiation, antibodies to two domains of PPARγ2 were prepared: (a) amino acid residues 4-26 (p4-26) which are specific for PPARγ2; and (b) amino acid residues 32 to 54 (p32-54) which is a domain shared by both PPARγ2 (56 Kda) and PPARγ1 (52 Kda). The anti-serum to mPPARγ2 was raised in rabbits injected with synthetic peptide antigen corresponding to either p4-26, or p32-54 of PPARγ2. Antisera were harvested every two weeks and their antigenicity against the respective peptides was determined. Antiserum collected at day 70 was the most antigenic and was subsequently harvested for antibody purification. Affinity purified anti- PPARγ2 was prepared from serum using the respective peptides linked to sulfo-link affinity columns (Pierce). As can be seen in Figure II.1, Western blot analysis of total protein extracts from undifferentiated and differentiated 3T3-L1 cells revealed that the PPARγ2 specific antibody (p4-26) detected a single band at 56 Kda (lanes 3 and 4); and that this band co-migrated with a protein produced from an in vitro transcription-translation reaction programmed with a vector containing the PPARγ2 open-reading frame (Figure II.1-lane 5). As expected, antibody prepared to the p32-54 domain of PPARγ2 detected both the larger PPARγ2 (56 Kda) and the smaller PPARγ1 (52 Kda) (Figure II.1-lanes 1 and 2). Clearly, the dominant protein in both undifferentiated and differentiated 3T3-L1 cells was the PPARγ2 isoform.

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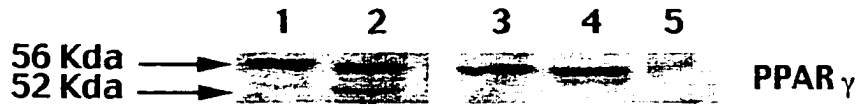

Figure II. 1. PPARγ protein expression in undifferentiated and differentiated 3T3-L1 cells.

Western analysis was conducted utilizing total cellular protein extracts (50 µg per lane) from undifferentiated (lanes 1 and 3) and fully differentiated (lanes 2 and 4) 3T3-L1 cells. After separating the proteins by polyacrylamide gel electrophoresis, lanes 1 and 2 were incubated with anti-PPARγ $_{32\text{-}54}$ which was raised against a peptide domain located in both PPARγ1 (52 Kda) and PPARγ2 (56 Kda); and lanes 3-5 were incubated with anti-PPARγ $_{4\text{-}26}$ which was raised against a peptide domain located in only PPARγ2 (56 Kda). Lane 5, which was incubated with anti-PPARγ $_{4\text{-}26}$, was loaded with a 5 µl aliquot (5ng) of an in vitro transcribed and translated reaction that employed PPARγ2 cDNA as a template. Note that the predominant form of PPARγ in both undifferentiated and differentiated 3T3-L1 cells is PPARγ2, and that the in vitro translated PPARγ2 co-migrates with the 56 Kda protein. The entire gel is shown in the appendix (figure II.1.B)

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RNA isolation and Northern analysis. The abundance of a-FABP, PPARγ2 and RXRα mRNA was determined by Northern analysis at the times indicated in the various figures. RNA isolation and Northern blotting were performed as described previously (25). Twenty μg of total RNA were loaded on a 1% formaldehyde-agarose gel as described (26) and transferred to a Zeta probe membrane (Biorad). The membranes were hybridized with cDNA probes labeled with α-^{32}P-dCTP (Dupont NEN). The full length cDNA for a-FABP was provided by D. Bernlohr, University of Minnesota (29). PPARγ2 cDNA was a generous gift by B. M. Spiegelman, Dana-Farber Cancer Institute, and RXRα cDNA was provided by T. Neuman, Colorado State University. Autoradiographs were digitally captured and quantified using the Optical Integrator and Image software from Ambis.

Cellular protein extraction and Western analysis. Total cellular protein extracts were prepared from undifferentiated and differentiated 3T3-L1 cells grown in a 100 mm petri dishes. The plates were first washed twice with PBS, and the cells were subsequently scraped from the plates using 0.6 ml of RIPA buffer (PBS, 1% NP40, 0.5 % sodium deoxycholate, 0.1% SDS, 0.25 mM PMSF, 5 mg/ml aprotinin, 1 mM Na orthovanadate) A cell lysate was prepared by passing the cells through a syringe fitted with a 21 gauge needle, the lysate was transferred to a microfuge tube, incubated on ice for 60 min, and subsequently was microfuged at 10,000 g for 20 minutes at 4°C. The supernatant containing the total cell lysate was saved and store at -80°C until use. Cytosolic and nuclear protein extracts were prepared according to the method of Sanchez et al. (28). Briefly, 3T3-L1 cells grown in 100 mm dish were rinsed with ice cold PBS, and scrapped into a microfuge tube with 1.0 ml PBS. The cells were pelleted by centrifugation (1000g, 10 min). The resulting cell

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pellet was resuspended in 250 µl of 10 mM HEPES, 1 mM EDTA, 0.5 mM DTT, 0.25 mM PMSF, 50 mM NaF, 2 mM Na meta-vanadate, and 5 mg/ml leupeptin and pepstatin (buffer A); and homogenized in a dounce homogenizer with 20 strokes. This homogenate was centrifuged at 1000g for 10 min. The resulting low speed nuclear pellet was utilized for protein extraction as described below; and the supernatant (S1) was processed as follows: 4M NaCl was added to the S1 fraction to make a final concentration of 0.5M NaCl and the mixture was incubated on ice for 1 hr with occasional vortexing. The S1 fraction was centrifuged at 100,000 g for 1 hr and the supernatant was saved as cytosolic extract. Extracts were aliquoted and store in liquid nitrogen until use. The low speed nuclear pellet was washed twice by resuspension in 250 µl buffer A containing 250 mM sucrose and pelleted at 1000 g for 10 min. The pellet was then resuspended in 100 µl buffer A and 100 µl 1M NaCl was added to make a final concentration of 0.5 M NaCl and incubated on ice for 1 hr with occasional vortexing. After salt extraction the nuclear pellet was centrifuged at 8000g for 30 min, the supernatant was saved as nuclear extract. Extracts were aliquoted and store in liquid nitrogen until use.

For Western analysis, proteins were separated by SDS-PAGE, transferred and immobilized on a nitrocellulose membrane at 4°C. The membrane was blocked by incubation with 5% non fat dry milk in phosphate-buffered saline (PBS) at 4°C over night. The membrane was then washed briefly in PBS, and incubated with rabbit antibody raised against rat PPARγ2 diluted 1/15,000; or against rat RXRα (Santa Cruz, CA) diluted 1:500 in PBS containing 0.1% Tween 20 (PBS-T). Incubation with antibodies and detection of the antigen-antibody complex were performed using the ECL kit (Amersham) according to the manufacturer's instructions, except that washes following secondary antibody incubation consisted of six changes of buffer over one hour.

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Immunocytochemistry. Undifferentiated and differentiated 3T3-L1 cells were grown in 12 well plates and induced to differentiate as described previously. At each time indicated in the figure, the cells were fixed in formalin for 15 min. Subsequently each well was rinsed twice with PBS, and treated with 0.03% H_2O_2 in methanol. After a 20 min incubation, the cells were washed three times with PBS containing 0.1% bovine serum albumin. Subsequently, the cells were incubated with 10% goat serum for 20 min, and then treated for 45 min with a 1:500 dilution of affinity purified anti- PPARγ2 (p4-26) (Affinity Bioreagents, Boulder, CO). After antibody incubation the cells were washed with PBS containing bovine serum albumin. Subsequently the cells were treated for 30 min with anti-rabbit IgG (Vector Lab Inc). Following three washes with PBS-albumin, antibody staining was performed using vectastain as described by the manufacturer (Vector Lab, Inc.). Nuclei were visualized by 1 min incubation with Harris's hematoxylin.

Gel mobility shift assay. Five μg of nuclear protein extract (29) was incubated in binding buffer (100 mM KCL, 0.1% NP40, 10% glycerol, 10 mM HEPES, 5 mM $MgCl_2$, 2.5 mM $ZnSO_4$, 0.8 mM DTT), with 1 μg of poly dA-dT, and 1 ng of γ-^{32}P-ATP labeled DNA probe (20,000 dpm). The 20 μl reaction was incubated on ice for 20 min. For cold competition, 200 ng of unlabelled ARE, PPRE, or GRE were added to the binding reaction and kept on ice for 20 min. For supershift, 2 μl of anti- PPARγ2 (p32-54) were added to the tubes at the end of the binding reaction, and incubated on ice for 30 min. Samples were then loaded onto a 4.5 % acrylamide gel containing 0.1% NP40. Electrophoresis was run at 200 v for 3 hr in a 40 mM Tris and 380 mM glycine buffer (pH 8.3) containing 0.1% NP40. Gels were dried and quantified by radioimagizing.

Ablation of the PPARγ2 gel shift by anti- PPARγ2 (p4-26) was achieved by incubating overnight of 5 μl affinity purified antibody with 5 μg nuclear protein

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extract in binding buffer. After the overnight incubation at 4°C, radiolabeled ARE (40,000 dpm) was added to the reaction mixture and the incubation was continued on ice for an additional 25 min. After incubation the mixture was separated by electrophoresis as described above.

The double stranded oligonucleotides used were (only one strand is shown, underlined sequences correspond to the DR-1 consensus motif):

ARE: 5'-CAGAAATGCACATTTCACCCAGAGAGAAGGG-3'

GRE : 5'-AGTTTTTGGTTACAAACTGTTCTTAAAACGAGG-3' .

PPRE: 5'- GATCTGTGACCTTTGTCCTAGTAAG 3'

II.4 RESULTS

PPARγ2 expression during 3T3-L1 differentiation. When 3T3-L1 preadipocytes undergo terminal differentiation to mature fat cells, the level of PPARγ2 mRNA increases significantly (9,10). However, the relationship between the level of PPARγ2 mRNA and PPARγ2 protein is unknown. This is particularly true for the early stages of 3T3-L1 differentiation. Using a two stage differentiation protocol (see METHODS), we discovered that total protein extracts from undifferentiated 3T3-L1 cells contained a significant quantity of PPARγ2 protein (see -48h in Figure II.2). In fact, the level of immuno-reactive PPARγ2 in preadipocytes was approximately 30% of that found in mature fat cells. Similarly, the 3T3-L1 preadipocytes contained a level of PPARγ2 mRNA that was comparable to the amount of PPARγ2 protein (Figure II.3). As expected, the level of PPARγ2 mRNA increased significantly in response to differentiation stimuli. However, by applying our two step differentiation protocol, we discovered that IBMX and dexamethasone were largely responsible for the increased abundance of PPARγ2 mRNA (Figure II.3 and Appendix Figures II.3B and II.3C).

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Figure II. 2. Increases in the cellular content of PPARγ2 protein during fat cell differentiation.

Undifferentiated 3T3-L1 cells were grown to confluence (-48 h) (lane 1 and 8) and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone (0 h) (lane 3 and 10), the cells were induced to differentiate by changing the media to one containing either insulin (1 μM) (lane 1-7), or insulin plus pioglitazone (10 μM, PIOG) (lane 8-14). Total protein extracts (20 μg per lane) were prepared for Western analysis at the hours indicated in the figure: -24h, (lanes 2 and 9); 2h, (lanes 4 and 10); 4h (lanes 5 and 11); 12h (lanes 6 and 12); 48h (lanes 7 and 13). The protein blots were incubated with anti-PPARγ $_{32\text{-}54}$. The 56 Kda mPPARγ2 is noted by the arrow.

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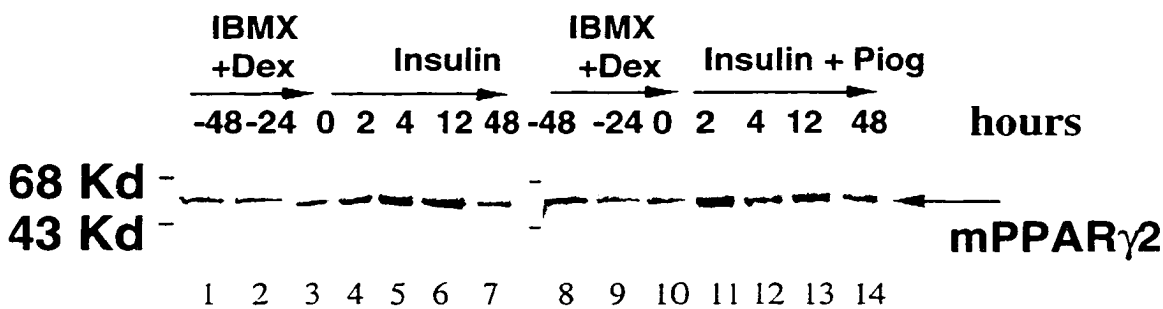

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Figure II. 3. Increases in PPARγ2 and a-FABP mRNA abundance during the early stages of preadipocyte differentiation.

Undifferentiated 3T3-L1 cells were grown to confluence (-48 h) and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin (designated with - symbol), or insulin plus 10 μM pioglitazone (designated with + symbol). Total RNA was extracted at the hours indicated in the figure, and the Northern blot was sequentially hybridized with cDNAs for mPPARγ2, a-FABP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The molecular sizes of the PPARγ2 and a-FABP transcripts are noted at the right.

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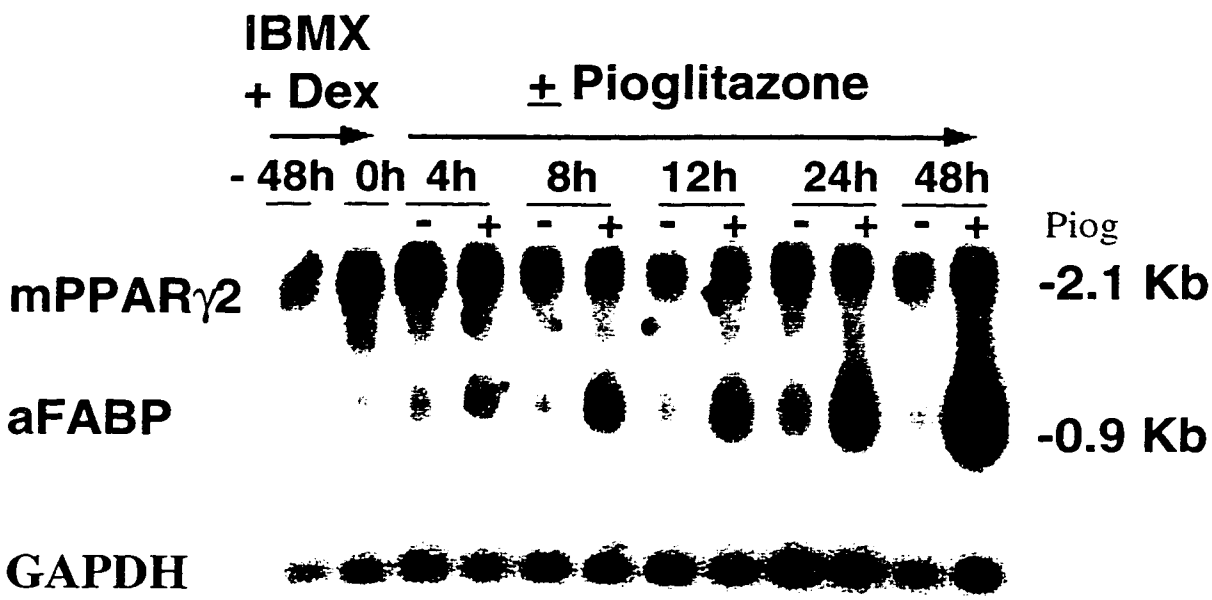

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Interestingly, the rise in PPARγ2 mRNA resulting from IBMX and dexamethasone treatment was not accompanied by an increase in PPARγ2 protein (Figure II.2). However, when the media containing IBMX and dexamethasone was removed and replaced by one that contained insulin, the amount of PPARγ2 protein increased 2-3 fold within 2-4 h after insulin exposure (Figure II.2 and Appendix Figures II.2B and II.2C). This increase in PPARγ2 protein was not enhanced by the pioglitazone, a potent enhancer of fat cell differentiation.

Western analysis of cytosolic and nuclear protein extracts from undifferentiated 3T3-L1 cells indicated that PPARγ2 protein was distributed evenly between the cytosol and nucleus (Figures II.4 and II.5 and Appendix Figure II.4C). When the undifferentiated 3T3-L1 cells were treated with insulin, the 2-3 fold increase in total cellular PPARγ2 protein that was noted previously (Figure II.2 and Appendix Figure II.2B) appeared to be restricted to the cytosolic fraction, while the nuclear fraction of PPARγ2 remained relatively constant in undifferentiated and differentiated 3T3-L1 cells (Figures II.4 and II.5). Even when the cells were treated with pioglitazone, which is a potent ligand activator for PPARγ2 (8,19), the amount of immuno-reactive PPARγ2 in the nucleus did not appear to increase (Figure II.4 and Appendix II.4C). However, one interesting observation associated with the pioglitazone treatment of 3T3-L1 cells was that the nuclear protein extracts from pioglitazone treated cells did not appear to contain the lower molecular weight PPARγ1, while nuclear extracts from cells treated with insulin contained the predominant

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Figure II. 4. Changes in nuclear and cytosolic content of PPARγ2 protein during the conversion of 3T3-L1 preadipocytes to mature fat cells.

Undifferentiated 3T3-L1 cells were grown to confluence (-2 days) and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin (designated with the - symbol), or insulin plus 10 μM pioglitazone (designated with the + symbol). **(A)** At the days indicated in the figure, cytosolic (Cyt) and nuclear (Nuc) protein extracts (10 μg per lane) were prepared for Western blot analysis of PPARγ2 using anti-PPARγ $_{32\text{-}54}$. **(B)** a-FABP protein abundance was determined at the days indicated. Lane NH shows the level of a-FABP in 3T3-L1 cells after they have undergone 2 days of IBMX and dexamethasone treatment followed by 5 days of no hormone or pioglitazone (NH) treatment. The abundance of a-FABP in rat adipose tissue protein extract is depicted in the "Ad" lane (10 μg).

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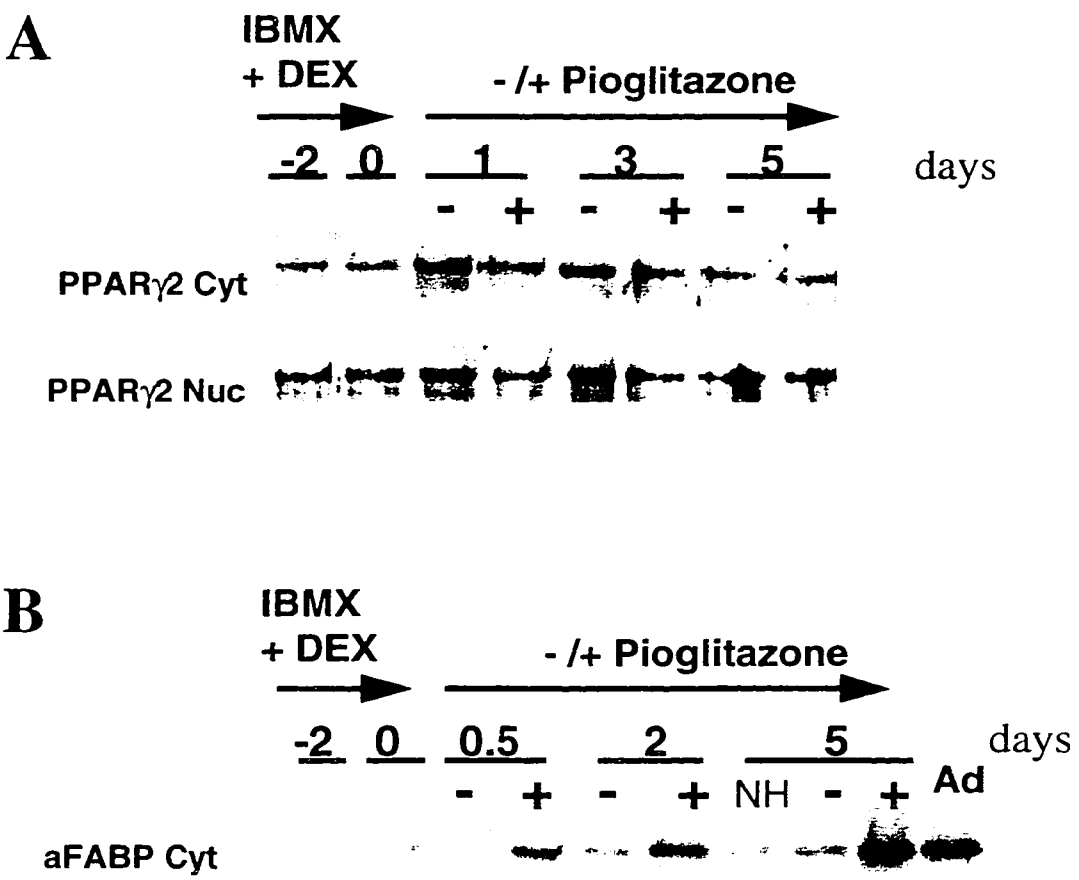

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Figure II. 5. Intracellular distribution and abundance of PPARγ2 protein in undifferentiated and differentiated 3T3-L1 cells.

Undifferentiated 3T3-L1 cells were grown to confluence (-2 days) and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing 1 μM insulin plus 10 μM pioglitazone. At -2, 0, 5, and 10 days of treatment, the cellular localization of PPARγ2 protein (red) was determined by immunocytochemical staining using PPARγ2 specific anti- PPARγ2 $_{4\text{-}26}$ (A). Preincubating the anti-PPARγ2 with its specific peptide completely blocked PPARγ2 protein staining (B). Nuclei were detected by hematoxylin staining (pale blue), and are marked by the large arrows. Lipid vacuoles were detected by oil red O staining (not shown) and are noted by the thin arrows.

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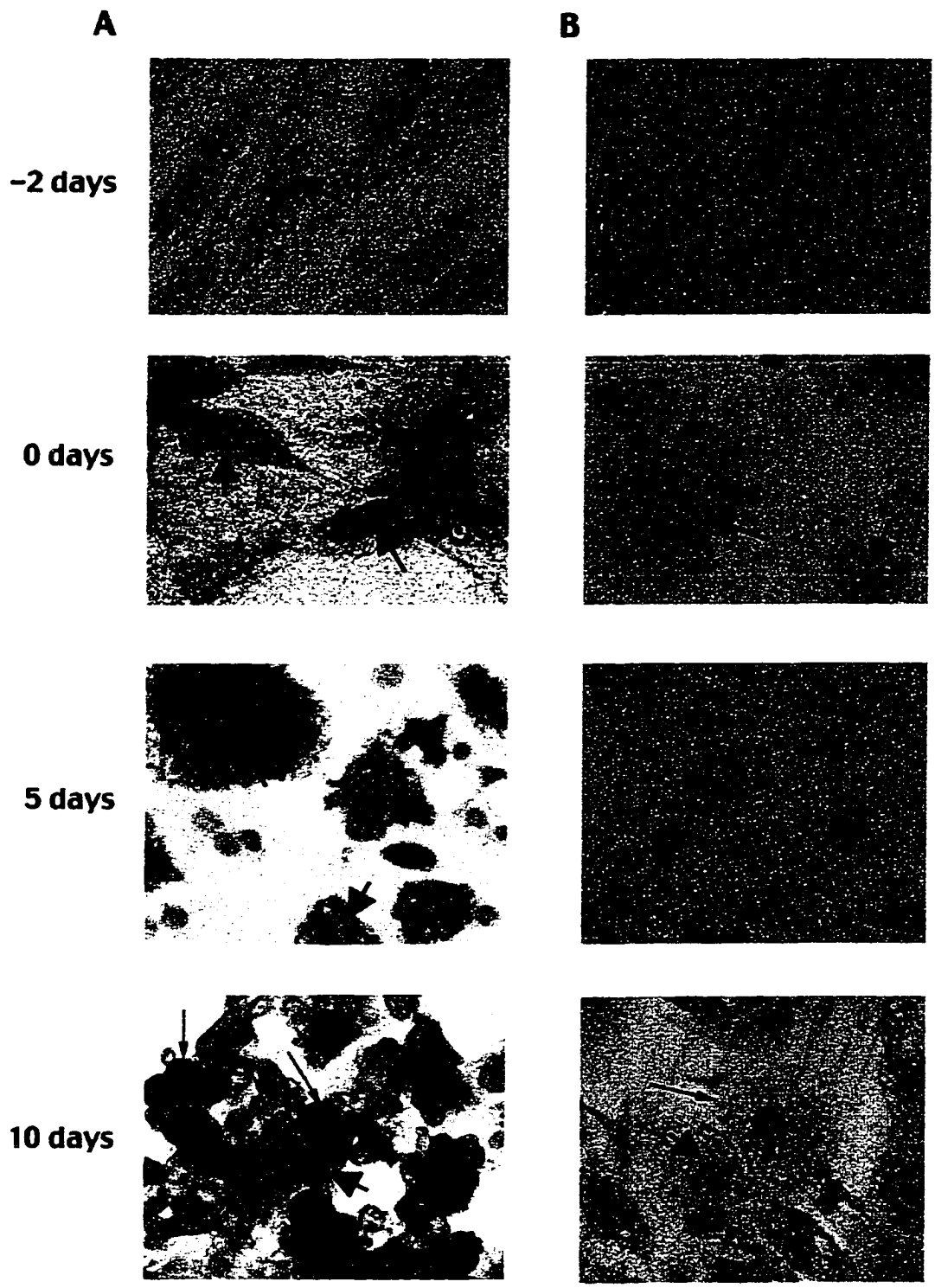

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PPARγ2 protein, and a small quantity of immuno-reactive PPARγ1 (Figure II.4-lanes 1 and 3). The cellular fractionation studies indicated that a significant quantity of PPARγ2 was located in the cytosol. To verify this observation, an antibody specific for the N-terminal peptide domain of PPARγ2 (p4-26) was employed in a collection of immunocytochemical studies. These studies supported our conclusion that undifferentiated 3T3-L1 preadipocytes contained both cytosolic and nuclear PPARγ2 (Figure II.5). The staining pattern for immuno-reactive PPARγ2 indicated that PPARγ2 protein was scattered diffusely throughout the cytosol (Figure II.5). However, as the preadipoctyes underwent differentiation to mature fat cells, the cytosolic content of PPARγ2 increased, and the cytosolic PPARγ2 appeared to be concentrated around the lipid vacuoles of the maturing fat cell (Figure II.5). The concentrating of the PPARγ2 around the lipid vacuoles is particularly noticeable at day 5 and day 10 (Figure II.5).

Expression of RXRα during fat cell differentiation. Because undifferentiated 3T3-L1 fibroblasts expressed significant levels of PPARγ2 mRNA and protein (i.e. 30% of fully differentiated cells) (Figures II.2-II.4), we have concluded that the expression of PPARγ2 is not the limiting factor for the initiation of terminal fat cell differentiation. However, PPARγ2 induces the transcription of fat specific genes (e.g. a-FABP) by binding to the ARE (DR-1) DNA recognition sequence as a heterodimer with the trans-factor RXRα. In light of the fact that PPARγ2 requires RXRα as a partner, we hypothesized that the stimulus for terminal fat cell differentiation may be the induction of RXRα expression, and that the acceleration of fat cell differentiation by pioglitazone may reflect an amplification in RXRα expression. Consistent with this hypothesis, preadipocytes did not contain measurable quantities of RXRα or a-FABP protein (Figure II.6); but treatment of undifferentiated 3T3-L1 cells with

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Figure II. 6. Pattern of RXRα and a-FABP protein expression as undifferentiated 3T3-L1 cells undergo conversion to mature fat cells.

Undifferentiated 3T3-L1 cells were grown to confluence (-2d) and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone (0d), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin (designated by the - symbol), or insulin plus 10 μM pioglitazone (designated by the + symbol). **(A)** Nuclear (Nuc) and cytosolic (Cyt) protein extracts (10 μg per lane) were prepared and utilized for Western blot analysis. Frames **(B)** and **(C)** depict the relative changes in RXRα and a-FABP protein, respectively, as undifferentiated 3T3-L1 preadipocytes undergo differentiation. Values are means ±SD for n=2-4 cell preparations. circles= insulin and squares= insulin plus pioglitazone.

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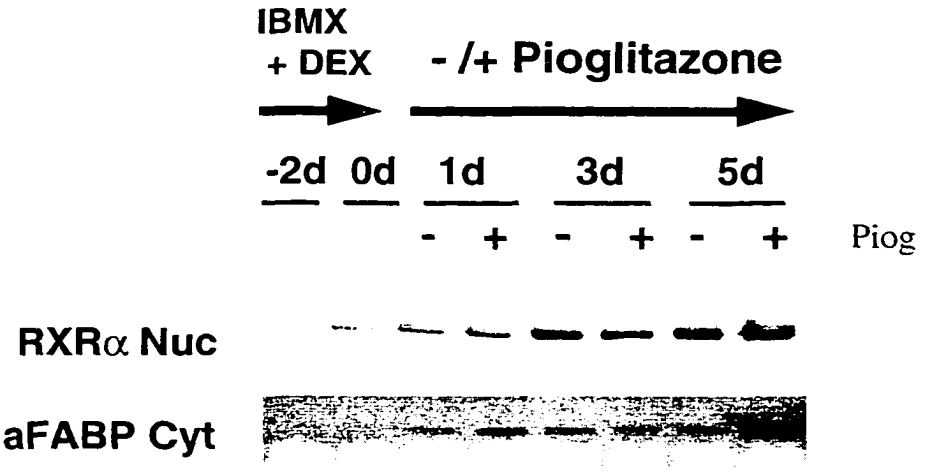
A



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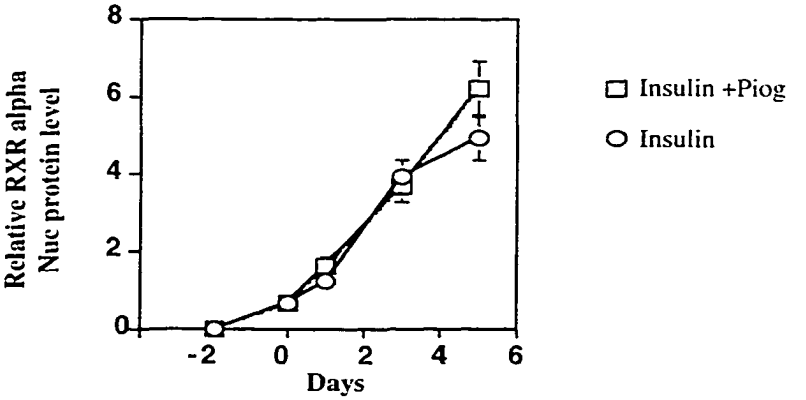

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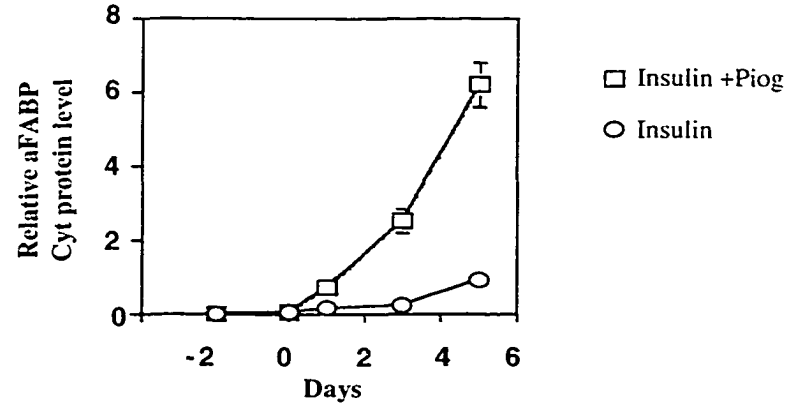

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Figure II. 7. Changes in RXRα mRNA abundance during the early phases of 3T3-L1 preadipocyte differentiation.

Undifferentiated 3T3-L1 cells were grown to confluence (-48 h) and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin (designated with - symbol), or insulin plus 10 μM pioglitazone (designated with + symbol). Total RNA was extracted at the hours indicated in the figure, and the Northern blot was sequentially hybridized with cDNAs for RXRα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The molecular size of the RXRα transcript is noted at the right.

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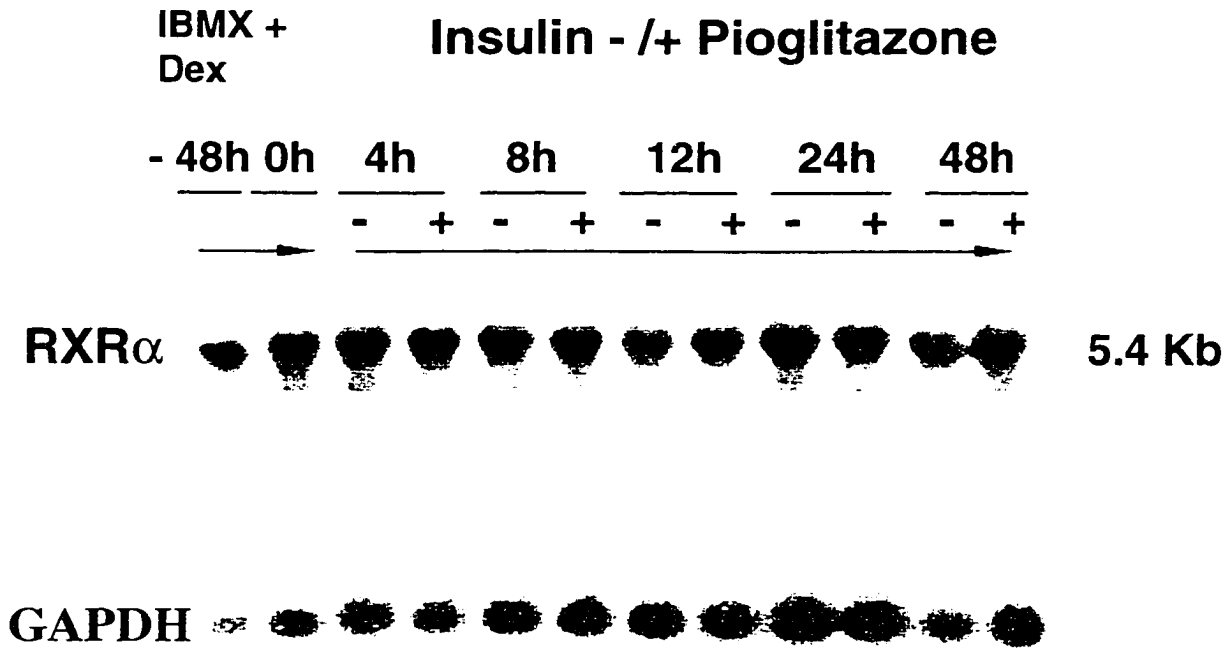

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IBMX and dexamethasone for 48 h greatly increased the cellular content of RXRα. Cell fractionation studies revealed that unlike PPARγ2, virtually all of the RXRα was located in the nuclear compartment and none was detected in the cytosolic compartment (Fig II.6D appendix). Moreover, the nuclear content of RXRα increased 6-fold during the subsequent 5 days differentiation (Figure II.6). This rise in nuclear RXRα content was paralleled by an increase in the cellular content of a-FABP mRNA and protein (Figures II.3, II.4 and II.6 and Appendix Figures II.4D and II.4E). Although it appears that RXRα is required for a-FABP expression and fat cell differentiation, pioglitazone did not enhance or accelerate the expression of RXRα (Figures II.6 and II.7).

One surprising finding was that Northern blot analysis of total RNA revealed that abundance of RXRα mRNA was high in undifferentiated 3T3-L1 preadipocytes (Figure II.7 and Appendix Figure II.7B), but RXRα mRNA was not translated into RXRα protein. RXRα protein was only expressed after the preadipocytes were stimulated to differentiate by treating them with IBMX and dexamethasone (Figure II.6). It would appear that the translation of RXRα mRNA is suppressed in the undifferentiated 3T3-L1 cell.

Pioglitazone enhances the PPARγ2/RXRα interaction with ARE. The previously described data indicate that pioglitazone does not induce PPARγ2 or RXRα gene expression, nor does it increase the cellular content of PPARγ2 or RXRα proteins. Nevertheless, pioglitazone did accelerate the differentiation process as revealed by the marked and rapid induction of a-FABP (Figures II.3, II.4 and II.6 and Appendix Figures II.3C and II.4D). Since pioglitazone is a well established high affinity, ligand activator of PPARγ2, we examined the ability of pioglitazone to stimulate PPARγ2 binding to its ARE (DR-1) recognition sequence (Figure II.8).

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Figure II.8A. Pioglitazone enhances PPARγ2 binding to the ARE (DR-1) of the adipose response element from the a-FABP gene.

Undifferentiated 3T3-L1 cells were grown to confluence and staged to differentiate as described in METHODS. After a 48 h staging period with IBMX and dexamethasone, the cells were induced to differentiate by changing the media to one containing no hormones (NH), 1 μM insulin (I), or insulin plus 10 μM pioglitazone (IP). After 12 hours (12 h) and 5 days (5d) nuclear protein extracts were prepared and utilized in a gel retardation assay using ^{32}P-labeled ARE (DR-1) from the adipose response element of the a-FABP gene. Nuclear protein extracts from cells treated with insulin plus pioglitazone were used in competition assays (lanes 10-12). Lane 10 is a 100-fold molar excess of unlabeled DR-1 oligo from the adipose response element, and lane 11 is a 100-fold molar excess of DR-1 oligo from the peroxisome proliferator response element. Lane 12 is a 100-fold molar excess of a glucocorticoid response element. PPARγ2 binding to the DR-1 oligo is depicted by the lower arrow. Treatment with anti-PPARγ $_{32\text{-}54}$ (lanes 5, 9) resulted in a supershifted band (upper arrow).

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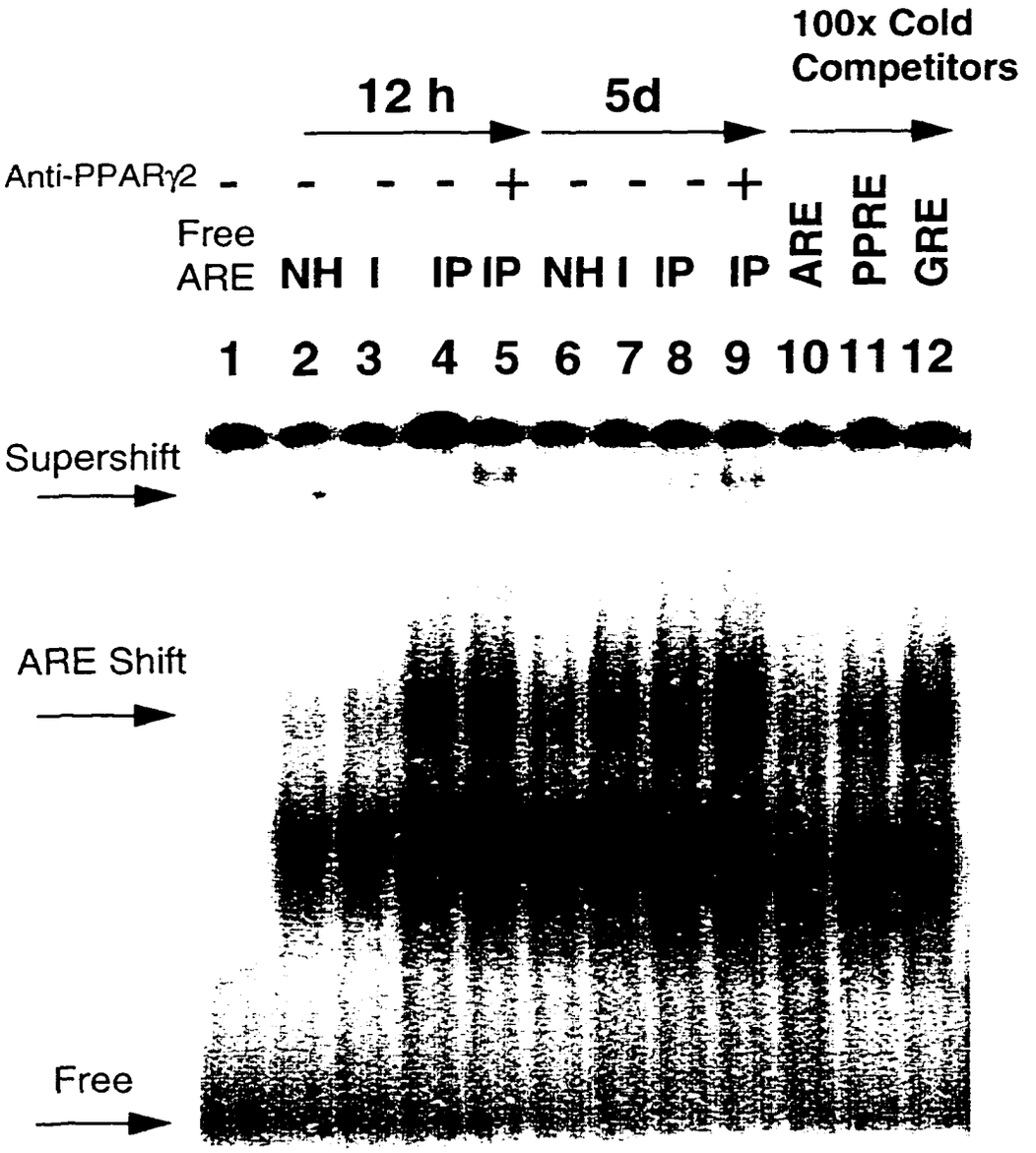

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Figure II.8B. Pioglitazone enhances PPARγ2 binding to the ARE (DR-1) of the adipose response element from the a-FABP gene.

Undifferentiated 3T3-L1 cells were grown to confluence and staged to differentiate as described in figure II.8A. Nuclear protein extracts were prepared from confluent untreated, undifferentiated 3T3-L1 cells (lane 2), and fully differentiated (10 days of pioglitazone and insulin treatment) 3T3-L1 cells (lanes 3-5). PPARγ2 interaction with the ARE (DR-1) of the adipose response element is depicted in lane 3; treating the nuclear extract with anti- PPARγ2 $_{4\text{-}26}$ nearly eliminated the shifted band (lane 4); but treating the nuclear extract with anti-PPARδ (lane5) had no effect on the PPARγ2 shift. Lane 1 depicts the free probe. ***Note**: treating the nuclear extract with preimmune serum (Appendix Figure II.8C) or with anti-PPARα antibody (Appendix Figure II.8E) had no effect on PPARγ2 shift.*

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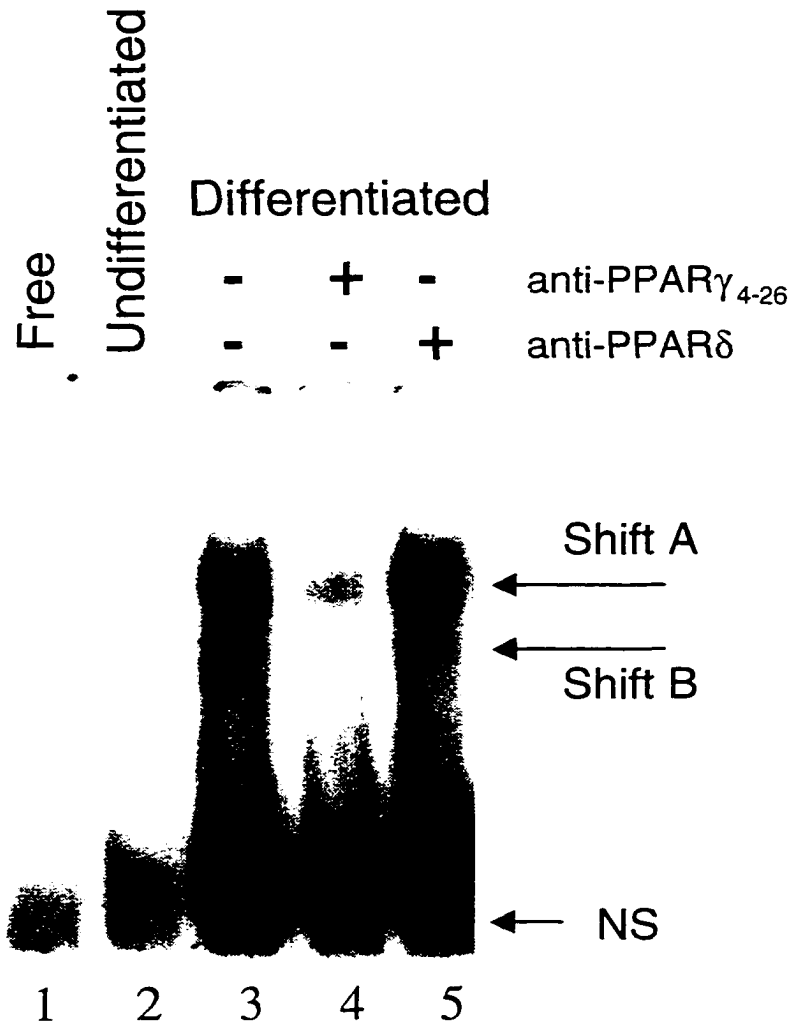

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As expected, nuclear proteins extracted from pioglitazone treated 3T3-L1 preadipocytes readily bound to the ARE (DR-1) recognition sequence of a-FABP (Figure II.8A). This increase in protein binding appeared to involve enhanced PPARγ2 binding because: (a) cold DR-1 competed for binding (Figure II.8A, lanes 10-11); (b) anti- PPARγ2/γ1 treatment yielded a supershift (Figure II.8A, lanes 5 and 9); and (c) prolonged incubation of the nuclear extract with PPARγ2 specific antibody abolished protein binding to the ARE (DR-1) (Figure II.8B, lane 4).

Relative to cells treated with insulin alone, pioglitazone increased PPARγ2 binding to the ARE (DR-1) of a-FABP by 5-fold within 12 h (Figure II.8A- lanes 3 and 4). However, after 5 days of insulin treatment, PPARγ2 binding to the ARE (DR-1) was comparable to that of pioglitazone (Figure II.8A- lanes 7 and 8). Apparently, sufficient endogenous ligand was produced after 5 days of insulin stimulation to fully activate PPARγ2. Also as expected, the binding of PPARγ2 to its ARE (DR-1) recognition sequence was paralleled by an increase in a-FABP mRNA and an increase in a-FABP protein (Figures II.3, II.4 and II.6 and Appendix Figures II.3C and II.4D). Finally, the ability of pioglitazone to stimulate PPARγ2 binding to its ARE (DR-1) recognition sequence was very rapid. As an example, nuclear extracts from 3T3-L1 preadipocytes staged to differentiate by treatment with IBMX and dexamethasone showed virtually no PPARγ2 binding to the ARE (DR-1), but PPARγ2 binding to the ARE oligo was detectable within 2-4 h of adding pioglitazone to the media, and near maximal PPARγ2 binding occurred after 12 h of pioglitazone treatment (data not shown).

II.5 DISCUSSION

Several years ago we reported that the insulin or IGF-1 induction of fat cell differentiation could be greatly accelerated by the insulin sensitizing agent,

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pioglitazone (21, 23). One of the most pronounced responses to this thiazolidinedione derivative was the greatly amplified level of expression for the adipocyte-specific gene, a-FABP (23). A collection of studies since this initial observation indicate that thiazolidinediones, as well as free fatty acids, enhance fat cell differentiation by functioning as ligand activators for a group of transcription factors termed PPARs. PPARs, notably PPARγ2, induce the transcription of adipose specific genes by binding to a DR-1 motif in the 5'-flanking region of the genes (11, 22, 24). The importance of PPARs in fat cell differentiation has been clearly demonstrated by ectopic expression of the PPARγ2 and PPARδ in CHO cells and muscle cells respectively (30, 31). In both of these cases, the cells began to express a-FABP and accumulate lipid in response to ligand activation of the PPARs (30, 31). In light of the pivotal role that PPARs play in fat cell differentiation and adipose specific gene expression, we hypothesized that insulin stimulated terminal fat cell differentiation by inducing the synthesis of PPARγ2; and that the thiazolidinedione, pioglitazone, accelerated fat cell differentiation by inducing the expression of PPARγ2 and/or its heterodimer partner RXRα. In addition, we hypothesized that PPARγ2, like other steroid-type receptors, existed in both the cytosolic and nuclear compartments, and that ligand activation by pioglitazone would enrich the nuclear content of PPARγ2.

The experimental approach to these hypotheses differed from that of most other investigators in that a two step differentiation protocol was employed. Most prior reports induce the conversion of 3T3-L1 preadipocytes to mature fat cells by first treating the preadipocytes with a combination of insulin, IBMX, and dexamethasone; and then subsequently removing the IBMX and dexamethasone after 48h. In order to separate the effects of IMBX/dexamethasone from those of insulin, we first treated the 3T3-L1

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preadipocytes with IBMX/dexamethasone for 48h, and then change the media to one containing insulin. In applying this two step differentiation approach, we discovered that the marked induction in PPARγ2 mRNA associated with fat cell differentiation (5,9,13) was not in response to insulin or pioglitazone; but rather the rise in PPARγ2 mRNA that occurred during fat cell differentiation appeared to be an early phase response to IBMX (presumably resulting from an increase in cAMP) and/or dexamethasone. Interestingly, the increase in PPARγ2 mRNA induced by IBMX/dexamethasone was not associated with an increase in PPARγ2 protein (Figures II.2 and II.4 and Appendix Figures II.2B and II.4C). However, when the IBMX/dexamethasone mixture was removed and insulin was added to the media, total cellular PPARγ2 protein increased 2-3 fold within two hours (Figure II.2 and Appendix Figure II.2B). These data suggest that during the early stages of fat cell differentiation, the amount of cellular PPARγ2 is in part governed by translation control mechanisms, and that these mechanisms may be insulin dependent.

Even though pioglitazone treatment of differentiating 3T3-L1 cells greatly enhanced the expression of a-FABP (Figure II.2), as well as increased the expression of lipoprotein lipase and fatty acid synthase (data not shown), pioglitazone had no effect on the abundance of PPARγ2 mRNA or on the total cellular content of PPARγ2 protein of differentiating 3T3-L1 cells (Figures II.2 and II.3 and Appendix Figures II.2C and II.3B). However, pioglitazone treatment of differentiating 3T3-L1 preadipocytes greatly stimulated PPARγ2 binding to its DR-1 recognition sequence of the a-FABP gene. Thus, the pioglitazone dependent acceleration of fat cell differentiation appears to be largely due to its function as a ligand activator of PPARγ2, rather than as an inducer of PPARγ2 expression.

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Because PPARγ2 has been considered the pivotal transcription factor for terminal fat cell differentiation (6,9-14), we were surprised to discover that 3T3-L1 preadipocytes contained significant quantities (i.e. 30-50% of fully differentiated cells) of PPARγ2 mRNA and protein. Equally surprising was the discovery that PPARγ2 existed in both the cytosolic and nuclear compartments of non-differentiating and differentiating 3T3-L1 cells; and that the insulin dependent increase in total cellular PPARγ2 occurred largely in the cytosolic compartment. Moreover, the PPARγ2 ligand activator, pioglitazone, did not increase the fraction of PPARγ2 located in the nuclear compartment. The cytosolic component of PPARγ2 appeared to be diffusely distributed throughout the cytosol of the undifferentiated 3T3-L1 cells, but as the preadipocytes underwent differentiation and accumulated lipid, the PPARγ2 became associated with the lipid droplets (Figure II.5). The significance of PPARγ2's association with the lipid droplets is unclear, but PPARγ2 is a receptor for free fatty acids and certain prostanoids. Thus, PPARγ2's association with lipid droplets may simply place it in closer proximity to its fatty acid ligand. Similarly, we can only speculate as to the function of cytosolic PPARγ2, but the relatively high cytosolic content of PPARγ2 in preadipoctyes combined with the observation that the 2-3 fold increase in total cellular PPARγ2 occurring during early differentiation is largely restricted to the cytosolic compartment strongly suggest that PPARγ2 may have a cytosolic function. In this regard, it is interesting to note that the 3'-untranslated region of the fatty acid synthase gene contains a consensus DR-1 recognition sequence for PPARγ2/RXRα. Since the rise in fatty acid synthase mRNA of differentiating 3T3-L1 cells is predominantely the product of an increased mRNA stability, one might speculate that PPARγ2 binding to the DR-1 of the 3'-untranslated region of the fatty acid synthase transcript could regulate the cellular abundance of fatty acid

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synthase mRNA by altering its stability. Whatever the function may be, the discovery that significant quantities of PPARγ2 exist in the cytosol of both preadipocytes and mature adipocytes suggests a new cellular role for PPARγ2.

While our data demonstrated that 3T3-L1 preadipocytes contained significant quantities of PPARγ2 protein, nuclear proteins extracted from undifferentiated preadipocytes were not able to bind to the DR-1 recognition sequence (Figure II.8), nor did pioglitazone treatment of preadipocytes stimulate a-FABP expression. If preadipocytes contain ample levels of PPARγ2, what then is the determinant for the expression of fat specific genes such as a-FABP? The answer to this question may lie in the temporal pattern of RXRα expression. Transcriptional activation of genes involved in the terminal stages of fat cell differentiation (e.g. a-FABP) requires that PPARγ2 bind to its response element (e.g. TGCACATTTCACC) as a heterodimer complex with RXRα (32, 33). However, even though preadipocytes contain a significant level of RXRα mRNA, they contain no detectable amount of RXRα protein. The reason why the RXRα transcript is translationally repressed in undifferentiated 3T3-L1 cells is unknown, but the nature of this mechanism would appear to play a pivotal role in the terminal differentiation of 3T3-L1 preadipocytes. Regardless of the mechanism, it is only when the preadipocytes are treated with IBMX and dexamethasone that the cellular content of RXRα begins to increase. Since the preadipocytes lack RXRα protein, PPARγ2 cannot form a heterodimer with RXRα and consequently it cannot interact with the DR-1 recognition sequence. Thus, the transcription of fat specific genes (i.e. a-FABP) is not initiated, and terminal fat cell differentiation does not occur.

While it is clear that the synthesis of RXRα is essential for the expression of a-FABP, and for the terminal differentiation of fat cells, the ability of thiazolidinediones such as pioglitazone to accelerate fat cell differentiation and

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increase insulin/IGF-1 sensitivity appears to be independent of RXRα expression and the nuclear content of RXRα protein (Figure II.6). Given that pioglitazone does not increase the expression of either PPARγ2 or RXRα during the early phase of terminal fat cell differentiation, the question continues to remain as to how pioglitazone accelerates the differentiation of 3T3-L1 preadipocytes, and induces the expression of a-FABP. The answer to this question appears to lie in the observation that pioglitazone is a high affinity ligand for PPARγ2 (11,22) and that ligand binding to PPARγ2 stimulates PPARγ2/RXRα interaction with its ARE (DR-1) DNA response element (Figure II.8), which in turn induces a-FABP transcription (23, 24). While this mechanism may explain how thiazolidinediones accelerate the transcription of genes involved in terminal fat cell differentiation, it does not explain how pioglitazone shifts the dose response curve for IGF-1 to the left (i.e. increased IGF-1 sensitivity) (21, 23). The explanation for the enhanced IGF-1 responsivity remains to be elucidated.

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Chapter III

The following chapter has been published in the Journal of Lipid Research: Vol 39 Issue 10 2048-2053 (1998 Oct) . The text and figures have been kept as they appear in the original publication, some figure legends have been modified as recommended by the graduate committee. P. Thuillier's contribution to this article constituted in RNA collection and northern analysis (Figure III.2) development of anti-PPARγ2 antibody for western blot analysis and assistance in western blot protocol (Figures III.3, III.4) and gel shift (Figure III.5)

Original Title and list of Authors as they appear in the published article are:

TITLE: A novel 3T3-L1 preadipocyte variant that expresses PPARγ2 and RXRα but does not undergo differentiation.

AUTHORS: Rebecca A. Baillie, Xiaoming Sha, Philippe Thuillier and Steven D. Clarke

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RUNNING TITLE: Preadipocyte differentiation and the role of PPARγ2.

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A novel 3T3-L1 preadipocyte variant that expresses PPARγ2 and RXRα but does not undergo differentiation.

III.1 ABSTRACT

In this report we described a novel adipocyte-like cell line derived from 3T3-L1 cells that continued dividing after the cells reached confluence, formed foci, and constitutively expressed adipose fatty acid binding protein (a-FABP) mRNA, but was not able to fully differentiate. Adipose-FABP protein levels in the variant were 30% of fully differentiated 3T3-L1 cells, and the level of this protein was not induced by insulin. We hypothesized that the variant, termed 3T3-L1/RB1 cells, lacked expression of either the peroxisomal proliferator activated receptor γ2 (PPARγ2) or retinoid X receptor α (RXRα) transcription factors or the activator necessary for terminal differentiation. However, western analysis showed that 3T3-L1/RB1 cells contained PPARγ2 and RXRα proteins at levels equal or greater than that of the parent cell line. Addition of a PPARγ2 activator ligand, pioglitazone, to the 3T3-L1/RB1 cell medium did not increase a-FABP mRNA or protein in these cells. In gel retardation assays, nuclear proteins from 3T3-L1/RB1 cells showed very little ability to bind the adipose response element of the a-FABP gene and the DNA binding was unaffected by treatment of the cells with insulin or insulin and pioglitazone. In summary, the variant 3T3-L1/RB1 cells contained a defective signaling system which did not permit ligand activation of PPARγ2 and terminal differentiation.

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III.2 INTRODUCTION

The conversion of preadipocytes to mature fat cells is a coordinated response to a collection of external stimuli including signals from cAMP, glucocorticoids, IGF-1, and arachidonate [1-6]. These signals initiate a cascade of transcriptional changes which lead to the down regulation of differentiation suppressors [7], and the up regulation of differentiation inducers [1]. Two transcription factors which are essential to terminal fat cell differentiation and the expression of fat specific genes are PPARγ2 and RXRα [8-11]. PPARγ2 is a ligand activated transcription factor which stimulates the transcription of fat specific genes (e.g. a-FABP) by forming a heterodimer with RXRα and subsequently binding to its DNA recognition site, DR-1 (direct repeat, one base pair spacer) [4,9]. The endogenous ligand activator for PPARγ2 appears to be a prostanoid with 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 [4]. However, the ligand domain of PPARγ2 can bind a number of different activating compounds including fibrates, ETYA, and the novel antidiabetic drugs, thiazolidinediones [12,13]. If PPARγ2, RXRα, or the appropriate PPARγ2 ligand are not present, then the expression of fat specific genes and terminal fat cell differentiation does not occur [6,11]. As an example, the ectopic expression of PPARγ2 in 3T3 fibroblasts did not initiate fat cell differentiation until a ligand activator for PPARγ2 was introduced [14]. Similarly, 3T3-L1 preadipocytes do not express a-FABP and terminally differentiate until RXRα expression has been induced by glucocorticoids [9,11]. Thus, it is generally accepted that the events of terminal fat cell differentiation and the expression of fat specific genes such as a-FABP require the presence of PPARγ2, a PPARγ2 ligand activator, and RXRα.

Recently we isolated a variant of the 3T3-L1 adipocyte cell line which lacked the ability to withdraw from the cell cycle and terminally differentiate. In this report we describe the variant (termed 3T3-L1/RB1), and we examine the

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hypothesis that the variant line does not express either PPARγ2 or RXRα, and/or the variant does not produce the ligand activator that is essential to terminal fat cell differentiation and the induction of the fat specific gene, a-FABP.

III.3 METHODS

Reagents. Dulbecco's modified Eagle's medium, fetal and newborn calf serum, and random prime and PCR labeling kits were purchased from Gibco BRL/Life Technologies (Gaithersburg, MD). PPARγ2 antibody was from Affinity Bioreagents, Inc. (Golden, CO). RXRα antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The cDNAs for a-FABP, lipoprotein lipase, RXRα and PPARγ2 were provided by D. Bernlohr, (St. Paul, MN), R. Eckel (Denver, CO), T. Neuman (Fort Collins, CO), B. Spiegelman (Boston, MA) respectively. The [α-^{32}P]-dCTP was purchased from Dupont/NEN (Boston, MA). The Enhanced Chemiluminesence kit was purchased from Amersham (Arlington Heights, IL). All other reagents were from Sigma Chemical (St. Louis, MO).

Cell culture. 3T3-L1 preadipocytes (ATCC #F8979) and 3T3-L/RB1 cells were grown in Dulbecco's modified Eagle's medium that contained 5% fetal calf serum. When the cells reached confluence, they were staged to differentiate by changing the medium to one containing 5% newborn calf serum plus 0.5 mM isobutyl-methylxanthine (IBMX) and 1 μM dexamethasone. IBMX and dexamethasone were removed after 48h (designated as time 0), and the medium was changed to one containing no hormones, 1 μM insulin, or insulin plus 20 μM pioglitazone. Cell protein was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

RNA isolation and Northern blotting. The relative abundance of mRNA encoding a-FABP, lipoprotein lipase, and PPARγ2 was determined by

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Northern analysis using total RNA isolated by the guanidinium isothiocyanate-phenol method [15]. RNA (10 μg) was size fractionated by electrophoresis in a 1.3% agarose gel containing 6.6% formaldehyde, 20 mM MOPS, 1 mM EDTA, and 1 mM sodium acetate. After electrophoresis, the RNA was electroblotted onto Zetaprobe GT® membrane (Bio-RAD, Richmond, CA) and fixed to the membrane by UV crosslinking and baking. The cDNA probes for the Northern blotting analysis were labeled using random prime or PCR labeling. Membranes were hybridized and washed as previously described [16]. The membranes were imaged and quantified using an Instantimager (Packard Instrument Company, Meriden, CT) or by exposure to Kodak X-Omat AR film (Rochester, NY) followed by visual imagizing using the AMBIS visualizing system.

Western analysis. Total cellular protein extracts were prepared at the various times cited in the figures. Cells on 60 mm dishes were rinsed twice with PBS and then scraped from the plates using 0.3 ml of RIPA buffer (PBS, 1% NP40, 0.5 % sodium deoxycholate, 0.1% SDS, 100 mg/ml PMSF, 5 mg/ml aprotinin, 1 mM Na orthovanadate). The lysates were passed through a syringe fitted with a 21 gauge needle, transferred to a centrifuge tube and centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant containing the total cell lysate was stored at -80°C until use. Nuclear protein extracts were prepared using the procedure from Dignam et al [17]. Proteins (25 μg) were separated by SDS-PAGE on a 10 % polyacrylamide gel, transferred and immobilized on a nitrocellulose membrane. The membrane was blocked by incubation with 5% non fat dry milk in phosphate-buffered saline at 4°C over night. The membrane was then washed briefly in Tris-buffered saline, and incubated with rabbit antibody raised against a-FABP diluted 1:1000, PPARγ2 diluted 1/15000 or RXRα diluted 1:1000 in Tris-buffered saline containing 0.05% Tween 20.

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Incubation with antibodies and detection of the antigen-antibody complex were performed using the enhanced chemiluminescence.

Gel retardation assay. Nuclear protein extracts (5 μg) isolated using the procedure from Dignam et al [17] were mixed in binding buffer (100 mM KCL, 0.1% NP40, 10% glycerol, 10 mM HEPES, 5 mM MgCl2, 2.5 mM ZnSO4, 0.8 mM DTT), 1 μg of Poly (dA-dT) in the presence of 20,000 cpm (1 ng) of γ-^{32}P-ATP labeled DNA probe in a total volume of 20 μl and incubated on ice for 20 min. Samples were then loaded on a 4.5 % polyacrylamide gel containing 0.1% NP40 and run at 200 v for 2 hr. in 40 mM Tris, 380 mM glycine, pH 8.3 buffer containing 0.1% NP40. Gels were dried and quantified by photoimagizing. The double stranded oligonucleotide used for gel shift assays was the adipose response element for PPARγ2/RXRα previously identified for the a-FABP gene [8]. The sequence of this response element is: 5'-CAGAAATGCACATTTCACCCAGAGAGAAGGG-3'.

III.4 RESULTS

During our studies of the mechanisms governing the conversion of 3T3-L1 fibroblasts to mature fat cells we noted that a small number of the cells no longer stopped dividing when confluence was attained. This sub-group of 3T3-L1 cells was named 3T3-L1/RB1, and they displayed a number of characteristics distinctly different from the parent 3T3-L1 cells. Specifically, the doubling time of the 3T3-L1/RB1 cells was 4-6h compared to 12-18h for the 3T3-L1 parent. In addition, unlike the 3T3-L1 cells which upon reaching confluence become rounded, develop a genotype characteristic of mature fat cells, and subsequently fill with lipid; the 3T3-L1/RB1 cells maintain their fibroblast-like shape, and do not develop a gene expression pattern that is characteristic of adipocytes (Figures III.1 and III.2). As an example, 3T3-L1

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Figure III. 1- The abundance of a-FABP transcripts in 3T3-L1 and 3T3-L1/RB1 cells.

The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 hours (METHODS). Following the 48 hour staging period, IBMX and dexamethasone were removed from the media, and the cells were cultured for additional 1, 3, and 5 days with no hormones (NA), insulin, or pioglitazone plus insulin. Transcript abundance was determined by Northern analysis. Panel A is cells treated with insulin. Panel B is cells treated with insulin and pioglitazone. Note the 10-fold difference in scale between panels A and B. A sample Northern is depicted in Figure 2. Results are representative of four experiments. Results shown as means $\pm$ SEM.

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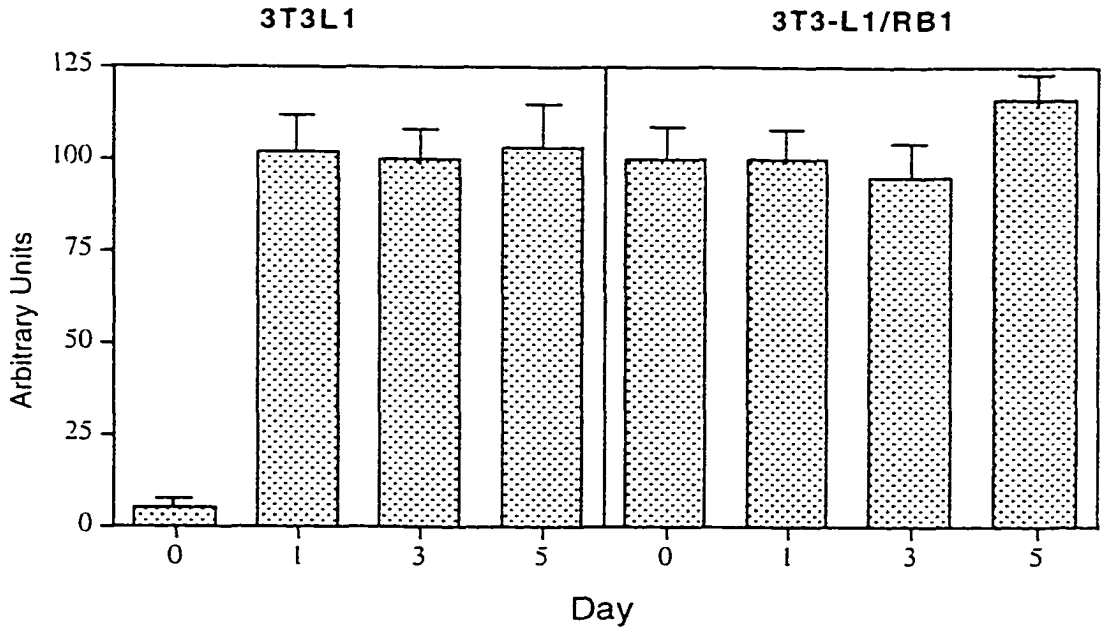
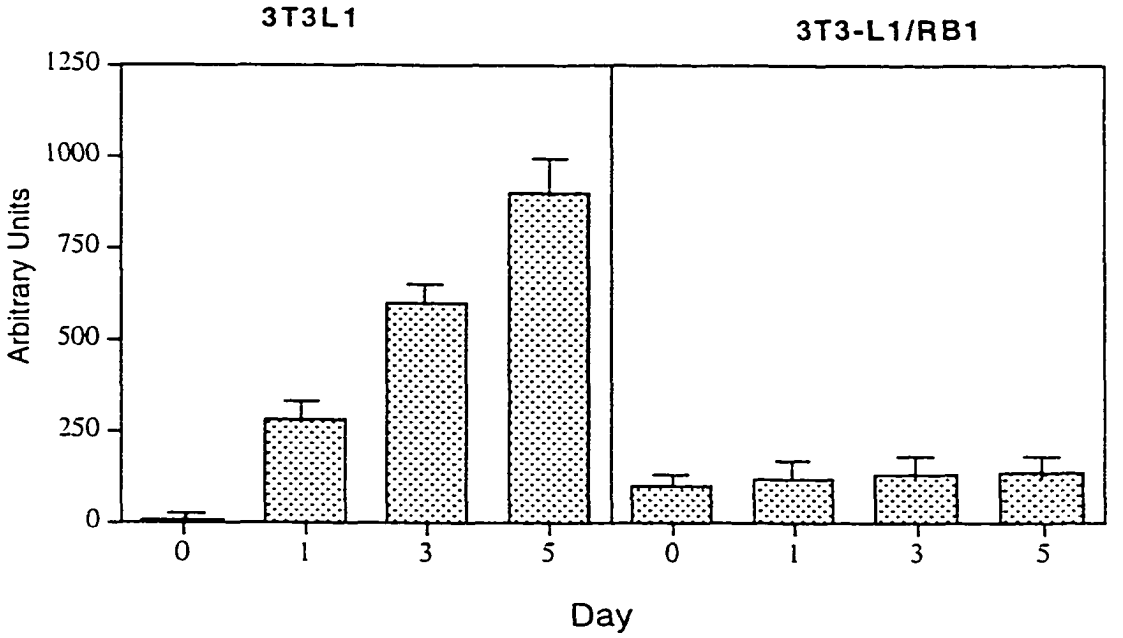
A. Insulin



B. Insulin+Pioglitazone



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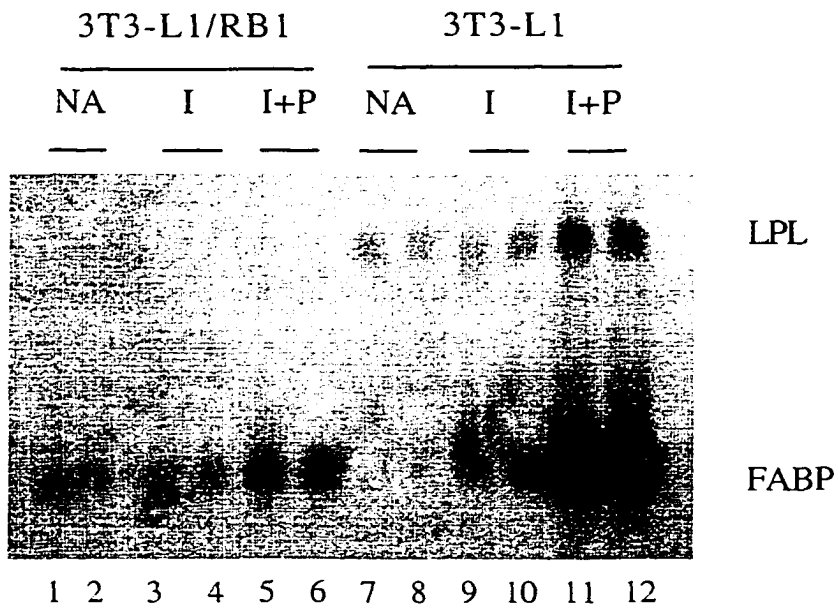

Figure III. 2- Adipose fatty acid binding protein and lipoprotein lipase mRNA quantity in 3T3-L1 and 3T3-L1/RB1 cells

The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 hours (METHODS). Following the 48 hour staging period, IBMX and dexamethasone were removed from the media, and the cells were cultured for an additional 3 days with no hormones, insulin, or pioglitazone plus insulin. Total RNA was extracted, and utilized for Northern blot analysis.

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fibroblasts do not express the adipocyte specific gene, a-FABP, until they have reached confluence and have been induced to differentiate by treatment with IBMX, glucocorticoid, and insulin (Figure III.1). Under these conditions, the differentiating 3T3-L1 cells form large lipid droplets, and greatly accelerate the expression of adipocyte genes such as a-FABP and lipoprotein lipase (Figure III.2). On the other hand, although the 3T3-L1/RB1 cells did contain a low number of triglyceride droplets (data not shown) and did express a low, constitutive level of a-FABP (Figures III.1 and III.2), exposing the 3T3-L1/RB1 variant to the differentiating stimuli of IBMX, dexamethasone, and insulin did not increase the abundance of a-FABP or lipoprotein lipase mRNA nor did the differentiation cocktail increase the cellular content of a-FABP protein (Figure III.3). While the 3T3-L1/RB1 variant had a slight response to pioglitazone in the representative Northern shown here, additional experiments showed that this response was not significant, and may have been due to residual 3T3-L1 cells in the culture.

We considered the possibility that the 3T3-L1/RB1 variant responded to differentiation stimuli at a much slower rate than did the 3T3-L1 parent line. To address this hypothesis, the 3T3-L1/RB1 cells were treated with the insulin/IGF-1 sensitizing agent pioglitazone [18,19]. Pioglitazone is a member of the thiazolidinedione family of antidiabetic compounds which functions as a ligand activator for the adipocyte transcription factor, PPARγ2. The consequence of ligand activation is an acceleration and amplification of adipocyte gene expression, e.g. a-FABP [5,6,10,19]. As expected, pioglitazone increased the abundance of a-FABP and lipoprotein lipase mRNA in differentiating 3T3-L1 cells by 10-15 fold (Figure III.2). However, pioglitazone treatment of 3T3-

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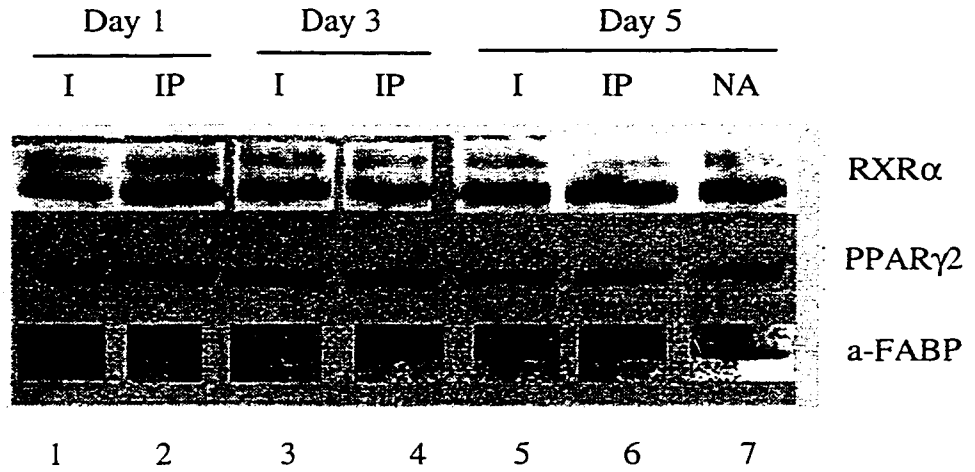

Figure III. 3- Total cellular content of PPARγ2, RXRα, and a-FABP in 3T3-L1/RB1 cells

The 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 hours (METHODS). Following the 48 hour staging period, IBMX and dexamethasone were removed from the media, and the cells were cultured for additional 1, 3, and 5 days with no hormones (NA), insulin, or pioglitazone plus insulin. Total protein was extracted, and utilized for Western analysis. The blots were incubated with antibodies for PPARγ2 or RXRα, or antisera against a-FABP. Results are representative of three experiments.

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L1/RB1 cells had no effect on lipoprotein lipase expression, and it exerted a very limited ability to increase the abundance of a-FABP mRNA, or the level of a-FABP protein in the 3T3-1/RB1 variant (Figure III.3).

Because the 3T3-L1/RB1 cells did not respond to pioglitazone, we hypothesized that the PPARγ2 receptor for pioglitazone, and/or its heterodimer partner, RXRα, may not be expressed in the 3T3-L1/RB1 variant. Consistent with our hypothesis, the 3T3-L1/RB1 variant was found by Northern analysis to contain undetectable levels of mRNA for PPARγ2 and RXRα. Since our previous studies indicated that the cellular content of PPARγ2 and RXRα proteins may be regulated at the level of translation rather than by mRNA abundance [20], the nuclear content of PPARγ2 and RXRα were quantified in both the 3T3-L1 parent and 3T3-L1/RB1 variant (Figure III.4). Surprisingly, the cellular level of PPARγ2 protein was comparable in both the parent and variant lines. Moreover, the amount of PPARγ2 and RXRα protein was not affected in either line by insulin or pioglitazone (Figures III.3 and III.4). Similarly both cell varieties contained RXRα, and like the PPARγ2 protein, RXRα levels were unaffected by insulin or pioglitazone (Figure III.4). Interestingly, the 3T3-L1/RB1 cells contained 2-3 fold more RXRα than the parent 3T3-L1, but this was not reflected in the mRNA abundance for RXRα.

Since both PPARγ2 and RXRα proteins were present in the 3T3-L1/RB1 cells at levels equal to or greater than the amounts found in their 3T3-L1 parent line, we hypothesized that the inability of the 3T3-L1/RB1 cells to express fat specific genes and to terminally differentiate into mature fat cells was perhaps due to a failure of PPARγ2 and RXRα to form a heterodimer and subsequently bind to the PPARγ2 response element. To address this hypothesis, electrophoretic mobility shift assays (EMSA) were employed using the adipose

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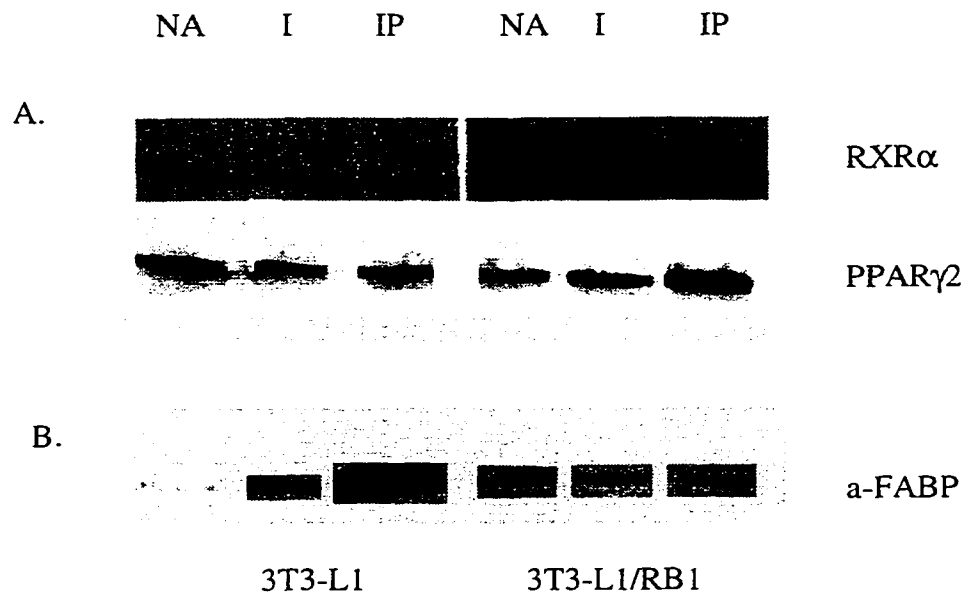

Figure III. 4-Nuclear content of PPARγ2 and RXRα in 3T3-L1 and 3T3-L1/RB1 cells

The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 hours (METHODS). Following the 48 hour staging period, IBMX and dexamethasone were removed from the media, and the cells were cultured for additional 3 days with no addition (NA), insulin, or pioglitazone plus insulin. Nuclear and total proteins were isolated and utilized for Western analysis. The blots were incubated with antibodies for PPARγ2 or RXRα or antisera against a-FABP. Panel A is nuclear protein extracts. Panel B is total protein extracts. Results are representative of three experiments.

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response element from the a-FABP gene, which is a well-established DNA binding site for the PPARγ2/RXRα heterodimer [4,8,9]. As expected, nuclear extracts from differentiating 3T3-L1 cells displayed a pronounced gel shift with the adipose response element (Figure III.5), while nuclear protein extracts from undifferentiated 3T3-L1 cells (i.e. no addition) had little ability to bind to the adipose response element of the a-FABP gene. On the other hand, nuclear proteins extracted from the 3T3-L1/RB1 variant treated with either insulin or pioglitazone displayed very little ability to interact with the DNA sequence for the adipose response element (Figure III.5). Thus, even though the 3T3-L1/RB1 variant contains ample quantities of PPARγ2 and RXRα protein, pioglitazone, the PPARγ2 ligand pioglitazone was unable to induce a PPARγ2 interaction with its cis-acting element.

III.5 DISCUSSION

The conversion of preadipocytes to mature fat cells is a coordinated response to a collection of external inducers [1,6,7]. At least three external factors are essential to the differentiation process: (a) cAMP [21]; (b) glucocorticoids [22]; and IGF-1 [2,3]. In addition, long chain fatty acids may function as differentiation inducers in some preadipocyte models [22]. The function of these inducing agents is to down regulate repressors of differentiation such as PREF-1 (preadipocyte factor 1), CUP (C/EBP undifferentiated protein), and RARγ (retinoic acid receptor gamma) [1,7,9]; while concomitantly increasing the expression of transcription factors essential for the transcription of genes characteristic of a terminally differentiated fat cell, e.g. C/EBP, RXRα, and PPARγ2 [5-11,23]. PPARγ2 and RXRα are one set of transcription factors whose synthesis is critical for the expression of fat specific

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Figure III. 5-EMSA

The 3T3-L1 and 3T3-L1/RB1 preadipocytes were staged to differentiate by treating them with IBMX and dexamethasone for 48 hours (METHODS). Following the 48 hour staging period, IBMX and dexamethasone were removed from the media, and the cells were cultured for additional 3 days with no hormone (NA), insulin, or pioglitazone plus insulin. Nuclear protein extracts were prepared and utilized in a gel retardation assay with ^{32}P-labeled ARE. Lane 1, free probe; lanes 2-4, 3T3-L1 cells; lanes 5-7, 3T3-L1/RB1 cells. Lanes 2 and 5 are no hormone; lanes 3 and 6 are insulin; lanes 4 and 7 are pioglitazone plus insulin.

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genes and terminal fat cell differentiation [14]. These two transcription factors stimulate the transcription of adipose specific genes by forming a heterodimer and subsequently binding to the DNA recognition site, DR-1 (direct repeat sequence with one nucleotide spacer) [8,9]. Like other members of the steroid receptor super-family, PPARγ2 contains a ligand binding domain, and binding of factors such fatty acids, fibrates, certain prostanoids, and thiazolidinediones to the ligand domain greatly enhances the interaction of PPARγ2 with its DNA recognition site [4,5,9,14], which in turn accelerates the transcription of adipose specific genes such as a-FABP [19,24].

In light of the essential role that PPARγ2/RXRα play in the expression of adipose specific genes and in the terminal differentiation of fat cells, we considered the possibility that the reason the 3T3-L1/RB1 variant did not differentiate may be because the expression of PPARγ2 and/or RXRα was repressed. However, Western blotting indicated that the total cellular and nuclear content of PPARγ2 and RXRα in the 3T3-L1/RB1 variant were equal to or greater than the amounts found in differentiated 3T3-L1 parent cells (Figure III.4). In light of the fact that the 3T3-L1/RB1 variant clearly contains PPARγ2 and RXRα, we evaluated the alternative hypothesis that the variant line may not produce the appropriate ligand needed to activate PPARγ2 and enhance its interaction with the DR-1. To address this alternative, the 3T3-L1/RB1 cells were treated with the high affinity ligand activator, pioglitazone. Pioglitazone is a novel antidiabetic agent that is a member of the thiazolidinedione family, and has the effect of increasing fat cell sensitivity to insulin and IGF-1 several fold [18,19]. In spite of the fact that the 3T3-L1/RB1 variant contained high levels of PPARγ2 and RXRα protein, pioglitazone did not enhance PPARγ2/RXRα interaction with its DR-1 recognition sequence from the a-FABP gene (Figure III.5). Consistent with this observation, a-FABP mRNA abundance was not

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significantly increased in 3T3-L1/RB1 cells treated with pioglitazone (Figure III.1). This outcome was very surprising because ectopic expression of PPARγ2 in NIH 3T3 fibroblasts, and subsequent treatment of the fibroblasts with pioglitazone led to near 95% conversion of the fibroblasts to mature fat cells [14].

The reasons for why 3T3-L1/RB1 cells do not terminally differentiate can only be speculated. One possibility is that these cells do not express the C/EBP family of proteins that may be needed to achieve full PPARγ2 responsivity. In this regard, ectopic expression in G8 myoblasts of either C/EBPα alone or PPARγ2 (plus activator) alone did not give rise to a fat cell phenotype, but co-expression of the two transcription factors led to 70% conversion of the cells to adipocytes [14]. A second possibility is that the 3T3-L1/RB1 cells contain a differentiation silencer or suppressor protein, e.g. high levels of PREF-1 or CHOP/gadd153 that could block the anti-mitogenic action of C/EBPα and lead to a failure of the cells to growth arrest [5-7,]. However the difficulty with both of these possibilities is that neither of them provide a good explanation for why PPARγ2/RXRα do not bind to the DR-1 recognition sequence derived from the a-FABP gene (Figure III.5). The inability of PPARγ2 to interact with its DNA recognition sequence even in the presence of the pioglitazone activator suggests that simple ligand binding to PPARγ2 may not be sufficient to elicit DNA binding. In support of this concept, pioglitazone treatment in the absence of insulin has been found not to induce the conversion of 3T3-L1 preadipocytes to mature fat cells (Clarke and Sha, unpublished data). In addition, the phosphorylation status of PPARγ2 has recently been found to regulate the ability of PPARγ2 to interact with its DR-1 recognition sequence [8,9]. Thus, it is feasible that the 3T3-L1/RB1 variant has a defect in the insulin or IGF-1 signaling cascade, or there may be an overexpression of an anti-adipogenic

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signal (e.g. PREF-1) which is blocking PPARγ2 activation. Regardless of the mechanism, it appears we have isolated a variant of the 3T3-L1 adipocyte line which expresses the trans-factors needed for terminal fat cell differentiation, but which does not undergo differentiation. This RB1 variant of 3T3-L1 cells should provide a useful tool for better understanding the mechanism by which PPARγ2 exerts its transcription response, and for elucidating regulatory steps in terminal fat cell differentiation.

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Chapter IV. Discussion

IV.1. Activation of differentiation

Insulin resistance is a fundamental component of NIDDM and is associated with a variety of other disorders such as obesity, hypertension and atherosclerosis. Obesity is the most common and costly nutritional problem in the United States, affecting approximately 33% of adults. Health costs directly attributable to obesity amount to approximately $68 billion per year. An additional $30 billion per year is spent on weight reduction programs and special foods (1). Obesity results from excessive development of adipose tissue. The last five years have yielded exciting information concerning the regulatory mechanisms of adipogenesis. The recent discovery of the aP2 enhancer and the cloning of mPPARγ2 have contributed to further understanding some of the molecular mechanisms of adipocyte differentiation. It was originally thought that an increase in PPARγ2 expression was sufficient for preadipocyte commitment to enter terminal differentiation (2). More recently, the discovery of high affinity ligand activators for PPARγ2 has resulted in an increasing search for the physiological importance of these ligands. It is now clear that PPARγ activation requires ligand binding. Among the ligands identified, the arachidonic acid metabolite 15-deoxy-Δ^{12-14}-prostaglandin J2 (PGJ2) and thiazolidinediones derivatives appear to be the strongest activators (3-8). However, despite it's high affinity for PPARγ, PGJ2's significance in vivo is still unknown. This is mostly due to the lack of direct evidence of PGJ2 action in vivo, combined with it's low levels of detection in the proposed targets tissues. Furthermore, our observations suggest that ligand activation of PPARγ is dependent on the concerted expression of RXRα. This result is not very

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surprising since it has been established that PPARγ binds to its DR1 only as a heterodimer with RXRs (9-12). More surprising is the high abundance of PPARγ detected in the cytosol. Because most transcription factors bind to the DNA to modulate genes transcription, their localization is usually mostl prominent in the nucleus. However, there are several examples of factors such as NFκB, the glucocorticoid receptor, and others which are also found in the cytosol (13, 14). This unusual compartmental localization is a unique mechanism of activation regulation of the factor that involves its translocation from the cytosol to the nucleus. Whether the cytosolic PPARγ corresponds to an active or inactive form of the receptor, or reflects a possible translocational regulation to the nucleus are questions that remain to be addressed. It will be important to determine whether the cytosolic PPARγ is similar to the nuclear PPARγ. Interestingly, cytosolic extracts from 3T3-L1 have the ability to shift ARE (fig II.8C Appendix). In addition, anti-PPARγ_2 antibody was able to supershift this complex, suggesting that PPARγ_2 exists in the cytosol in an active binding form. However, our data indicate that RxRα is not present in the cytosol (figure II.6B). This suggests that PPARγ_2 could heterodimerizes and bind to ARE with some other factor not yet determined. RxRβ and specially RxRγ are good candidates for that interaction as they also heterodimerize with PPARs and bind with high affinity to the DR1 (11). Indeed, the shift obtained with cytosolic extracts appears to have a faster migration than the shift obtained with nuclear extracts (Appendix figure II.8.C, lanes 2 and 4 versus lane 3 and 5 respectively). This suggests that the cytosolic complex detected by gel shift could be smaller than the nuclear complex. Unfortunately, there is no information regarding RxRγ protein levels in the cytosol of 3T3-L1 cells undergoing differentiation. Hence it might be of interest to determine whether RxRγ is expressed and present during the differentiation process and whether it might play a role in dimerizing with

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PPARγ_2. The segregation of PPARγ_2 in the cytosol by another protein would provide a regulatory mechanism, allowing PPARγ_2 release from the cytosol and activation into the nucleus. The presence of RxRγ in cytosolic extract and/or gel shift could be easily determined by Western blot analysis and supershift respectively, using an anti-RxRγ antibody. It is also possible, that the cytosolic form of PPARγ could be associated with a set of cytosolic proteins that could play a role in the regulation of its translocation. Such a model has been described for the glucocorticoid receptor (GR) (13). The GR is present in the cytosol as a heteromeric complex associated with a variety of heat shock proteins (hsp) (13, 15). The synthesis of hsps increases upon heat stress, a feature presumably important for the survival of cells and organisms (15). These hsps were able to delocalize the unliganded GR to become tightly bound within the nucleus under heat shock or chemical stress. Reportedly, the heat shock response was altered during 3T3-L1 differentiation (16), suggesting a possible regulatory mechanism of fat cell differentiation involving these factors. Furthermore, hsp90 was recently found to interact with PPARγ (personal communications J. Vanden Heuvel; Pennsylvania State University). Hence, in addition to its ligand activation, the mechanisms by which ARF6 is activated to bind to ARE6 could involve a mechanism of translocation regulation involving hsps, as suggested above. PPARγ high abundance in the cytosol could also reflect a role in the stability of certain newly expressed mRNAs. Two possible targets could be FAS or a-FABP mRNA known to be regulated primarily by transcription during the early stages of differentiation and then mostly regulated by stabilization following terminal differentiation. Hence, more investigation will be necessary to determine the mechanisms and the different level of regulation, including the natural ligands responsible for the activation of adipocyte differentiation. Our data also suggest that PPARγ induction takes place after

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removal of dexamathosone (Figures II.2 and II.4A). This suggests an inhibitory effect of dex on the expression of PPARγ. New evidence by Glorian et al. (17) implies that glucocorticoid represses the stimulation of PEPCK gene transcription by thiazolidinediones and fibrates. Hence, glucocorticoid could similarly have an inhibitory effect on PPARγ induction during adipocyte differentiation. Recent work from Lazar et al. (18) and Moller et al. (19) suggests a third level of regulation for PPARγ activation through MAP kinase activation and down-regulation of phosphatase 2A, resulting in a non active phosphorylated PPARγ. Interestingly, activation of MAP Kinase was mediated by PGF2α, another arachidonic acid metabolite, suggesting that the balance between PGF2α and PGJ2 signaling may play a key role in the development of obesity and diabetes. It would be interesting to determine which of the active or non-active forms of PPARγ2 are more abundant in the cytosol and whether nuclear activation results from a change of phosphorylation in the cytosol. More studies will clearly be needed to test this hypothesis.

IV.2. PPARs involvement in Cancer

Although PPARγ2 was originally described as an adipogenic specific factor, it is clear now that it is expressed in other tissues. Thus, its role might not be limited solely to adipogenesis. A role for PPARs in carcinogenesis has been considered due to their involvement in regulating growth and differentiation,. PPARγ and PPARs have been recently associated with inflammation (20) and with several type of cancers, including colon (21), liposarcoma (22) and hepatic cancer (23). Inflammation is thought to be required for tumor promotion since most tumor promoters induce inflammation and many compounds that act as anti-promoters reduce imflammation (24). In two recent studies, PPARγ was implicated as a key regulator in inflammation, associated with activation of monocytes stimulated with

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TPA (20) and macrophages stimulated by thioglycollate (25). Co-treatment of TPA-stimulated monocytes with PPAR ligands (e.g. troglitazone, 15Δ-PGJ2 or NSAIDs) significantly reduced cytokine production (20). In a separate study, macrophages co-treated with thioglycollate to induce nitric oxide synthase and PPARγ ligands (i.e. 15Δ-PGJ2 or BRL49653), exhibited significantly reduced levels of nitric oxide synthase, cytokines and TNFα (25). In addition to modulating differentiation and inflammation, activation and subsequent induction of transcriptional activity of PPARs appears to directly effect cell growth, apoptosis and carcinogenesis. The role of PPARs in carcinogenesis has been most extensively studied in rodent liver. In hepatocarcinogenesis, PPARα ligands (peroxisome proliferators) act as nongenotoxic carcinogens and induce events such as increased cell proliferation and inhibition of apoptosis of initiated cells (26). The specific role(s) of PPARα, the predominant isoform in liver, in hepatocarcinogenesis was recently demonstrated in PPARα null mice (27) where the peroxisome proliferators Wy-14,643 was ineffective at inducing hepatocarcinogenesis. Activation of PPARα in liver is likely to affect multiple events involved in carcinogenesis, including elevated free radical generation via enhanced peroxisomal β-oxidation, altered apoptosis and proliferation. However, some peroxisome proliferators (i.e. certain endogenous polyunsaturated fatty acids including linoleate, arachidonate and the fatty acid derivative, perfluorinated decanoate) have no effect or are weak promoters of rodent hepatocarcinogenesis (28). Inhibition of apoptosis as a mechanism of PPARα promotion of liver carcinogenesis was recently supported by a study on cultured hepatocytes. Treatment with Wy-14,643 resulted in apoptosis which was prevented when cultures were transfected with a dominant negative PPARα with a mutation in the ligand binding domain (29). The authors suggest that PPARα may cause apoptosis in hepatocytes by inducing the release of a soluble factor such as tumor necrosis factor (TNF)-α.

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A limited amount of research has shown an association of potent PPAR ligands with inhibition of extrahepatic cancers. Original studies by Abou-El-Ela et al. (30), O'Connor et al. (31) and more recently by Reddy et al. (21) suggest that two components of fish oil, the n-3 polyunsaturated fatty acids eicosapentanoate and decosahexaenoate, are potent activators of PPARα and inhibit tummorigenesis in mammary gland, pancreas and colon (21, 30, 31). Furthermore, linoleate and conjugated linoleate are two groups of fatty acids that activate PPARα (34), and inhibit carcinogenesis in rodent mammary gland, colon, forestomach and skin (32-33, 35-38). In vitro studies suggest that these fatty acids have varying but strong affinities to PPARα and to PPARγ (39). Thus, anti-tumor promoting fatty acids may inhibit carcinogenesis via PPAR activation and these effects could be modulated by the molar ratios of each PPAR isoform.

A role for PPARγ in colonic cancer was recently suggested since a significant increase of PPARγ mRNA and protein was found in rat colonic tumors induced by azoxymethane as compared with normal surrounding tissue (40). In addition, the PPARγ ligand troglitazone reduced tumor growth of transplantable prostate tumors derived from PC3 cells (41). In human colon cancer (CaCo-2), transfected with a PPRE-luciferase reporter plasmid and treated with 15Δ-PGJ2, luciferase expression was induced two fold. It was suggested that elevated levels of PPARγ in colonic tumors may explain why the NSAID prostaglandin synthesis inhibitors are chemoprotective in skin and colon carcinogenesis, i.e. they reduce the level of the prostaglandin ligands for PPARγ activation. In colon cancers, increase in PPARγ expression was associated with differentiation and surprisingly, PPARγ1 was found in higher abundance in tumors when compared to PPARγ2. The significance of PPARγ1 increase in cancer is not known since no function or role has been documented. However, the authors (40) suggest that expression of PPARγ1could be used as a marker of tumor progression.

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There is also evidence that PPAR and their ligands may play a role in skin cancer. Tumor promoters activate phospholipase A2 (24), one of the rate limiting steps in fatty acid release and subsequent metabolism. Due to substrate specificity and relative abundance in membrane phospholipids, the most prevalent fatty acids released are arachidonic and linoleic acid. Arachidonate can be metabolized to prostaglandins (PG) or hydroxyeicosatetrenoic acid (HETE) by cyclooxygenases (COX) and lipoxygenases (LOX), respectively. Linoleic acid can also be metabolized by LOX to hydroxyoctadecadienoic acid (HODE). As described previously, several of these metabolites, referred to as eicosanoids, have been shown to be potent activators of PPARs (42) and some of them have been associated with proliferation, differentiation and inflammation in mouse skin. For example, PGE2 is critical for 12-O-tetradecanoylphorbol-13 acetate (TPA)-induced hyperplasia (43). Inhibition of PG synthesis with non steroidal anti-inflammatory drugs (NSAIDs) results in tumor development in the skin, as well as colon models of carcinogenesis (24). Interestingly, some NSAIDs such as indomethacin, which block prostaglandin synthesis by inhibiting COX-1 and COX-2, have also been shown to act as ligands and activators of PPARγ (44). LOX inhibitors also inhibit skin tumor promotion. The linoleate metabolite 13(S) HODE is a newly identified high affinity ligand for PPARγ and is expressed at high levels in the skin. In addition, 13(S) HODE affects the expression of keratins involved in differentiation in murine keratinocytes (45) and it can reverse inflammation associated with psoriasis (a common skin disorder) in guinea pig epidermis (46). The mechanisms by which specific lipids contribute to tumor development while other lipids seem to have a protective effect is not well understood. Activation of transcription factors such as PPAR may represent one converging pathway linking cellular processes involved in cell differentiation, proliferation, inflammation and cancer with lipids that are known

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to modulate these processes. A variety of compounds, including several naturally occurring polyunsaturated fatty acids [linoleic acid (33) and conjugated linoleic acid (32)], eicosanoids (24), and analogs of retinoic acids (47), inhibit mouse skin tumor promotion. Because several of these compounds are known ligands and activators of PPARs, PPARs may represent a critical link between action of these compounds on skin carcinogenesis and on downstream altered gene expression.

Recent studies have suggested a role for PPARs in keratinocyte differentiation (48, 49). Addition of PPARα ligands (clofibrate, oleate, linoleate or eicosatetraynoate) to human keratinocytes cultured in low calcium non-differentiating media, induced the expression of involucrin and transglutaminase, two gene markers of keratinocyte differentiation. In addition, clofibrate was as effective at enhancing differentiation if cultures were grown in high calcium, differentiation-inducing media. The PPARγ ligand, prostaglandin (PGJ_2), on the other hand, had no effect. Furthermore, consistent with the effect of differentiation on proliferation, DNA synthesis was markedly reduced in clofibrate-treated cultures. This suggests a regulatory role for PPARα in keratinocyte growth and differentiation (49).

Based on these studies, it seems likely that PPARs may play a role in skin carcinogenesis. One particular interest will be to determine whether PPARγ and/or PPARα play a role in skin differentiation and tumor prevention. One of the first steps will be to determine the patterns of PPAR expression and activity in the epidermis after single or multiple treatment with tumor promoters and at specific stages of carcinogenesis. Papillomas and carcinomas can easily be generated by treatment of mice with the phorbol ester tumor promoter TPA (32). In addition, several well established keratinocyte cell lines derived from papillomas and carcinomas could be examined for their PPAR levels and activity. Although PPARs appear to be elevated in skin cancer (personal

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communications J. Vanden Heuvel; Pennsylvania State University) it is not known whether this elevation of PPARs levels is accompanied by an increase in transcriptional activity on specific target genes yet to be determined. However, one of the effects of TPA in the epidermis is to stimulate arachidonate release from phospholipids. Subsequently these phopholipids are metabolized via cyclooxygenase and lipoxygenase pathways, generating metabolites such as PGF2α and PGE2. Interestingly, it has been long known that while PGE2 inhibits TPA-induced tumor promotion, PGF2α enhances tumor production by 60% (24). Hence, the recent reports that PGF2α inhibits PPARγ activation in adipocyte (18, 19) suggest that an elevation of PPARγ in skin tumors could correlate with an attempt of the cells to compensate their inability to differentiate by increasing PPARγ expression. Because PPAR ligands have been shown to induce differentiation of multiple tissues including: adipose tissue, breast cancer, prostate cancer, macrophages and monocytes (22, 25, 30, 35, 41) via a PPAR mediated mechanism, it is anticipated that a similar effect could take place in skin. Therfore, if PPARs can induce differentiation of cancer cells it wouldl be of interest to determine whether this effect can be preventive and might also contribute to a reversal of tumor establishment. Generation of transgenic PPAR mice with expression targeted to the skin, using a loricrin or keratin promoter (specific to the superbasal layer of the epidermis), could provide answers to these questions. It would be expected that if PPARs play a preventive role in tumor development, these transgenic mice would be resistant to typical TPA tumor promotion protocols. Inversely, a transgenic with a dominant negative PPAR targeted to the skin would be expected to have an increased sensitivity to tumor promoters and an apparition of tumors earlier than in wild type animals. In these type of studies, it would be important to record the changes of proliferation markers (such as BrdU labeling index, cell number),

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differentiation (expression of keratin, loricrin, filaggrin etc.), and inflammation (expression of lipid metabolizing genes) in either transgenic models. It would also be important to determine the influence of specific PPAR ligands on these events. Of particular interest would be to determine the mechanistic basis of dysregulation of PPAR expression and activity in epidermal carcinogenesis.

The continuing growth of reports involving PPARs has broadened our understanding of their role and mode of action in the cell. Originally limited to a role in lipid metabolism and adipocyte differentiation, PPARs are now believed to exert their actions in multiple tissues and have more recently been associated with several pathological conditions including cardiovascular disease and cancer. In addition, these reports have generated an overwhelming enthusiasm in the field, forecasting more exciting discoveries in the future regarding the roles and function of PPARs.

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V APPENDIX.

V.1 Additional figures

-Figure II.1B: PPARγ protein expression in undifferentiated and differentiated 3T3-L1 cells.

-Figure II.2B: Total PPARγ levels during adipocyte differentiation (quantified)

-Figure II.2C: Total PPARγ levels during adipocyte differentiation (summary)

-Figure II.3B: PPARγ mRNA levels during adipocyte differentiation (graph)

-Figure II.3C: a-FABP mRNA levels during adipocyte differentiation (graph)

-Figure II.4C: Relative cytosolic and nuclear PPARγ levels during adipocyte differentiation (graph)

-Figure II.4D: :a-FABP protein levels during adipocyte differentiation (graph)

-Figure II.4E: Changes in a-FABP cytosolic content.

-Figure II.6D: Relative cytosolic and nuclear RXRα and a-FABP levels during adipocyte differentiation (western blot)

-Figure II.7B: RXRα mRNA levels during adipocyte differentiation (graph)

-Figure II.8C: Gel shift and supershift of cytosolic, nuclear extracts and in vitro translated PPARγ and RXRα. Preimmune control.

-Figure II.8D: Products of in vitro translation of PPARγ and RXRα.

-Figure II.8E: Inhibition of ARE shift by anti- PPARγ_{4-26} antibody but not by anti-PPARα, anti-PPARδ or preimmune serum.

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Figure II.1B PPARγ protein detection in undifferentiated (U) and differentiated (D) 3T3-L1 cells with anti-PPARγ $_{32-54}$ or anti-PPARγ $_{4-26}$ antibody. 50 μg of total protein were loaded per lane and 5 μl of in vitro translated PPARγ2 (5 ng) was loaded in the lane designated TNT. (This figure represents the entire gel from figure II.1). The band detected in the TNT reaction with a molecular weight of approximately 40-45 Kda could be a proteolytic truncated form of PPARγ2 containing the antigenic N terminal of PPARγ2. The band detected by anti-PPARγ $_{32-54}$ and designated γ1 likely represents the PPARγ1 isoform of 52 Kda. Alternatively, it could represent an intermediate form generated by proteolysis of PPARγ2 which would migrate at the same expected size of PPARγ1. However, the fact that the PPARγ1 isoform is not detected with anti-PPARγ $_{4-26}$ antibody (lanes 3 and 4) suggests that it is missing the N-terminal first amino acids containing the PPARγ2 specific antigenic domain, which is in agreement with PPARγ1 bonafide structure. The PPARγ2 form detected in differentiated cells (lane 4) migrates as a doublet which likely corresponds to the inactive phosphorylated and active dephosphorylated form of PPARγ2. Note that the smaller (active) PPARγ2 form is not detected in the undifferentiated cells (lane 3).

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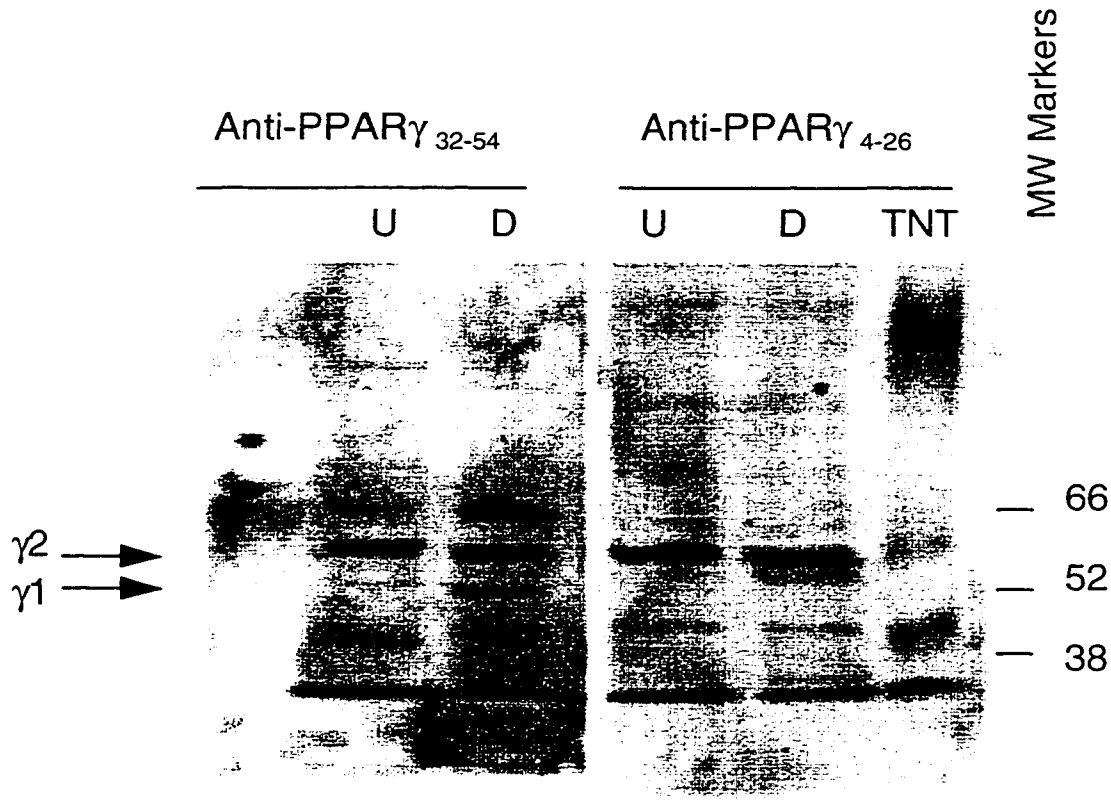

Figure II.1B. PPARγ protein detection in undifferentiated(U) and differentiated (D) 3T3-L1 cells with anti-PPARγ $_{32-54}$ or anti-PPARγ $_{4-26}$ antibody (*see legend opposite page*)

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B

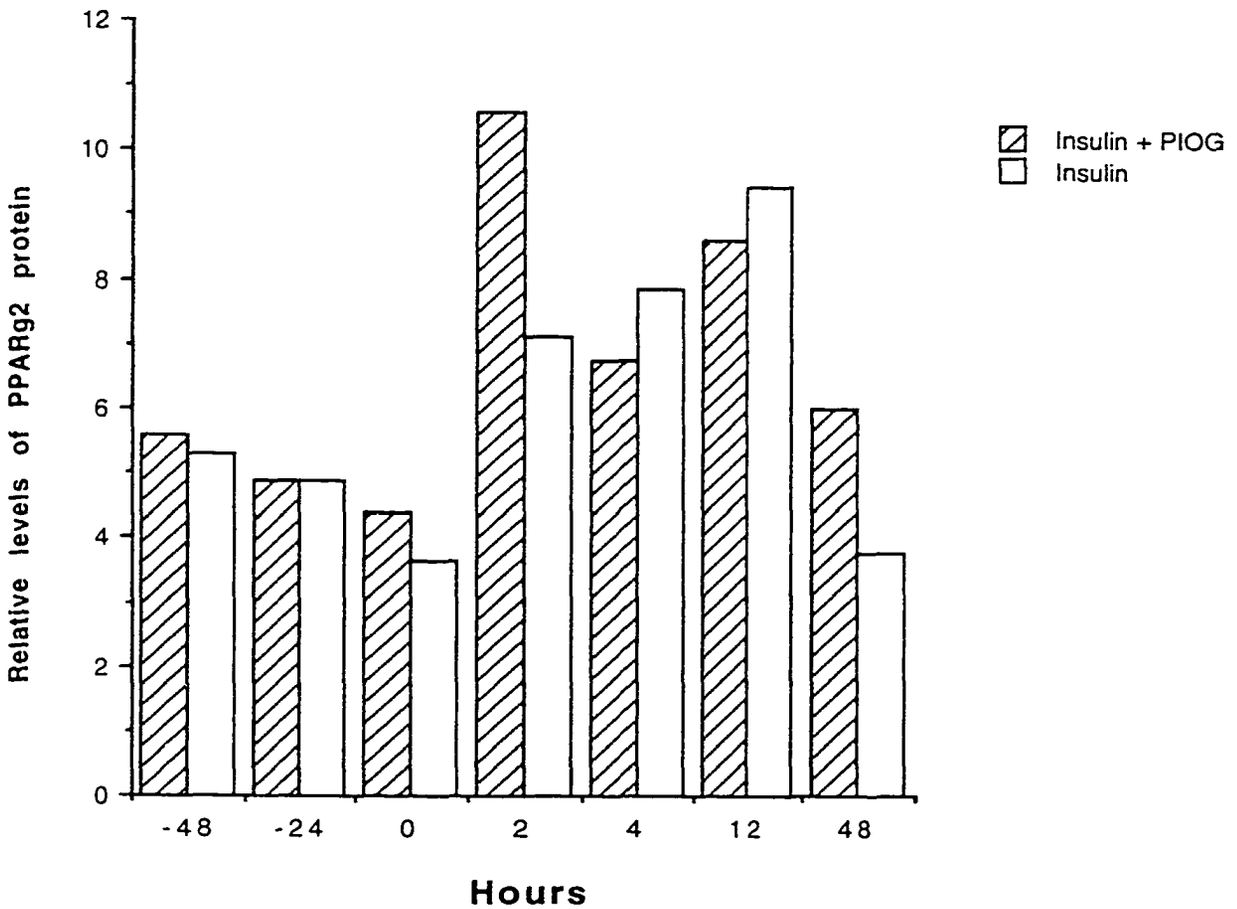

Figure II. 2B. Total PPARγ2 levels during adipocyte differentiation. Western blot shown in figure II.2 was captured optically using a video camera linked to a computer. The image was then analyzed using the Ambis program, and each band detected on the gel was quantified according to its intensity.

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C

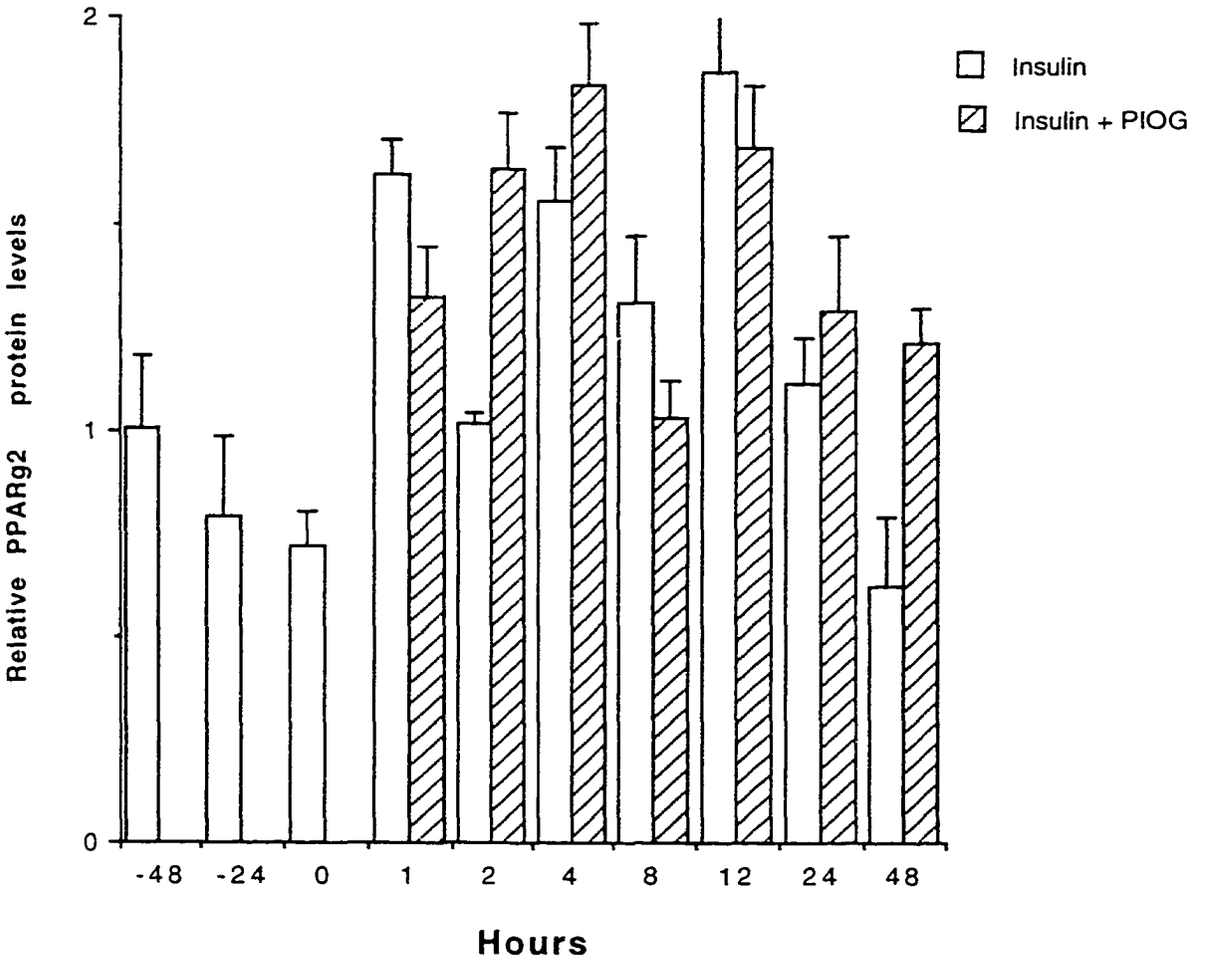

Figure II. 2C. Total PPARγ2 levels during adipocyte differentiation. Western blots from seven different experiments were captured optically using a video camera linked to a computer. The image was then analyzed using the Ambis program, and each band detected on the gel was quantified according to its intensity. The intensity of the band detected at 0h was arbitrarily given the value 1 and values from other bands were calculated relative to the value of the 0h band. Each data point represents the mean ± SD of at least three separate determinations

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Figure II. 3B-3C. Increases in PPARγ2 and a-FABP mRNA abundance during the early stages of preadipocyte differentiation. Undifferentiated 3T3-L1 cells were grown to confluence (-48 h) and staged to differentiate as described in METHODS (Chapter II). After a 48 h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin or insulin plus 10 μM pioglitazone. Total RNA was extracted at the hours indicated in the figure, and the Northern blot was sequentially hybridized with cDNAs for mPPARγ2, a-FABP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The intensity of the bands shown in a representative Northern in figure II.3 were quantified using the Ambis program. Loading errors were corrected relative to GAPDH and were normalized to the -48h value for PPARγ2 mRNA (fig II.3B) and to the 0h value for a-FABP mRNA (fig II.3C) (i.e., the 0h value was arbitrarily given the value 1 and values from other bands were calculated relative to the value of the 0h band). Each data point represents the mean ± SD of 2-4 individual experiments.

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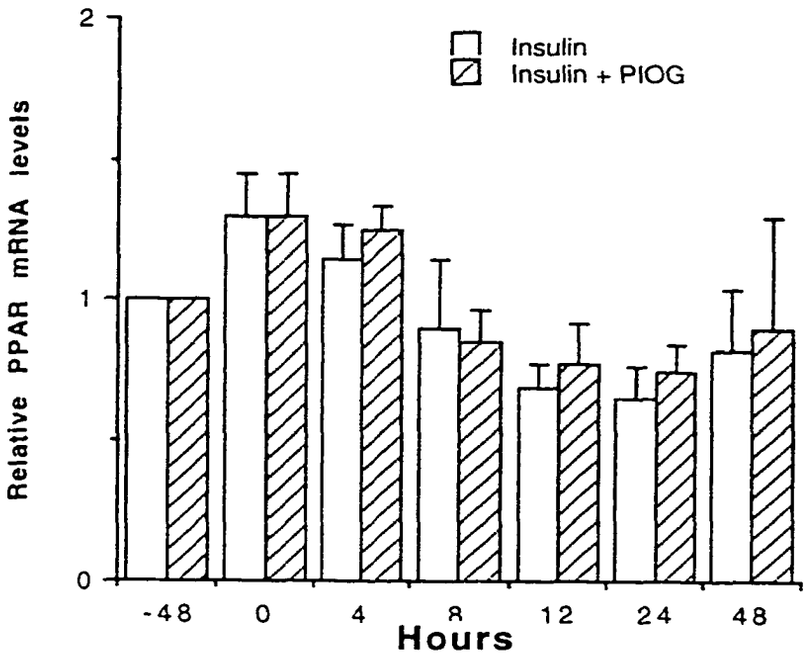
II-3B



II-3C

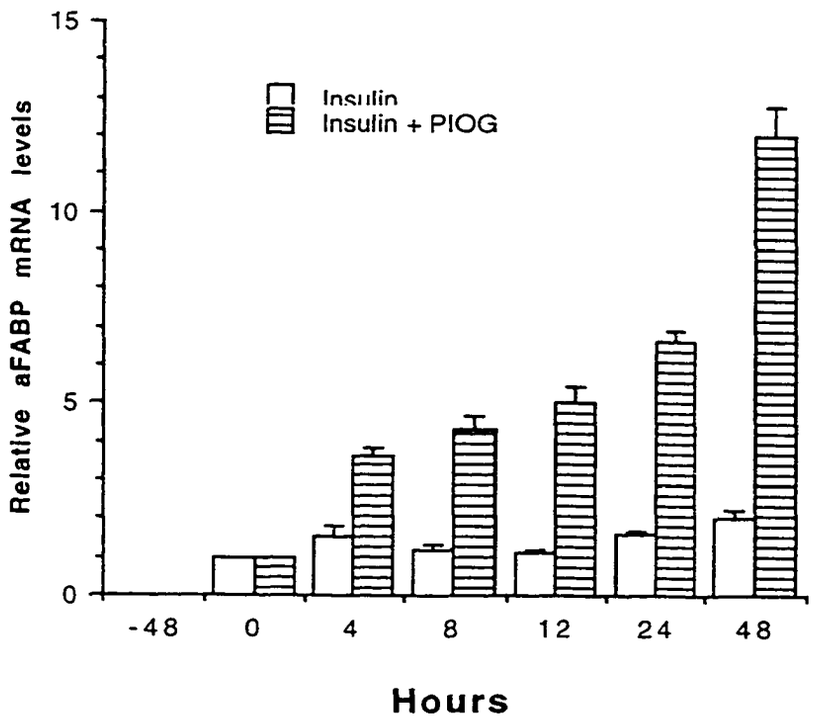


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Figure II.4C-II.4D. Changes in nuclear and cytosolic content of PPARγ2 protein during the conversion of 3T3-L1 preadipocytes to mature fat cells. Undifferentiated 3T3-L1 cells were grown to confluence (-2 days) and staged to differentiate as described in METHODS (Chapter II). After a 48 h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin, or insulin plus 10 μM pioglitazone. At the days indicated in the figure, cytosolic and nuclear protein extracts (10 μg per lane) were prepared for Western blot analysis of PPARγ2 using anti-PPARγ $_{32\text{-}54}$. The graphs represents the relative nuclear (4C) and cytosolic (4D) PPARγ2 levels from the Western blot shown in figure II. 4A.

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Figure II.4C Nuclear PPARg2 levels

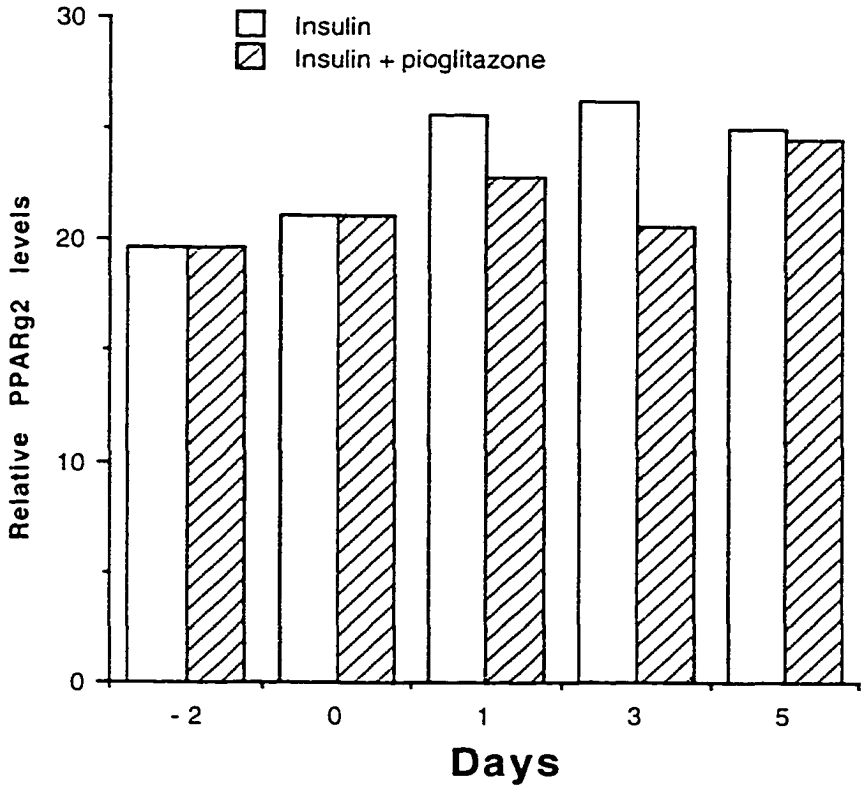
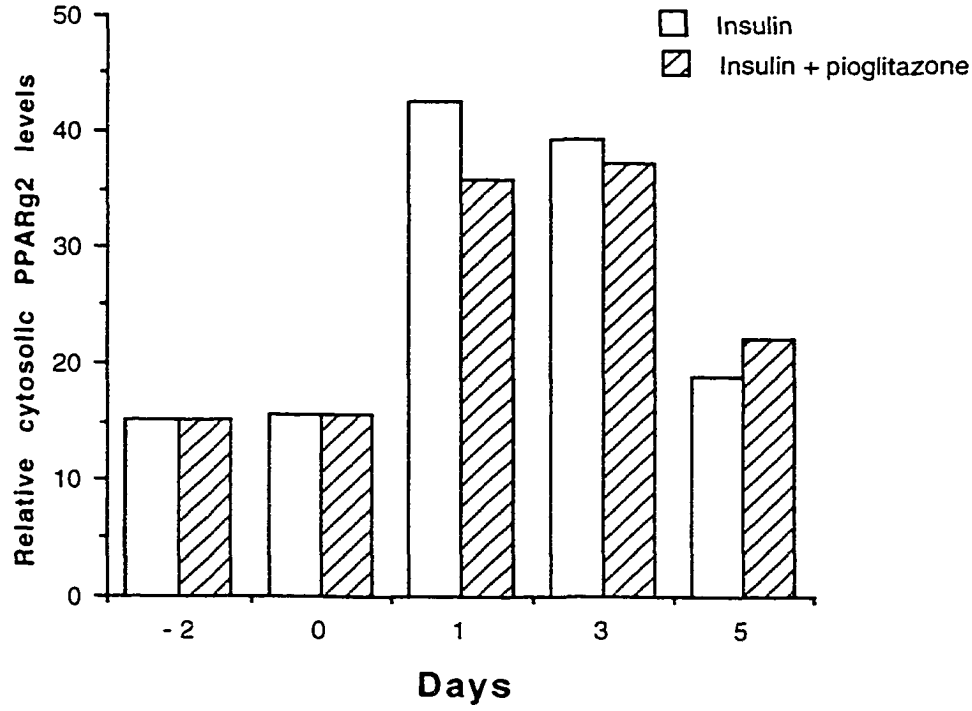


Figure II.4D Cytosolic PPARg2 levels



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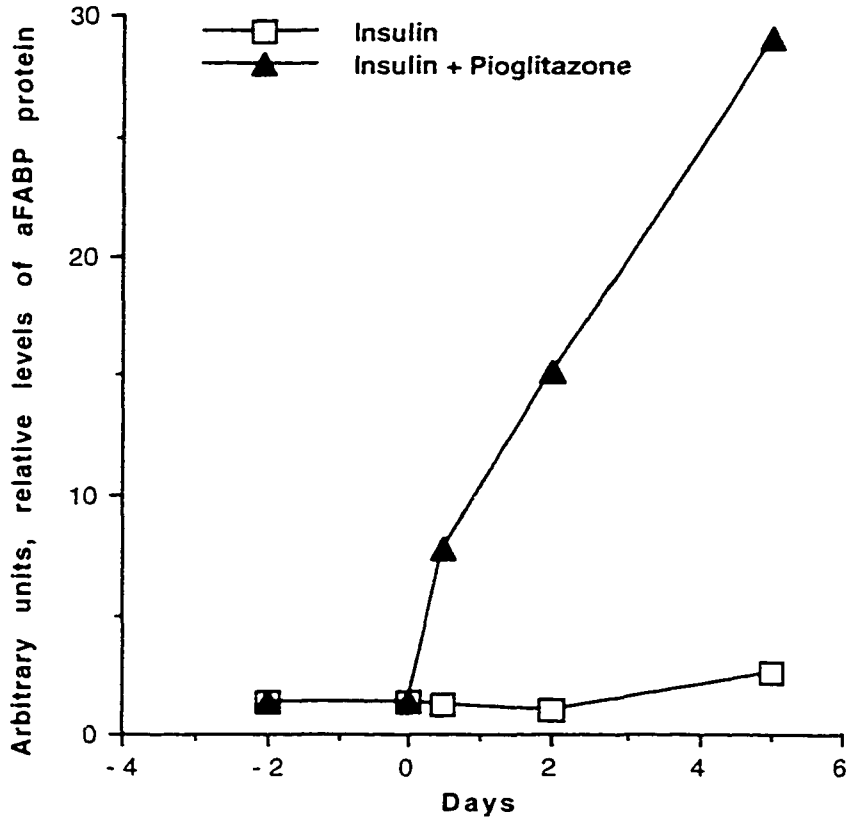

Figure II.4E. Changes in a-FABP cytosolic content during the conversion of 3T3-L1 preadipocytes to mature fat cells. Undifferentiated 3T3-L1 cells were grown to confluence (-2 days) and staged to differentiate as described in METHODS (Chapter II). After a 48 h staging period with IBMX and dexamethasone (0 days), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin, or insulin plus 10 μM pioglitazone. a-FABP protein abundance was determined at the days indicated in the figure. The graph represents the relative cytosolic a-FABP levels from the Western blot shown in figure II. 4B.

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Figure II.6D. RXRα and a-FABP protein levels in nuclear and cytosolic fractions during adipocyte differentiation. Nuclear (lanes 2-5) and cytosolic (lanes 7-10) extracts from 3T3-L1 preadipocytes induced to differentiate with insulin were prepared at the time indicated: preadipocytes (lane 2 and 7 , -48h); post-staged cells (lanes 3 and 8, 0h); 1 day (lanes 4 and 9, 1d); 3 days (lanes 5 and 10, 3d); 10 ng TNT in vitro transcribed/ translated RXRα (lane 1); molecular weights indicated in Kda on the left side of the figure were determined from overnight exposure of the ^{14}C-MW markers (lane 6). Proteins (75 μg) were separated on a 12% acrylamide SDS gel and electroblotted on PVDF membrane. Top panel, the membrane was immuno-blotted with anti-RXRα antibody (1/500). After stripping it, the membrane was then immunoblotted with anti-a-FABP antibody (1/1000), bottom panel. As shown, RXRα was only detected in the nuclear fractions and a-FABP was only detected in the cytosolic fractions.

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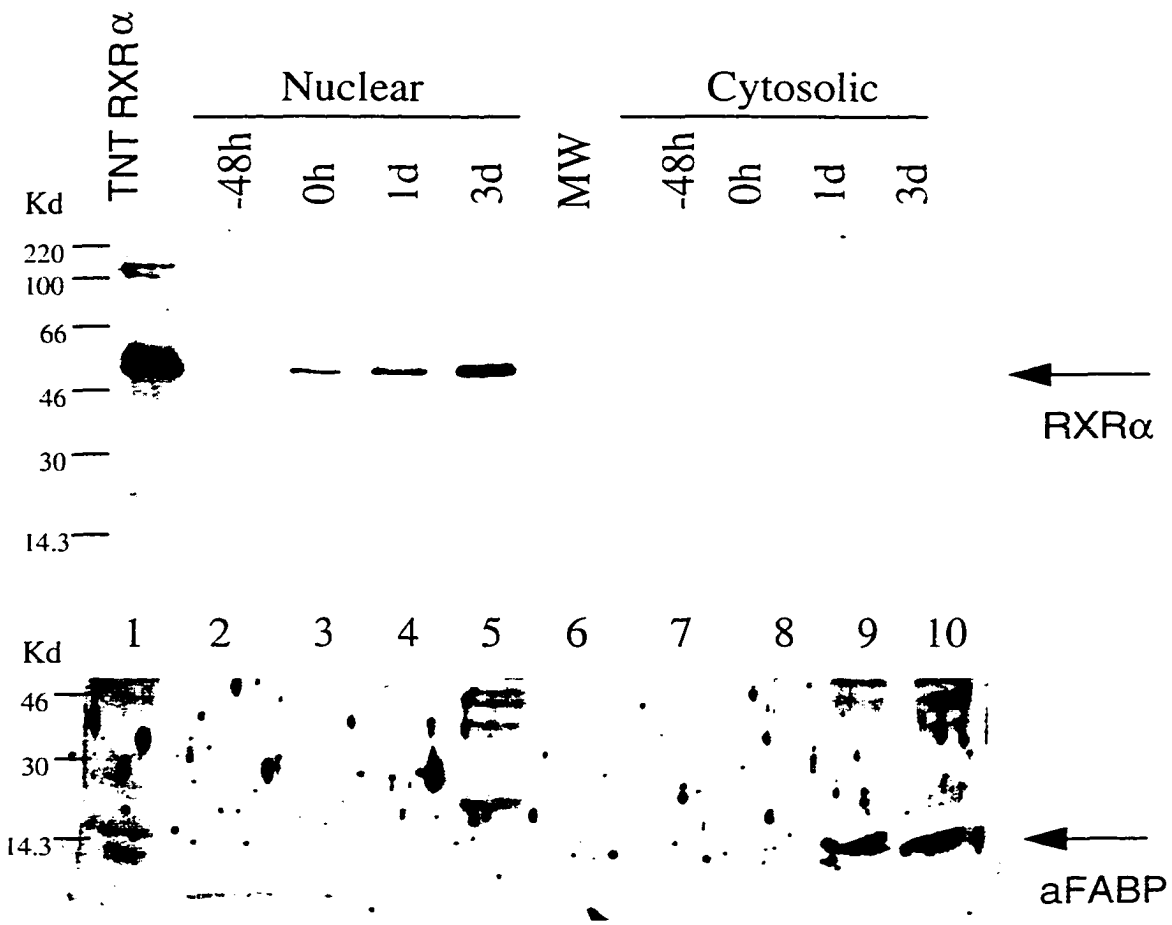

Figure II.6.D. RXRα and aFABP protein levels in nuclear and cytosolic fractions during adipocyte differentiation. (*See legend opposite page*)

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Figure II. 7B. RXRα mRNA abundance during the early stages of preadipocyte differentiation. Undifferentiated 3T3-L1 cells were grown to confluence (-48 h) and staged to differentiate as described in METHODS (Chapter II). After a 48 h staging period with IBMX and dexamethasone (0 h), the cells were induced to differentiate by changing the media to one containing either 1 μM insulin or insulin plus 10 μM pioglitazone. Total RNA was extracted at the hours indicated in the figure, and the Northern blot was sequentially hybridized with cDNAs for RXRα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The intensity of the bands shown in a representative Northern, in figure II.7, were quantified using the Ambis program. Loading errors were corrected relative to GAPDH and were normalized to the -48h value (i.e., the 48h value was arbitrarily given the value 1 and values from other bands were calculated relative to the value of the 48h band). Each data point represents the mean ± SD of 2-4 individual experiments.

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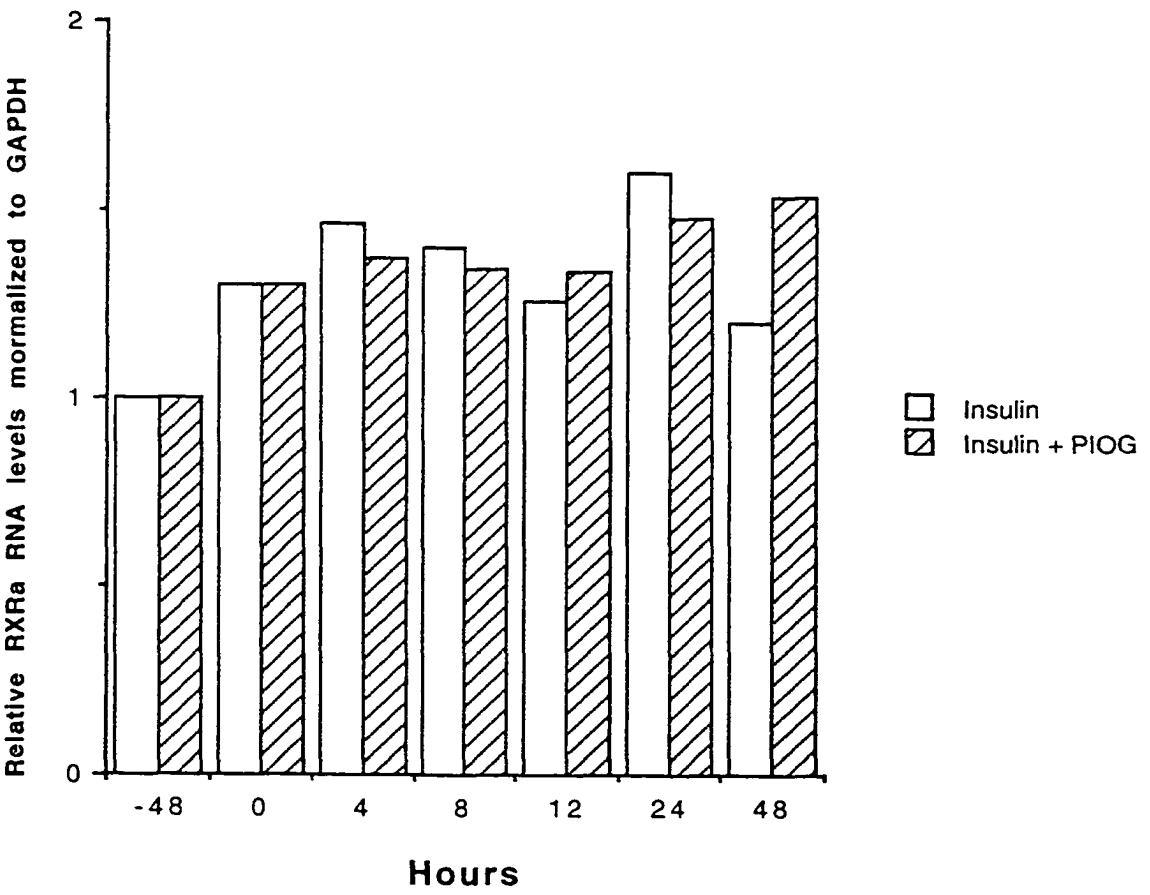

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Figure II.8C. EMSA with cytosolic, nuclear extracts and in vitro translated PPARγ and RXRα, and supershift with anti-PPARγ antibody. 2.5 μg of cytosolic (C) or nuclear (N) extracts isolated from differentiating 3T3-L1 cells treated with insulin (Ins) (lanes 2, 3, 6, 7) or insulin + piog (Ins/P) (lanes 4, 5, 8, 9) were incubated with a γ^{32}P radiolabeled ARE oligonucleotide in the absence (lanes 2-4) or presence (lanes 6-9) of anti-PPARγ antibody (1 μl). In lanes 10-12, 5 μl of in vitro translated PPARγ and RXRα were incubated with the γ^{32}P radiolabeled ARE (lane 10) or with 1 μl preimmune serum (lane 11) or with 1μl anti-PPARγ antibody (lane 12). The complexes formed were resolved on a 4.5% acrylamide gel which was dried and exposed to film overnight. An arrow (shift A) indicates the shift generated with nuclear extracts as depicted in figure II.8B. Interestingly, cytosolic extracts were able to bind to the ARE (lanes 2 and 4), and the protein-DNA complex formed appeared to migrate faster than the shift obtained with nuclear extracts (lanes 3 and 5). Presence of anti-PPARγ antibody in the binding reaction resulted in the migration of an additional complex of slower mobility (supershift) and was detected with cytosolic and nuclear extracts as indicated by an arrow (lanes 6-10). In vitro translated PPARγ and RXRα (from pV-Sport1-PPARγ2 and CMV-RXRα as described in figure II.8D), generated a shift (lane 10, shift TNT) which migrated with the same apparent mobility of the nuclear shift (lane 3 and 5). Addition of anti-PPARγ antibody produced a supershift (lane 12) co-migrating with the supershift detected with cytosolic or nuclear extracts. Addition of preimmune serum had no effect (lane 11). An arrow at the bottom of the gel indicates the location of the free probe (free). NS, non specific binding.

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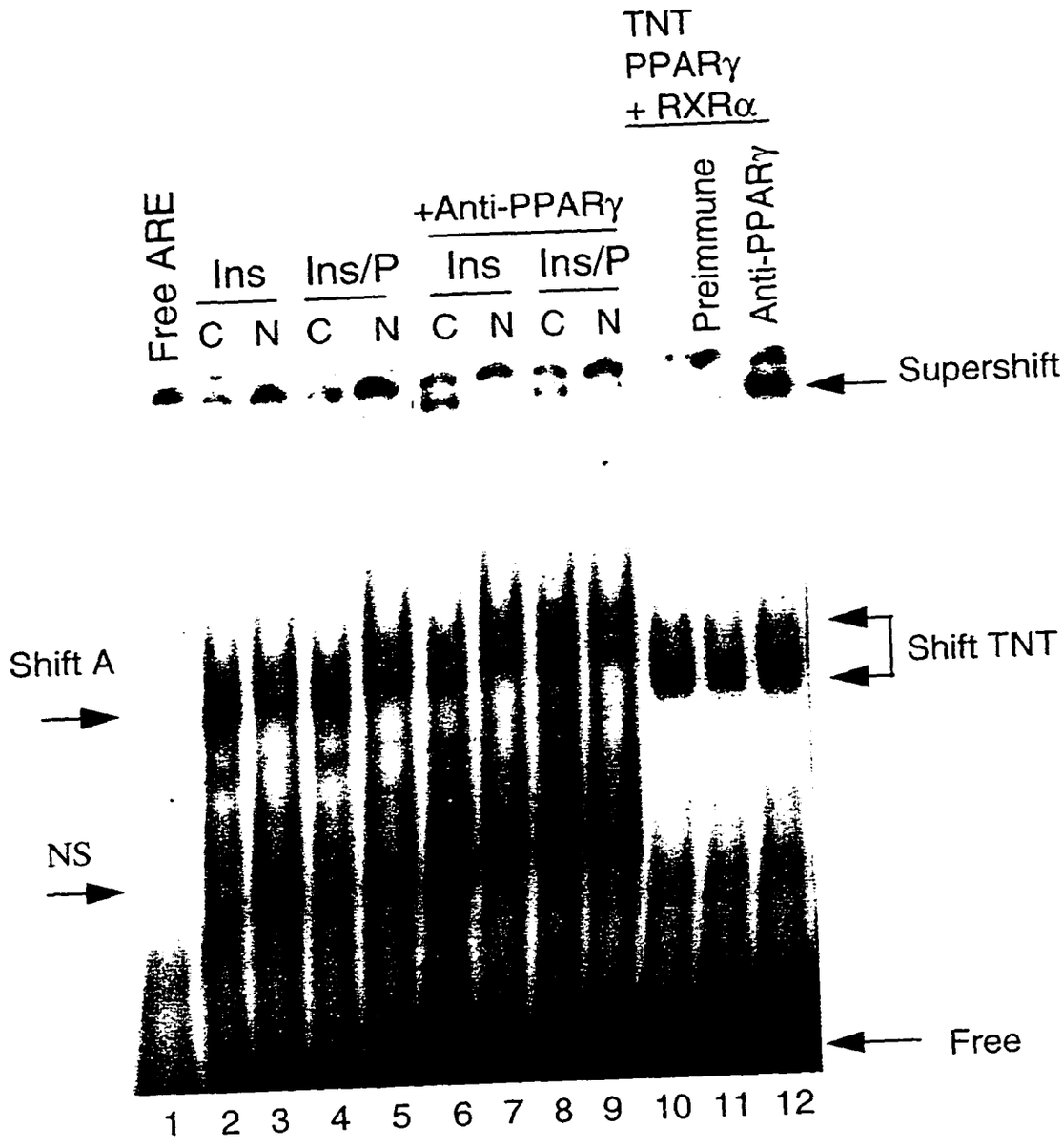

Figure II.8C. EMSA with cytosolic, nuclear extracts and in vitro translated PPARγ and RXRα ,and supershift with anti-PPARγ antibody .(*See legend opposite page*)

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Figure II.8D. In vitro translation of PPARγ and RXRα. PPARγ and RXRα were translated in the presence of ^{35}S-Methionine with the TNT in vitro transcription/translation kit (Promega). PPARγ (lane 1 and 4) was generated from the pV-Sport1-PPARγ2 plasmid in the presence of SP6 RNA polymerase. RXRα was generated from either a pV-Sport1-RXRα or a CMV-RXRα plasmid in the presence of SP6 (lane 2) or T7 (lane 3) RNA polymerase respectively. The Luciferase protein (Luc) of molecular weight 62 Kda was also produced in the presence of either SP6 or T7 RNA polymerase (lane 5 and 6 respectively). Five µl of each translation reaction (containing approximately 5 ng of translated product), was run on a 12.5% acrylamide SDS gel as indicated in the figure (except lane 4 which had 10 µl of TNT translated PPARγ). The gel was dried for 20 minutes at 65°C and exposed to a film for 2 hours. The two major products translated from the pV-Sport1-PPARγ2 plasmid, PPARγ2 (γ2) and PPARγ1 (γ1), are indicated on the left of the figure.

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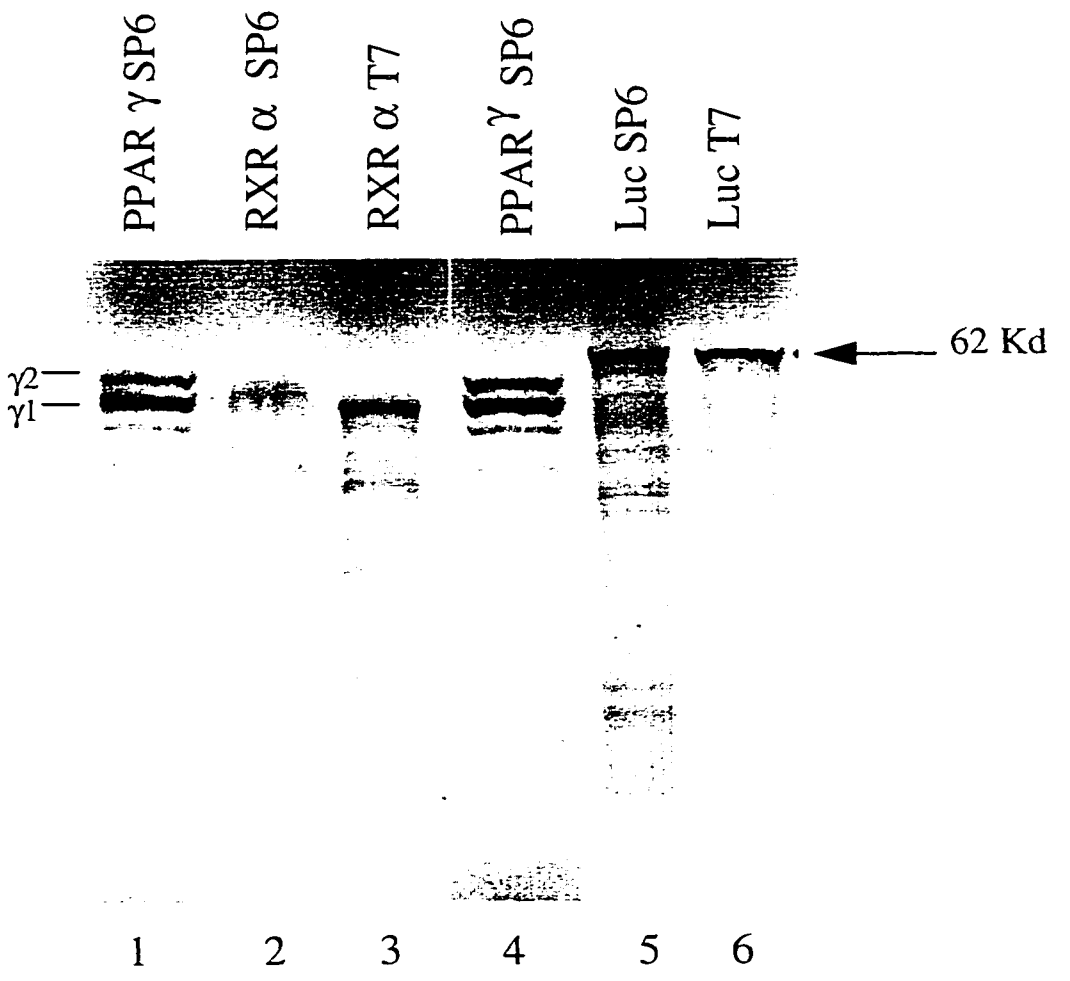

Figure II.8D. In vitro translation of PPARγ and RXRα. (*See legend opposite page*)

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Figure II.8E. Anti-PPARγ $_{4-26}$ antibody, but not anti-PPARα , anti-PPARδ antibodies or preimmune serum, inhibits PPARγ shift Undifferentiated 3T3-L1 cells were grown to confluence and staged to differentiate as described in figure II.8A. Nuclear protein extracts were prepared from confluent untreated, undifferentiated 3T3-L1 cells (lane 2), and fully differentiated (10 days of pioglitazone and insulin treatment) 3T3-L1 cells (lanes 3-10). Nuclear extracts were incubated with preimmune serum or antibodies against PPARα, PPARδ, or PPARγ2 for 2 hr (2 hr Ab, lanes 4-7) or overnight (o/n Ab, lanes 8-10) at 4°C prior to the incubation with ^{32}P-radiolabeled ARE. PPARγ2 interaction with the ARE (DR-1) of the a-FABP gene is depicted in lane 3; treating the nuclear extract with 1 μl of anti- PPARγ2 $_{4-26}$ for 2 hr decreased binding by 50% (lane 7) and nearly eliminated the shifted band after overnight incubation (lane 8). However, treating the nuclear extract with preimmune serum (lane 4), anti-PPARα antibody (lane 5, 10) or anti-PPARδ antibody (lanes 6, 9) had no effect on the PPARγ2 shift. Lane 1 depicts the free probe. NS, non specific binding.

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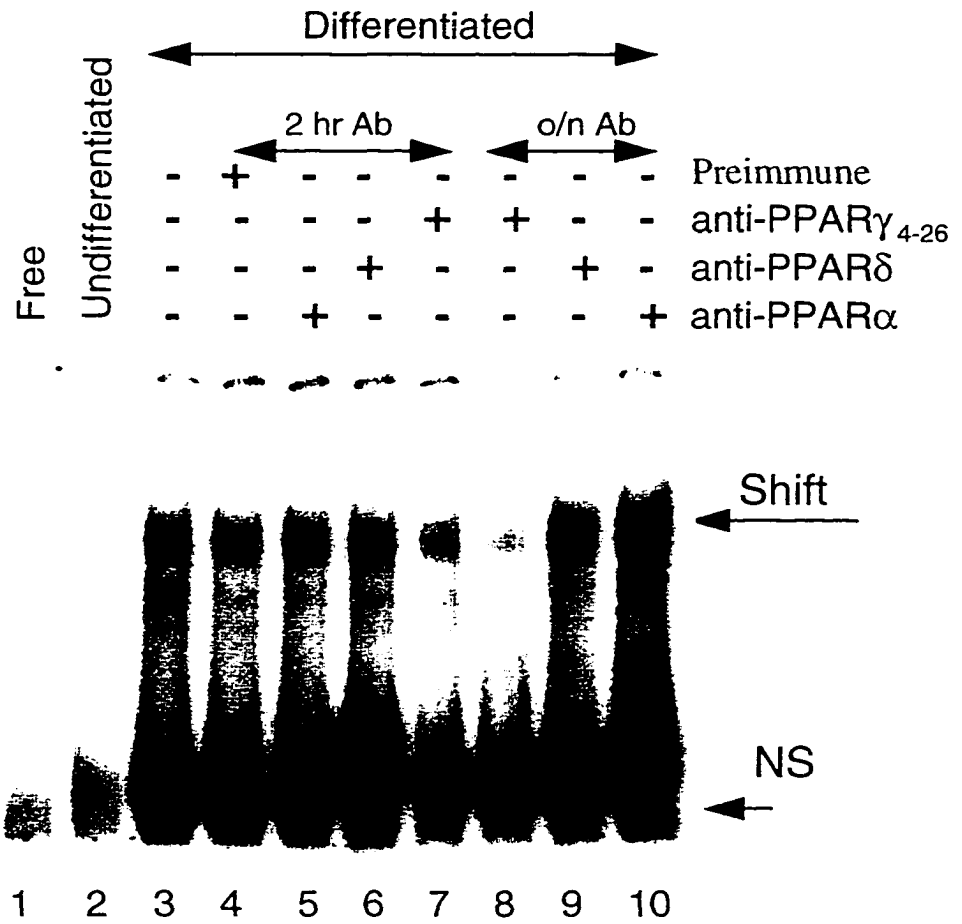

Figure II.8E. Anti-PPARγ$_{4-26}$ antibody inhibits PPARγ2 binding to the ARE (DR-1) of the adipose response element from the a-FABP gene. (*See legend opposite page*)

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V.2 Sequencing of the 5' flanking region of the a-FABP gene

The following sequence represents the 5' flanking region of the a-FABP gene from bases -8146 to +98. The region of this gene spanning from -8146 to -869 bp was sequenced using the Sequenase DNA Sequencing Kit 2.0 (USB). Detection was achieved by ^{35}S-labeling or by silver staining using the Silver Sequence DNA sequence system (Promega) of the -8146 to +98 bp 5' flanking region of a-FABP cloned into the PGEM-3Z plasmid (Promega). The sequence determination was performed between November 1992 and July 1995. Two original primers oriented toward each other were designed at the 5' end (primer #1, -8146 SP6 primer from the plasmid sequence) and in the -869 bp region of the gene (primer#5, -806 to - 822) as shown on map1. Two additional primers were made upon the publication of the sequence from the ap2 enhancer (-5400 -4900)(Ref 12 Chapter I), with one primer going toward the 5' end (primer #3, -5368 to -5386) the other toward the 3'-end of the a-FABP 5' flanking sequence (primer #4, -4973 to -4955). Each primer allowed the sequencing of about 250 to 350 bases. New sequences were entered manually in a computer and overlaps were determined using the Mac Dnasis program. From the obtained sequence, a new primer was designed using the Mac Dnasis program in a region located between 30 to 60 bases, 5' from the last base determined (as shown for primer #2). In most cases the 5' sequence obtained with this new primer overlapped the last bases of the 3' sequence obtained with the previous primer. In some cases no overlaps were obtained and a new primer located further 5' of the newly determined sequence was designed. This strategy was applied from four different initial starting points within the a-FABP 5' flanking sequence and going toward the following directions: 1) from -8146 toward the

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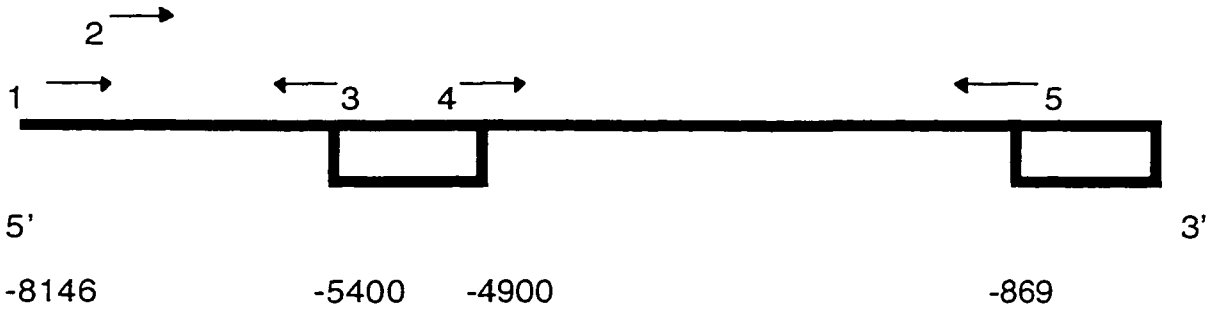
3'-end, 2) from -5400 toward the 5'-end, 3) from -4900 toward the 3'-end , 4) from -806 toward the 5'-end.

The entire sequencing was achieved by the contribution of the following people under Philippe Thuillier, Steve Clarke and Rebecca Baillie's supervision and training:

Wendy Crow: 1993-1994 Sequence from -8146 to -7200 , from -6400 to -5400, from -4900 to -4200, from -3500 to -863

Amy Blake: 1994-1995 Sequence from -7200 to -6400 and -4200 to -3500



Map 1

The start site is indicated by the symbol " ↑ " (ATAGCA)

5'

-8146 GTGCTC ATAGATCAGT CTGTCAGCGC TGGGAAAGGA TGCTGGCGCT

-8100 TCCCTTGACT CACCCAAGAG AGGAAAAAAC CCACATTTTA TAAAAACATG

-8050 TTCCCGTTGT AGCAAAGGGT AAGAAACAGG CCTGCACAGA GGACGTTTCC

-8000 TTGGGCTGCT ACTCTTTCCT CTGAGAACGG AAAGAAACGC TACCACCGGA

-7950 AGTAACGAAA GAAACACTAC ACAATAAGCA ACACAGTGGG ACTGCTGGCC

-7900 CCCTCTCCCA GTCCAATGTA AATGTATTCT AAAGAATAAG TTTAAAACAG

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-7800 CTGACTTCAA TATAGAAATG AGTTTGCAAA TGGTATTTTT TTTTAACCAA

-7750 ATGCTTTAAA TGATTTTTTT TTCCAGAAAA AAAGGTGGGA CAAACAAAAT

-7700 TCCTAATCAA CTTATAGCAA ATGATGCCTA GCTCTTAGCA CACATTTGAC

-7650 AATGAACGGT TGATTCGGAT GTTATAAGAA GGTGGGCTGA TTTCGCCTTA

-7600 GATGTGACTC CATGGTGATT TCTGTTAGCA ATATGTTAGA CATTGACTAC

-7550 ATCTACATCA TCTAACTACC TCTAATCTAA CTCTTATCTA ATAATGTGTG

-7500 GGTGTGATGG TGCTGGTGGT CTGATCCCAG CCATCACTCA GGAAGCATGA

-7450 AGCAGGCTGT TCTAGGTAAA TTCAAGGCCA GCCTGGATCT ACATAGTTTT

-7400 AGAATAGCCA GAGCTTTATA GTGAGACACG CAAAACAAAT GAATAAATAA

-7350 AATCCCTGGA ATCCAGAAGT ACTTCACAGA TTGGCTTTTG GGGAGTGGAG

-7300 TCAATCGTTT GCCTCCATAT GTAAACAATG AGCTCTCAGG AGAGTGGGAT

-7250 GGAAGTCTAA GGAGAAAGTG CTGTTCCCAA TGCATGTATA TCTCCCACAC

-7200 AGAACCCGAA GGAACTTTGT AGGACAGTTT TGAAGGTGCC AGGCCCATGC

-7150 AGGCTGTGTG AGCTGCTACA TGAGGTGGTG GTTATGTGAT TTCCACCTGT

-7100 GGAGACACAC TGGCACTAAG AAAACTTTGC AATGGTAATA TTTTAGATGT

-7050 TGAAATGTGA GAATAGAGAA CCCCAGCCTA TGAGYAGCAG TTTTCAATAG

-7000 ATATTACCAA AGACGTTTGA TAGTGATTAT GCTTTCTTGA TAGGTCTGTG

-6950 GAGAATGCCT GAGACCTCTG GAAAGGCAAA CACATTCTTC TTGGTTCATC

-6900 GCACATTGCT TCACACACTG CTGGTCTTAG GCTTCTTGGT GTGTACAGCA

-6850 TAGGAAGGGG GTGTCATAAA GGTGGCCTGG GATTGGTGGA TTTTTTGGCC

-6800 CGATCTGGAG CAAGCTCAGG AATTGGTAGG ATTTTGTGGC TTCAGCTTGG

-6750 CCACCTGACT TGAGGGTATG GAAACCACTG CTATATCAGT TAGGGTCGTC

-6700 TACGGGTGGG GCCTGGCCAG TCATACCTTA AGGGGCTTAC ACATCTGCTG

-6650 GGATAGCCAG AGAGACACTG CTATTTTTGA AAGCATTTCA AACATCCCCA

-6600 TTTGGGGCCT GGAGATGTAA CTAAATGCTG ??GTATTTTC TCAGCCTGTA

-6550 CAGGGTCCTA GCTCAGTCAT TACAGATGAT GTACCTTGCC GAGGTGTCAG

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-6350 ACGTCCCCAA CCTTGGGGGA AAATAACTCT TATTCTGATT CATGCCCATC

-6300 CTGACAATTT GTTGTGTATT TCATTAATTT CTTGAAGTTG TTCTCAAGAT

-6250 GTGAAGAATG TCCTCTCACG CCGCAGTCAC CAGTGCAGAG AACCCAGTCC

-6200 TCTGAAAGTC TAAGACCACA GAGATTGTTC AAGCATGGGT ATTTTGGG?A

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-5950 CAGCTGAAGG AAGGGGAGTG TTGTCGATAT CAATCAGCCA GCTAAGGTTT

-5900 GCAAGCTCGT GAGCAGGGGA GAAGAATTTC AGCGCGTCTA ACCCCAGGGT

-5850 TTGAGGATCT TTCTAGCCTG TGATGTGATT TATAATACAA TTAACAAAAT

-5800 ATGTCACAGG CATCTTATCC ACCCACTGAC TGTAAATGTG AATGCAATGG

-5750 CACTTCTAGT GTATTGGTGT CTTTAGAGAA CAGAATTCCA GCAGGAATCA

-5700 GGTAGCTGGA GAATCGCACA GAGCCATGCG ATTCTTGGCA AGCCATGCGA

-5650 CAAAGGCAGA AATGCACATT TCACCCAGAG AGAAGGGATT GATGTCAGCA

-5600 GGAAGTCACC ACCCAGAGAG CAAATGGAGT TCCCAGATGC CTGACATTTG

-5550 CCTTCTTACT GGATCAGAGT TCACTAGTGG AAAGTGTCAC AGCCCAAACA

-5500 CTCCCCCAAA GCTCAGCCCT TCCTTGCCTT GTAACAATCA AGCCGCTCCT

-5450 GGATGAACTG CTCCGCCCTC TGTCTCTTTG GCAGGGTTGG AGCCCACTGT

-5400 GGCCTGAGCG ACTTCTATGG CTCCCTTTTC TGTGATTTTC ATGAGTTTAC

-5350 TGAGCTCTTT TCCCCCGCTT TATGATTTTC TCTTTTTGTC TCTCTCTTGC

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-5000 ACTCAGAGTG TCTAAGATTA CAGGCAGAGT GGTTCCTGTG TCTGTTCTCT

-4950 AGCCTTTC**TC TGGA**AACCCA CACAAGCTGT TTCTCCTACT AGAATGCCAC

-4900 CGTTTACATA GTGGTGGCCT GATTTCTACT TTCAGAACCC TTGAGCAGTT

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-450 TAAACA CTAT TCTAACAAAA GAGCCATTAA GACTAAGAAT CTCTAAGATA

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-150 CAAAGTTAGA AAATTTCTAT TAAAAACATG ACTCAGAGGA AAACATACAG

-100 GGTCTGGTCA TGAAGGAAAT GATCTGGCCC CCATTGGTCA CTCCTACAGT

-50 CACATGGTCA GGGCATCTTT AAAAGTGAGC TATCTGGACT TCAGAGGCTC

0 ↑ATAGCACCCT CCTGTGCTGC AGCCTTTCTC ACCTGGAAGA CAGCTCCTCC

+50 TCGAAGGTTT ACAAAATGTG TGATGCCTTT GTGGGAACCT GGAAGCT T +98

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