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**Genetic Manipulation of the Adipose Tissue via
Transgenesis**

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Title:

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TRANSGENESIS**

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A. Abstract

The long term goal of this study was to reduce caloric and fat content of beef and other red meats by means of genetic modification of the animal such that fat would not be accumulated. This was attempted by introducing into the germ line myogenic regulatory genes that would convert fat tissue to skeletal muscle. We first determined the consequences of ectopic expression of the myogenic regulatory gene *MyoD1*. It was found that deregulation of *MyoD1* did not result in ectopic skeletal muscle formation but rather led to embryonic lethalties, probably due to its role in the control of the cell cycle. This indicated that *MyoD1* should be placed under stringent control to allow survival. Embryonic lethalties were also observed when the regulatory elements of the adipose-specific gene adipsin directed the expression of *MyoD1* or *myogenin* cDNAs, suggesting that these sequences are probably not strong enough to confer tissue specificity. To determine the specificity of the control elements of another fat specific gene (adipocyte protein 2-aP2), we fused them to the bacterial β -galactosidase reporter gene and established stable transgenic strains. The expression of the reporter gene in none of the strains was adipose specific. Each strain displayed a unique pattern of expression in various cell lineages. A most exciting results were obtained in a transgenic strain in which cells migrating from the ventro-lateral edge of the dermomyotome of developing somites to populate the limb buds with myoblasts were specifically stained for lacZ. Since the control sequences of the adipsin or aP2 genes did not confer fat specificity in transgenic mice we have taken both molecular and genetic approaches as an initial effort to identify genes important in the conversion of a multipotential cell such as C3H10T1/2 cell to adipoblast. Several novel adipocyte cell lines have been established that differ in the expression of transcription factors of the C/EBP family known to be markers for adipocyte differentiation. These studies revealed that one of the genetic programming changes which occur during 10T1/2 conversion from multipotential cell to a committed adipoblast is the ability to induce C/EBP α gene expression. It is expected that further analysis of

this gene would identify elements which regulate this lineage-specific expression. Such elements might be good candidates in future attempts to convert adipoblasts to skeletal muscle cells in vivo.

B. Objectives

The major objective of the proposed research was to alter the fat content of farm animal, by introducing into the germ line of animals myogenic determination genes (that are able to convert in vitro adipocytes to skeletal muscle cells), and target their expression to the adipose tissue. Thus, the expected reduction in fat tissue will be associated with an increase in muscle content. We planned to study the biological function of and in vivo, using transgenic mice. One way to ascertain the developmental role of / as well as the interaction between the different myogenic regulatory genes, is to alter their regulation and to study the developmental consequences of having them expressed in tissues or stages where they are not normally active. To this end, three sets of experiments were proposed:

1. Construction of fusion genes containing the full length mouse / cDNAs behind an adipocyte-specific promoter sequences.
2. Examine the ability of the constructed genes to convert in vitro cells of different embryonic origin to skeletal muscle cells.
3. Analyze the physiological consequences of / expression in the adipose tissue of transgenic mice.

C. DESCRIPTION OF THE SCIENTIFIC FINDINGS

I. In vivo studies:

1. Ectopic expression of leads to embryonic lethalties.

Before attempting any genetic manipulations in the adipose tissue, it was important to determine the tolerance of mouse embryos to ectopic expression of. The idea was that if mice can tolerate expression of this gene in non-skeletal muscle tissues, it would indicate that its function is not dominant.

The results of this study are described in detail in manuscript #1.

. Attempts to produce transgenic mice expressing the and cDNAs driven by the adipsin promoter.

Adipsin is mainly synthesized in adipose tissues and is found in the bloodstream. This protein, the expression of which is sharply down-regulated in several models of rodent obesity, has properties suggesting it may be a systemic regulator of lipid metabolism and energy balance. As a first step in targeting the expression of *MyoD1* and *myogenin* to the adipose tissue, we have cloned the adipsin promoter (Platt et al., 1989; kindly provided by B. Spiegelman) next to the full length cDNAs. As expected, transfection of these constructs into the multipotential 10T1/2 cells resulted in their conversion to adipocytes. The DNA fragments carrying each fusion gene were microinjected into mouse fertilized eggs. Forty five offspring were analyzed by southern blots of tail DNA for the presence of the injected DNA. None were transgenic. The most likely interpretation of these results is that the promoter sequences employed lack negative regulatory elements that suppresses transcription in the inappropriate tissues, and therefore, allows the ectopic expression of myogenin and *MyoD1* leading to embryonic lethalties as described above. Alternatively, the adipsin promoter is tissue specific but the myogenic conversion of adipocytes to skeletal muscle cells is not compatible with normal development. These experiments demonstrated that to be able to manipulate

the adipose tissue in vivo, we need to define regulatory DNA elements that are specific to this tissue. The identification and characterization of these elements is quicker achieved in vitro, in adipogenic and non-adipogenic cells in culture than in vivo in transgenics.

. Analysis of transgenic mice carrying the 5.4 kb of the aP2 promoter in front of the β -galactosidase reporter gene.

The adipose conversion of pre-adipocyte cells (oB17, 3T3-L1 and 3T3-F442A) represents a valid model to delineate in vitro the development of adipose tissue in vivo. This process is accompanied by a significant transition in the cellular pattern of protein synthesis that reflects the transcriptional activation of adipose-related genes. In that respect, differentiation of adipocytes can be divided into early and late events that are regulated differently. Early events are triggered by growth arrest and characterized by the activation of a set of genes, among which are pOb24 and lipoprotein lipase genes. Terminal differentiation is characterized by the activation of aP2 and GPDH, that probably play important roles in adipose cell physiology. The cytosolic aP2 protein is an adipocyte-specific lipid-binding protein as suggested by its ability to bind fatty acid.

A 5.4 kb DNA fragment of the promoter region of the aP2 gene was isolated in the laboratory of Bruce Spiegelman. It was shown that several binding sites for the transcription factors c-jun and C/EBP as well as short sequence elements found in other adipocyte gene promoters (termed fat-specific elements) are found in close proximity to the transcription initiation site (Graves et al., 1991). We therefore, decided to examine whether these sequences would confer adipose-specific expression to the bacterial β -galactosidase (lacZ) reporter gene in transgenic mice. The use of the lacZ reporter gene can provide a three-dimensional picture of promoter activity along with the high resolution of the β -gal signal, allowing detection of promoter activity at sites that contain low levels of transcripts and in few cells.

The activity of the 5.4 aP2lacZ construct was first tested by transient transfection assays in a variety of adipocyte and other cell types (e.g., 3T3 L1 and 10T1/2). Activity was significantly higher than a promoterless lacZ plasmid. However, it showed no apparent specificity. The construct worked about as well in non-adipocytes (10T1/2) cells as in 3T3L1 fat cells. Since transient transfection assays not always reveal cell type specific expression, we used these same sequences to generate stable transgenic strains. Four independent transgenic strains were produced. They carried 2-10 gene copies of the introduced gene.

Transgenic founder animals were mated to normal mice and analyzed for lacZ expression at various developmental stages (Fig. 1B-I):

(1) Mice of transgenic strain 5.4aP2-1. Several patterns of staining were observed in 11.5 days p.c. embryos (Fig. 1B) most likely reflecting multiple integration sites:

a. A basic pattern of staining that included all the groups of stained cells that was found in all other embryos of this strains. This pattern was characterized by a strong staining in axial structures such as the spinal cord (from the level of the hind limb buds to the bottom of the hindbrain) and somitic derived cells (all sclerotomal cells along the entire body and single spindle shaped cells in the central part of the myotome). Staining was also found in the head region: the mesenchyme or brain wall, the roof of the midbrain and less so in the forebrain. Strong staining was found in the mesenchyme adjacent to the eye and adjacent to the nasal cavity.

In addition to the above stained regions, prominent mesenchymal staining was found in other parts of the body including the maxillar and mandibular processes and the anterior half of the forelimb buds. Interestingly, stained cells were found also in the endodermal lining of the digestive tract but not in cells lining the tracheal analge.

b. The second pattern of stained embryos of this strain was very similar to the above but without stained cells near the eye and different staining in the limb buds.

c. In these embryos the basic pattern was essentially very similar but without stained cells in the endoderm or sclerotome.

d. The basic pattern was very similar but the number of stained cells in the somites was considerably smaller and there appeared a strong staining in the neck region posterior to the otic vesicles.

(2) Embryos of the 5.4aP2-2 strain. At 11.5 days p.c embryos displayed only a weakly stained spot in the region of the neck (Fig. 1F).

(3) 5.4aP2-3 embryos. Two staining patterns could be discerned (Fig. 1G,H):

a. A prominent staining in muscle. The staining in the fore and hind limb buds coincided with that obtained in mice transgenic for the enhancer-promoter of the myogenic regulatory gene fused to lacZ. Interestingly, more stained cells were found in thoracic and abdominal somites compared to cervical and lumbar somites. Stained cells could also be found in the neck and mastication muscles. Expression in the neural system included cells in the spinal cord and the brain (Fig. 1G).

b. In these embryos there were less or even no staining in the pre-muscle cell masses. For example, no stained cells could be detected in the limb buds. However, stained cells were found in the anterior edge of the maxillar process and in a group of cells in the ectodermal apical region in both the fore and hind limb buds (Fig. 1H).

An example of lacZ staining in 16.5 days p.c. embryo is shown in Fig. 1I. In newborn mice of this strain staining was confined to the central nervous system. In addition, there was staining in cardiomyocytes in the atria and ventricles. We could not detect stained cells in the adipose tissue.

(4) Embryos of transgenic strain 5.4aP2-4. Here we performed a more detailed analysis of expression during development:

At the 18-20 somite stage (Fig. 2A) there were two prominent spots of stained cells around the ocular vesicles. These spots spread posteriorly and ventrally to the maxillar process. This characteristic 'mask' was preserved also at later embryonic stages. In the visceral arches stained cells could be seen in the hyoid arch and in the region that will develop to the third and fourth visceral arches. There were few stained cells in the ventral part of the four

branchial somites and two symmetrical rows of stained cells lying ventrally from somites that demarcate the dorsal aorta.

At 25 somite stage (9.5 days p.c.) (Fig. 1B) the pattern of staining changed completely from the previous one (there are only 5-10 hours difference between the two) by the appearance of stained cells (i) in the neural tube. (ii) in six somites lying above the fore limb buds (Fig. 2C) and (iii) in the dorsal part of the fore limb buds. The staining found previously in the hyoid arch disappeared.

At 10.5 days p.c. (32-35 somites) (Fig. 2D-F) the aorta staining disappeared. Small groups of stained cells were found in the ventral part of most somites except for cervical and tail somites. The stained cells in the fore limb buds assume the characteristic positions of the premuscle cell masses while at the hind limb buds the pattern depicts the process of premuscle cell repopulating of the limbs.

Additional specificity appeared in close vicinity of or within the wall of blood vessels in the head and neck regions. This pattern of expression in the wall of blood vessels is an interesting feature that is maintained at later stages and in the newborn transgenics (Fig. 2G-I). In newborn transgenics the staining is confined to arteries and most likely also to capillaries. Strong expression of the transgene was also found in stromal cells of the thymus, weaker expression was found in some muscle fibers and in terminal end buds within the parenchyma of the mammary gland. In all these analyses, we did not see expression in the adipose tissue.

The pattern of somitic and limb bud expression in transgenic strain 5.4aP2-4 at early stages of development is unique and follows very nicely the expected pattern of migratory myoblasts: those myogenic precursor cells that leave the somites to populate the limb buds with myoblasts. It is well established in avians, but not yet in mammals, that cells in the lateral portion of the somite are unique in that although determined, they do not express any marker specific for myoblasts. There is a need to define gene products that make these cells different from other types of myoblasts. The lacZ staining in this strain clearly provides a molecular marker for migratory myoblasts for the

following reasons: (1) the temporal and spatial pattern of expression is that expected of such cells. (2) They originate in epithelial cells of the ventral lateral lip of the somite. (3) The expression of the transgene is transient. (4) Cells expressing lacZ do not form fibers. In fact immunohistochemical analyses demonstrated that lacZ staining and desmin or muscle actin staining is mutual exclusive. (5) Cells expressing the transgene are present in the VLL of somites between the limb buds. However, these cells do not migrate anywhere. In vitro studies with isolated somites revealed that these cells do migrate when cultured in close proximity of developing limb buds. (6) There is a clear border for the activation domain at the anterior end. It is less clear at the caudal end.

Recent evidence implicate Pax-3 (a member of transcription factors containing paired domain) gene in the development of migratory myoblasts (Bober et al., 1994; Goulding et al., 1994; Williams and Ordahl, 1994). The striking difference between the pattern of expression of our transgene and Pax-3 is its restricted mode of expression. Pax-3 is expressed in paraxial mesoderm prior to segmentation, in the neural crest and in spinal cord. In the somite, expression is found in the prospective dermomyotome and later in the myotomes and myoblasts cells in the limb that are derived from dermomyotome. The fact that Pax-3 expression is not limited to muscle may indicate a more general role in migration.

This transgenic strain may be very useful in future studies for the isolation and characterization of the genomic sequences that flank the transgene. We expect that such studies will result in the isolation of a novel gene specific to this sub population of myogenic cells.

4. Analysis of transgenic mice carrying the aP2 enhancer in the opposite orientation in front of a minimal aP2 promoter fused to the lacZ reporter gene.

It has been reported that an enhancer element located between -4.9 and -5.4 kb upstream from the aP2 start site, when linked to a minimal aP2 promoter sequences (-63 to +21) directed very high level of chloramphenicol

acetyl transferase (CAT) expression specifically to adipose tissue in transgenic mice (Ross et al., 1990). These results indicated that this enhancer element is a major determinant of gene expression for the aP2 gene. To better characterize this element at the tissue and cellular level we have fused the aP2 enhancer (kindly provided by B. Spiegelman) to the lacZ reporter gene and produced four independent transgenic strain with this gene construct. Analysis of lacZ expression revealed three patterns of expression:

- (1) In transgenic strains EP-aP2-1 and EP-aP2-2 (two independent strains), lacZ stained cells were detected in many regions of the brain and in single stained cells in the spinal cord. No staining could be detected in any fat tissue.
- (2) In transgenic strain EP-aP2-3, lacZ stained cells were found only in the spinal cord. There was no staining in any fat tissue.
- (3) In transgenic strain EP-aP2-4, staining was found in the brain, spinal cord and in the heart (Fig. 1A). However, there was no staining in any fat tissue.

Summary of in vivo studies:

In contrast to our initial expectations, the available genetic elements involved in adipocyte cell differentiation (those of the two adipocyte specific genes adipsin and aP2) could not be employed to convert the adipose tissue to skeletal muscle. This is mainly due to the ubiquitous mode of expression of these elements during embryogenesis and their questionable role in the establishment of the adipocyte cell lineage. Therefore, the project had to deviate slightly from its immediate goals and define more critically the genetic programs responsible for adipoblast determination by establishing unique cell lines, as described in detail bellow.

II. In vitro studies:

Introduction

Multipotential embryonic cells exhibit choices during development that progressively restrict their genetic potential. These decisions are translated into patterns of gene expression unique to each differentiated cell. The 10T1/2 cell

line, which converts to stable muscle, adipocyte and chondrocyte lineages after treatment with demethylating agents, has been used extensively to identify genetic changes occurring when a cell is determined to a specific differentiation pathway (Taylor and Jones, 1979). We have used these multipotential cells as a model system to examine changes in gene expression which occur when cells are determined to the adipocyte lineage.

The genetic programming changes responsible for adipoblast determination have remained ill-defined since this work was initiated. Correlative studies have pointed to a temporal cascade of three members of the C/EBP transcription factor family as integral to the adipocyte differentiation process (Cao et al., 1991); however, their role in lineage determination has remained controversial. Recently, transfection experiments using the C/EBP gene family suggest that forced expression of either C/EBP α or β together with appropriate hormone treatment leads to the conversion of uncommitted cells to the adipocyte lineage (Wen Chen et al., 1995). We have taken both genetic and molecular approaches to investigate the molecular events which occur during determination to the adipocyte lineage. Through the isolation of several novel adipocyte cell lines, we have confirmed the status of C/EBP α as central to the identity of cells in the adipocyte lineage. Comparison of these adipocyte cell lines with both the multipotential parental 10T1/2 cells and with cells committed to an alternative lineage, the 10T1/2 derived 23A2 muscle cell line, has revealed differences in the expression of transcription factors known to be markers for adipocyte differentiation.

1. Isolation of novel adipocyte cell lines

We have found that the C3H10T1/2 cell line, even in the absence of azacytidine treatment, converts at a detectable frequency to adipoblasts as judged by the emergence of adipocyte colonies after treatment with differentiation hormones (1 in 600 colonies for insulin/dexamethasone treatment; 1 in 100 for indomethacin/ dexamethasone treatment). We present here the isolation and initial characterization of two novel adipoblast cell lines

selected from the C3H10T1/2 population through repeated differentiation treatment and subsequent cloning. A regimen employing insulin and dexamethasone treatment was used to isolate the 10T82A cell line (figure 1A). 10TM42 was cloned using a similar protocol with the exception that Indomethacin and dexamethasone were used in the differentiation paradigm (Knight et al., 1987). The standard protocol used for differentiating adipoblasts into differentiated adipocytes is presented in figure 1B. These cells appear morphologically similar to the parental 10T1/2 cells while proliferating; in contrast, differentiated 10T82A and 10TM42 cells exhibit characteristics morphologically similar to other differentiating adipocyte cell lines (figure 2 and 3 respectively). Both of these lines continue to display the adipocyte phenotype after differentiation treatment through numerous passages in culture. We conclude that the 10T82A and 10TM42 cells are determined adipoblasts which have undergone stable heritable genetic changes which render them capable of initiating the fat program under the appropriate differentiation conditions. Interestingly, no matter which hormone treatment was used in selection of the cell line, both of the subsequent lines were capable of differentiation in response to either set of hormones suggesting that determination to the adipocyte lineage results in acquisition of the signaling systems for both insulin and indomethacin. Alternatively, both these pathways may converge on a common set of intracellular signaling molecules.

We have found that the C30A azacytidine-derived adipoblast cell line differentiates at a low frequency when treated with standard differentiation media (Konieczny and Emerson, 1984). The equivalent azaC-adipoblast line TA1 (Chapman et al., 1987) obtained from another laboratory yielded muscle along with fat when treated with differentiation media. Therefore, we also isolated a new adipoblast cell line IB1 which converts to fat at a frequency approaching 100% based on morphological criteria such as accumulation of lipid droplets (figure 4) and staining with oil red O (data not shown). These cells are hormone-independent in their differentiation properties. In addition to morphological criteria, we have used northern analysis to determine biochemical differentiation using a probe for the adipocyte-specific aP2 gene

kindly provided by Dr. Bruce Spiegelman (Bernlohr et al., 1984) (data not shown). A summary of the differentiation properties of these novel cell lines and their method of isolation is presented in figure 5. These cell lines are valuable resources with which to explore adipoblast determination and subsequent adipocyte differentiation.

The 10T82A and 10TM42 cell line are expected to be highly related to the parental 10T1/2 cells; therefore, they may prove to be more useful tools in future cDNA selection strategies than azacytidine-derived 10T1/2 adipoblast cell lines which contain numerous genetic changes. These cell lines can be used to construct cDNA libraries which can then be subtracted of sequences present in the parental 10T1/2 cell line. The final library could be used as a resource for: (1) identifying genes capable of conferring the adipocyte potential through transfection studies. (2) characterizing adipoblast-specific genes individually and as a population, thereby developing markers for the adipoblast state and (3) screening for sequences containing structural domains conserved in a number of developmentally regulated transcription factors, such as the bHLH domain found in the myogenic regulatory factors.

We expect that this future approach will result in the isolation of novel genes specific to determined adipoblasts which can be used to investigate the molecular mechanisms responsible for lineage determination and transduction of the lineage choice during terminal differentiation.

2. Characterization of 10T82, 10TM42, and aza-adipoblast IB1: Background

Previous studies have implicated the C/EBP family of transcription factors (α , β , δ) in the adipocyte differentiation process (Cao et al., 1991). A depiction of these results derived from western blot analysis using the 3T3L1 adipocyte model system is shown in figure 6 and summarized below (Cao et al., 1991).

C/EBP δ is expressed early after differentiation treatment in 3T3L1 cells and appears to be directly regulated by glucocorticoids (Cao et al., 1991). Its limited expression pattern on Northern analysis led to a hypothesis invoking its

expression as perhaps the initiator of the adipocyte differentiation program. C/EBP β is also expressed early after hormone treatment and appears to be directly regulated by cAMP levels (Cao et al., 1991). 3T3L1 cells require treatment with IBMX, a phosphodiesterase inhibitor, for differentiation, a requirement not shared by the 10T1/2 adipocyte system. The numerous cell types in which C/EBP β is expressed seemingly precluded its involvement as a tissue-specific regulator of adipocyte differentiation. However, recent experiments suggest that C/EBP β is capable of initiating the adipocyte differentiation program in 3T3 cells not committed to the adipocyte lineage (Wen Chen et al., 1995). This result was not observed by another group performing similar, but not identical experiments (Freytag et al., 1994).

C/EBP α appears relatively late after hormone treatment has been initiated, which led to the suggestion that a temporal cascade of C/EBP transcription factors produces the final differentiated adipocyte phenotype. C/EBP transcription factor binding sites are found in many adipocyte-specific differentiation genes (Christy et al., 1989). C/EBP α has been convincingly demonstrated by several groups to be a prerequisite for adipocyte differentiation: (1) antisense experiments have shown that knockout of C/EBP α results in a block of the differentiation process (Samuelsson et al., 1992; Lin and Lane, 1992); (2) introduction of C/EBP α into adipoblast cells results in the initiation of the differentiation process (Umek et al., 1991; Lin and Lane, 1994); and finally (3) recent transfection experiments using C/EBP α expression vectors have identified C/EBP α as capable of initiating the adipocyte differentiation program in multipotential cells such as 10T1/2 cells (Freytag et al., 1994; Wen Chen et al., 1995). We were not able to successfully convert multipotential 10T1/2 cells to the adipocyte lineage using expression vectors for any of the C/EBP transcription factors (data not shown); however, such negative results are difficult to interpret. As it was known at the time of these studies that C/EBP β expression extends to many tissue types, we limited our analysis of these lines to studies with the other family members C/EBP α and C/EBP δ . The recent results of Wen Chen et al. (1995) suggest that the expression pattern of C/EBP β may be relevant to adipogenesis; future

experiments will be needed to characterize the expression pattern of C/EBP β in these lines.

3. C/EBP α expression in 10T1/2-derived adipoblast cell lines

Using western blot analysis, we have found that all differentiating 10T1/2 cell lines, 10T82, 10TM42, and aza-IB1, express C/EBP α (figure 7). In contrast, the parental 10T1/2 cells do not produce C/EBP α protein in response to differentiation hormones. Therefore, the ability to turn on the C/EBP α gene is correlated with conversion to the adipocyte lineage regardless of how the cell lines were initially selected.

There are some subtle differences amongst the cell lines in terms of C/EBP α expression. Both the 10T82 and the aza-adipoblast IB1 cell lines have hormone-independent expression of C/EBP α almost immediately after the cells withdraw from the cell cycle. The zero times on the figures refer to 2 days postconfluency (figure 7). In the 3T3L1 system, C/EBP α expression is observed only after several days of hormone treatment. Therefore, the spontaneous and azacytidine conversion process may have led to some deregulation of the normal controls on C/EBP α expression, perhaps bypassing the signaling systems normally required for its expression. However, one interesting difference does exist between these two lines; IB1 will accumulate lipid and take on the morphological characteristics of adipocytes in the absence of hormone while 10T82A, despite the deregulated expression of C/EBP α , will not differentiate in the absence of hormones. Thus, other molecular events are required for 10T82A differentiation in addition to C/EBP α expression. We speculate that demethylation of the genome brought on by azacytidine treatment leads to a continuum of adipocyte cell phenotypes from completely hormone-regulated where C/EBP α would remain under hormonal control to total hormone independence, such as in the IB1 line, where C/EBP α and other factors required for differentiation would no longer be under hormonal control. However, the dramatic differentiation phenotype of the IB1 cell line is still under cell-cycle control as C/EBP α is not expressed in this, or any of the cell

lines until confluence is reached. Note the lack of C/EBP α expression in protein prepared from proliferating IB1 cells (P in figure 7). This is not surprising as deregulated cell-cycle expression of C/EBP α would have precluded isolation of the cell lines as it is known that high level expression of C/EBP α leads to withdrawal from the cell cycle (Umek et al., 1991). Cells with this property would not be available for selection cloning.

The 10TM42 cell line expresses C/EBP α only in response to hormones; specifically indomethacin and dexamethasone together are required for strong induction of the C/EBP α protein. Surprisingly, C/EBP α expression drops between 2 and 6 days of hormone treatment, a result which contrasts with the results seen for the other cell lines where C/EBP α expression continues to increase as differentiation progresses. The significance of this result remains difficult to interpret. Perhaps these cells are using an alternate pathway to maintain their differentiation state. More experiments will have to be performed to fully assess the importance of this result.

4. C/EBP δ expression

C/EBP δ is inducible by insulin and dexamethasone in 10T1/2 multipotential cells (figure 8), suggesting expression of C/EBP δ is not sufficient to initiate the adipocyte differentiation program, at least in a multipotential cell. Further, there was no clear correlation amongst the lines between C/EBP δ expression level and extent of adipocyte differentiation. There also was no consistent correlation between C/EBP α and C/EBP δ expression amongst all the cell lines, although generally an inverse correlation was noted between high level C/EBP α and C/EBP δ expression.

10T82A expresses high levels of C/EBP δ after hormone treatment; in addition there is a small amount of C/EBP δ protein present two days postconfluency (day 0 in the figure) prior to hormone treatment. These cells also exhibited deregulation of C/EBP α expression. Characterization of the regulatory region of the C/EBP α gene has identified a potential C/EBP binding site, leading to speculation that auto-regulation or perhaps cross-regulation by other members of the C/EBP gene family may be involved in control of this

gene (Christy et al., 1991). Deregulated C/EBP β expression may be affecting the expression pattern of the C/EBP α gene in 10T82 cells or vice versa. However, this is not a universal correlation as the IB1 cells also have deregulated C/EBP α expression but do not exhibit corresponding deregulated C/EBP δ levels under these same circumstances. Further, the 10TM42 cell line express C/EBP δ protein, albeit the levels are very low, in the absence of hormones, but require hormone treatment for the expression of C/EBP α and differentiation. Only a slight induction of C/EBP δ occurs in response to Indomethacin and dexamethasone treatment.

In 10T1/2 cells, C/EBP δ expression is quite high and extends at nearly maximal levels through day 4 after initiation of treatment. In contrast, C/EBP δ expression decreases to near background levels in 10T82A cells by day 4. We speculate that the high levels of C/EBP α protein produced in 10T82A cells may negatively affect C/EBP δ expression. This would be consistent with the observation that C/EBP δ levels are only slightly inducible in IB1 cells (compare IB1s with the parental 10T1/2s) where C/EBP α is highly expressed. Whether negative feedback regulation by C/EBP α on the expression level of C/EBP δ is in fact operative awaits further experiments.

Finally, continued treatment with dexamethasone rather than withdrawal after two days slightly inhibits differentiation in the 10T82A and 10TM42 cells (data not shown), suggesting that continued expression of C/EBP δ may actually hinder progression of differentiation. As this protein has been shown to interact with C/EBP α binding sites and associate with all the C/EBP family members (Cao et al., 1991), it is possible that it competes either directly with C/EBP α for access to the promoters of differentiation genes or indirectly through dimerization.

The results indicate that the regulation of these two C/EBP family members is quite complex and differs between the various adipocyte lines.

4. Expression of C/EBP genes in muscle cells

10T1/2 cells provide a unique system in which to compare gene expression between cells that are derived from the same lineage but which have been determined to a particular differentiation pathway. The 10T1/2-derived adipocyte cell lines all shared the ability to express C/EBP α which is not found in the 10T1/2 parent cells. C/EBP δ expression is shared by both the adipocyte lines and by 10T1/2 cells, albeit at varying levels. We were curious as to whether 10T1/2 cells committed to a different lineage, such as the azacytidine-derived muscle cell line 23A2 (Konieczny and Emerson, 1984), would express any of the C/EBP genes. These muscle cells, in contrast to their parental 10T1/2 cells, express myogenic regulatory transcription factors, members of the basic helix-loop-helix (bHLH) gene family (Vaidya et al., 1989). We predicted that C/EBP α would not be expressed in these muscle cells as its presence had not been noted in this cell type before (Figure 9). However, we were surprised by our observation that C/EBP δ is no longer expressed in these committed muscle cells despite being expressed in 10T1/2 cells in response to differentiation hormones. This lack of C/EBP δ induction could be due to either the absence of transcriptional regulatory factors required for C/EBP δ expression or a result of the loss of required signaling systems which normally lead to C/EBP δ expression after treatment with differentiation hormones. Further, C/EBP β is present in the control 10T1/2 and 10T82A cells but is not expressed in the 23A2 muscle cells. The results indicate that lineage choice in the 10T1/2 system leads to altered gene expression patterns which involve both the acquisition and loss of specific sets of transcription factors. A summary of the expression patterns observed for the C/EBP genes in these experiments is presented in Figure 10.

5. Regulation of the C/EBP α gene in 10T82A adipocytes

C/EBP α expression was shared by the 10T1/2-derived adipocyte cell lines 10T82A, 10TM42, and aza-IB. In addition, C/EBP α is found in other

adipocyte cell lines such as 3T3L1 and 3T3F442A cells (Cao et al., 1991). Therefore, we hypothesized that the C/EBP α gene contained regulatory elements which mediated the tissue-specific and differentiation-specific expression of this gene in adipocytes. Further, we speculated that the determined adipoblast cell lines had acquired a lineage-specific transcription factor (or factors) which was directing C/EBP α expression in these cells (figure 11). This factor would not be expected to be present in the multipotential 10T1/2 cells.

To investigate this possibility, a series of reporter plasmids (figure 12) were constructed containing various amounts of the rat C/EBP α gene regulatory region extending from -5700 bps. to -300 bps upstream, to +130 bps downstream of the transcription start site (the rat C/EBP α gene was the generous gift of S. McKnight). The C/EBP α regulatory regions were placed upstream of either the CAT or the β -galactosidase reporter gene. Transient transfection analysis was performed with the C/EBP α CAT reporter plasmids in the 10T82A adipocyte cell line both with and without differentiation treatment (Figure 13). The highest CAT expression occurs with the most truncated C/EBP CAT reporter plasmid -300C/EBP. This level of expression is 2-3 fold above that seen with the control plasmid TKcat, a relatively weakly expressed promoter. Further, no differences in expression were found between cells maintained in control media and those shifted to differentiation media.

These results prompted us to examine the expression of these constructs in the parental 10T1/2 cells in an attempt to distinguish basal from tissue-specific expression. As seen in figure 14, there was essentially no difference between the expression of -300CEBP in the 10T1/2 cells and the 10T82A adipocytes. Other constructs including that containing the longest 5' regulatory region, -5300C/EBP, were also tested and similar results were observed between the two cell lines (data not shown).

Several interpretations of these results are possible. (1) The regions of the C/EBP α gene used in the constructs may not contain sufficient information to direct tissue-specific or differentiation-specific expression. (2) The conditions used for differentiation may not have been optimal for achieving high level

C/EBP α expression during transient transfection analysis. (3) Regulation of C/EBP α may rely on chromatin configuration and accessibility rather than tissue-specific transcription factors.

These possibilities may be addressed in future experiments. More of the C/EBP α gene will need to be cloned and tested for tissue-specific expression both in transient as well as stable transfection experiments. Expression of the reporter plasmids described here, as well as those containing additional sequence from the C/EBP gene, should be tested in transgenic mice as this system can yield results when tissue-culture systems fail. These experiments may also be used to investigate the role of chromatin configuration in C/EBP α expression. More extensive testing with the transient system should also be performed to perhaps better balance the requirements of a confluent system for expression (C/EBP α) and the necessity of growing cells for optimal transient expression.

Summary of in vitro studies:

We have used the multipotential C3H10T1/2 cell line as a model system to examine genetic changes that occur when embryonic cells are determined to a specific differentiation pathway. These cells convert to stable muscle, adipocyte and chondrocyte lineages after treatment with DNA-demethylating agents. We have taken both molecular and genetic approaches in our initial efforts to identify genes important in the conversion of a multipotential cell to an adipoblast. Several novel adipocyte cell lines have been established by selection for 10T1/2 adipocyte variants that exhibit unique differentiation properties. Comparison of these adipocyte lines with both the parental 10T1/2 cells and with cells committed to an alternative lineage, the 10T1/2-derived 23A2 muscle cell line, has revealed differences in the expression of transcription factors known to be markers for adipocyte differentiation. Using western blot analysis, we have found that all differentiating 10T1/2 adipocyte lines express C/EBP α , a transcription factor which binds to regulatory regions of fat-specific genes and is required for differentiation in 3T3L1 adipocytes. In contrast, neither the parental 10T1/2 cells nor the 23A2 muscle cells express detectable

C/EBP α in response to differentiation hormones. Therefore, one of the genetic programming changes which occurs during 10T1/2 conversion from a multipotential cell to a committed adipoblast is the ability to induce C/EBP α gene expression. Further work is being directed towards identifying elements on the C/EBP α gene which regulate this lineage-specific expression. Interestingly, 10T1/2 cells and 10T1/2-derived adipocytes share some common signal transduction properties as both express another marker of fat differentiation, C/EBP δ in a similar manner during hormone treatment; 23A2 muscle cells, however, have lost the ability to express high levels of C/EBP δ . These results demonstrate that lineage choice in the 10T1/2 system involves both the acquisition and the loss of specific sets of transcription factors.

D. References

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Figure Legends

Fig. 1. Expression of the bacterial β -galactosidase revealed by *in toto* histochemical staining of transgenic embryos.

(A) Transgene expression in 25 somites stage (9.5 dpc) embryo from strain EPaP2-1. Transgene is widely expressed throughout developing central nervous system. Some stained cells can be seen also within the heart.

(B - I) Embryos obtained from three founder males carrying the 5.4aP2-lacZ transgene. (B) Three 11.5 dpc embryos of strain 5.4aP2-1 displaying distinct patterns of expression, suggesting multiple integration sites of the transgene.

(C - E) Higher magnification micrographs of each embryo shown in (B). (D) The basic pattern of expression in strain 5.4aP2-1. Stained cells form two symmetrical rows lying along the ventral side of the neural tube, from the bottom of the hindbrain to the level of the hindlimb bud. Strong staining is observed in somitic derivatives. Practically all sclerotomal cells along the embryonic body are stained. Single spindle shaped cells in the central parts of myotomes are also stained.

Stained cells are seen also in the head, both in mesenchyme and in the brain wall. Staining is concentrated in the region of the roof of midbrain and less concentrated staining can be seen in the region of forebrain. Prominent groups of heavily stained cells can be seen in the mesenchyme in the head and visceral arches. The mesenchyme of the anterior part of forelimb buds also contains many stained cells.

The patterns of β -gal staining represented in (C) and (E) appear to be the parts of the pattern observed in (D).

(F) Embryo of 11.5 dpc age from founder #2. Small group of stained cells is located in mesenchyme in the cervical region.

(G, H) Two 11.5 dpc embryos of 5.4aP2-3 strain, suggesting multiple integration sites of the transgene. (G) Most prominent groups of stained cells within the premuscle cell masses of limb buds. Stained cells are found also throughout the mesenchyme of the cephalo-cervical region. Staining in the somites; more stained cells located in thoracic compared to cervical or lumbar somites. Stained cells are found in different regions of developing central

nervous system. (H) Compared to the pattern shown in (G), staining is less pronounced in somites, head and neck regions. There is no staining of premuscle cell masses in limb buds. Some groups of stained cells are unique to this embryo: one lies along the anterior edge of the maxillary process and another in the apical ectodermal ridge stained in fore and hind limb buds. (I) A 16.5 dpc embryo of strain 5.4aP2-3 displaying staining in muscle cells throughout the body.

Fig. 2. Developmental expression of the reporter gene in embryos of strain 5.4aP2-4.

(A) A 9.0 dpc embryo (20 somites). Stained cells form a prominent spot around the ocular vesicles. This characteristic "mask" is maintained throughout development. Stained cells appear in the ventral parts of brachial somites. Two rows of stained cells posterior to these somites delineate the nephric ducts.

(B) Lateral view of the forelimb bud region of 9.5 dpc (25 somites) embryo. The onset of migration of stained cells from the somites into the limb bud mesenchyme. The nephric staining persists.

(C) Transverse section through the brachial somite of 25 somite embryo showing the stained cells within the epithelial layer in the ventral extremity of the dermomyotome. One stained cell can be seen in the mesenchyme lateral to the somite.

(D - F) A 10.5 dpc (36 somites) satge embryo. (D) Whole embryo. (E) Magnified lateral view of the midbody region (F) Dorsal view of the hindlimb bud region. Some stained cells appear in the ventral part of the spinal cord. The nephric staining disappears. A prominent group of stained cells are located in the ectoderm covering the region of future cervical sinus. Scattered stained cells accompany brain blood vessels. Prominent staining in the putative premuscle cells mass within the forelimb bud mesenchyme. Staining in brachial somites disappears while

thoracic somites contain few stained epithelial cells on the lateral side of the ventro-lateral lip of the dermomyotome. Migration of stained cells from somites into the hindlimb bud is very prominent.

(G) A 11.5 - 12.0 dpc (48-50 somites) stage embryo. Staining of cells accompanying blood vessels is more prominent in head and neck region. The staining in the limb buds mesenchyme is confined to the most proximal parts of premuscle cell masses.

(H) Fetus of 16.5 dpc. Staining in the developing muscles disappears. Most prominent is the staining of smooth muscle cells in *tunica media* of large blood vessels and arterioles.

(I) The head of newborn transgenic pup. A prominent vascular expression of transgene, that persists also in adult animals.

Figure 3 A. Selection strategy for isolation of novel adipocyte clones from multipotential 10T1/2 cells. A similar strategy was used for isolation of the 10TM42 cell line where Insulin was replaced by 125 uM indomethacin treatment. B. Differentiation protocol used throughout these experiments.

Figure 4. Top: Nondifferentiating 10T1/2 cells after 9 days in adipocyte differentiation medium. Bottom: Insulin-responsive 10T82A adipocytes treated with insulin and dexamethasone for 12 days. Adipocyte morphology was confirmed with oil red O staining (data not shown).

Figure 5. Indomethacin-induced 10TM42 cells treated for 6 days with indomethacin and dexamethasone. Top; a mixed population of cells prior to final subcloning. Bottom: center of a differentiated clone.

Figure 6. Azacytidine-derived IB1 adipoblast cell line after treatment with differentiation hormones. Subsequent studies show that hormones are not required for this differentiation to occur. Top: Differentiated IB1 cells after 8 days

hormone (insulin/DEX) treatment. Bottom: Edge of an IB1 clone during early selection passages.

Figure 7. Summary of selection screen and differentiation properties of the resulting cell lines. IB1 was clonally selected 4 times, 10T82A 6 times and 10TM42 4 times.

Figure 8. Summary of results from Cao et al. (1991) on expression patterns of the C/EBP gene family in 3T3L1 cells, another tissue-culture model system for adipocytes.

Figure 9. C/EBP α expression in parental 10T1/2 cells and in the novel adipoblast cell lines 10T82A, 10TM42 and IB1. Whole cell protein samples prepared from cells treated with differentiation or control media were used for western blot analysis with an anti-C/EBP α polyclonal antiserum. Polyclonal antisera specific for each of the C/EBP proteins was the generous gift of S. McKnight (Cao 1991). The more slowly migrating protein band is full-length C/EBP α ; the lower band is a proteolytic 29 kd fragment often seen in cellular preparations (Landschulz 1987). The protein isolation and western protocol employing the ECM chemiluminescence system was similar to that used by Cao et al. (1991). Cells were treated with differentiation hormones two days post-confluency. Control cells (C) were also confluent but did not receive hormone treatment. Day 0 protein samples were collected on the day the hormone treatments were initiated. Protein was also prepared from proliferating IB1 cells (P). INS:insulin; DEX: dexamethasone; INDO: indomethacin; ID/D: indomethacin and dexamethasone treatment; I/D or I-D: insulin and dexamethasone treatment.

Figure 10. C/EBP δ expression is inducible in 10T1/2 and 10T1/2-derived adipoblast cell lines. Top set of panels: The same set of whole-cell protein samples used in figure 7 was also probed with anti-C/EBP δ polyclonal antisera (Cao 1991). C/EBP δ runs as a doublet at approximately 26 kd. The C/EBP α

western blot results shown in figure 7 are presented in the set of bottom panels for direct comparison (the 29kd portion of the C/EBP α blot is not present in this figure).

Figure 11. 23A2 aza-myoblasts do not express the C/EBP gene family in response to either adipogenic hormones or muscle differentiation media. Whole cell protein was prepared from 10T1/2-derived aza-myoblasts that were treated with either insulin/dex (I/D), indomethacin/dex (ID/D) or muscle differentiation medium (MDIF) of 2.5% horse serum substituting for 10% fetal calf serum. Day 0 was again two days post confluence. Protein prepared from differentiated 10T82A cells was used as a positive control for C/EBP α , while 10T1/2 protein lysates provided both a negative control for C/EBP α and a positive control for C/EBP δ . Top panel: C/EBP α western blot; Middle panel: C/EBP δ western blot; Bottom panel: Anti-C/EBP β antisera (Cao 1991) obtained from S. McKnight was used in this series of experiments. All the samples were from the same experiment allowing cross-analysis between blots.

Figure 12. Expression profile of the C/EBP transcription factors in multipotential 10T1/2 cells as well as the 10T1/2-derived adipoblast and myoblast cell lines.

Figure 13. A model for the molecular mechanism responsible for C/EBP α expression in the 10T1/2-derived adipoblast cell lines. A transcriptional activator is present in the adipoblast lines that is absent in the multipotential 10T1/2 cells. This factor interacts with the C/EBP α gene and activates its expression. C/EBP α expression then leads to the activation of adipocyte-specific differentiation genes.

Figure 14. Preparation of C/EBP α gene reporter plasmids. A restriction map of the rat C/EBP α gene was established and then used to produce appropriate deletion fragments of the C/EBP α 5' regulatory region for cloning into CAT and β -galactosidase vectors. All the C/EBP α fragments were joined to the reporter

plasmids at +130 of the C/EBP α gene where an NcoI site was mapped. The arrows indicate the transcriptional start of the C/EBP α gene.

Figure 15. Transient transfection of 10T82A adipocytes with C/EBP α CAT reporter plasmids. Transient transfections were performed similar to Goldhamer et al. 1991???. In order to reach confluence as required for adipocyte differentiation, the cells were initially plated at 5×10^5 cells per plate and transfected the following day. The transfection mix was left on the cells overnight and rinsed off the next day with replacement of the media. The following day differentiation hormones were added to the appropriate plates and the cells were then harvested 2 days later. CAT assays were performed (Goldhamer 1993) and the results normalized to % conversion / 50ugs of cell lysate/ per hour of reaction time. All the assays were performed in the linear range of the assay. pOCAT contains no promoter and represents the background activity for the plasmid. TKcat and EMSV contain the thymidine kinase promoter and EMSV enhancer, respectively, directing CAT expression and serve as positive controls for transfection efficiency. -300, -1000, and -5300 are C/EBP α gene fragments of the respective lengths cloned upstream of the CAT reporter gene. The 2 bars for each reporter plasmid represent lysates prepared from control cells (-DIFF) and cells treated with differentiation media containing insulin and dexamethasone (+DIFF).

Figure 16. The C/EBP α promoter is active in 10T1/2 adipocytes and 10T1/2 multipotential cells. Experiments with the 10T1/2 cells were conducted similar to those presented in figure 13. The results for 10T82A are the same as those presented above.

Fig 1

Fig 2

Fig. 3A

Selection for spontaneous adipoblast cells from C3H10T1/2 cell line

Result

8 fat-containing areas on the
dishes were originally isolated

5/3000 still contained some fat in the
dishes in the next round of selection

10T82A has been subcloned
by selection 6 times

Adipocyte Differentiation Protocol

Fig 4

10T1/2 F2 fibroblasts - confluent + differentiation hormones

10T82A(2) adipoblasts - confluent + differentiation hormones

Fig 5

MA(2): 10T1/2 cells selected for differentiation with Indomethacin treatment

Fig 6

IB1 : azacytidine-derived adipoblast + differentiation hormones

Fig 7

Establishment of 10T1/2- derived adipoblast cell lines

Members of the C/EBP gene family expressed in 3T3L1 cells*

*results from Cao 1991

Fig 9

10T1/2-derived adipoblast cell lines express C/EBP α protein

Fig 10

C/EBP δ protein expression is inducible in 10T1/2 and 10T1/2-derived adipocyte cell lines

<u>INS/DEX</u>					C		<u>INS/DEX</u>					C				
0	1	2	4	8	0	2	2	6	2	6	0	1	2	4	8	P

C/EBP δ

C/EBP α

Cell line

10T1/2

INDO/DEX
selected
M42

INS/DEX
selected
10T82A

aza-induced
adipoblast
IB1

ID=Indomethacin
C=control
INS=insulin
D=dexamethasone

Fig 12

Fig. 13

Fig 14

Restriction map of C/EBP α regulatory region

Fig 15

Transient transfection of C/EBP α CAT constructs into 10T82A adipocytes

Fig. 16

The C/EBP α promoter is active in 10T1/2 adipocytes and 10T1/2 multipotential cells

E. Description of cooperation

The cooperation between the two research groups has been very useful and mutually beneficial. At Fox Chase, Dr. Emerson focused mainly on the isolation and characterization of genetic elements involved in skeletal muscle and adipose tissue determination using invitro methodologies: cell lines, transient and stable transfections, gene cloning and construction of fusion genes. In Israel, Dr. Shani concentrated on the production and analysis of expression of most of those gene constructs in transient and stable transgenic mouse strains. There was a continuous flow of materials (plasmids, sequences, embryos, transgenic strains) and information both ways. A member of Dr. Emerson (Dr. David Goldhamer) spent a month in Israel to become familiar with the transgenic world. Dr. Shani visited the laboratory of Dr. Emerson twice during this period for discussions, exchange of views and planning future experiments. The two groups intensively employed the e-mail and fax to transfer data, ideas and interpretations. The list of publications demonstrates the strong collaborative links between the two groups.

F. Evaluation of research achievements

Our 1st, 2nd and 3rd objectives were entirely accomplished. The gene constructs in *MyoD1* and *myogenin* cDNAs were driven by the adipsin promoter were tested invitro, in adipose and non-adipose cell lines and attempts were made to produce transgenic mice. The results suggest that such constructs could convert 10T1/2 cells into adipoblasts. However, no live transgenics could be produced probably due to the ubiquitous expression of these elements in other cell lineages. To evaluate the tissue specificity of another adipose control elements, of the aP2 gene, we produced 4 transgenic strains carrying the entire 5.4 kb promoter sequences and 4 transgenic strains carrying only the enhancer and promoter sequences in front of the lacZ reporter gene. Analysis of the temporal and spatial patterns of expression of all

8 strains during mouse development demonstrated no adipose specificity. Each strain displayed a unique pattern of expression, indicating that these elements are weak and subjected to chromosomal position effects.

Due to the fact that the control elements of two known adipose specific genes failed to allow the production of live transgenics when fused in front of the myogenic regulatory cDNAs, led us to a slight deviation from the original objectives. Instead of exploiting the available control gene sequences, we had to define new genetic elements that are involved in the determination of cells to the adipocyte lineage. By establishing novel adipocyte cell lines from the multipotential 10T1/2 cells the differential role of three members of the C/EBP gene family of transcription factors during the conversion of 10T1/2 cells to adipoblasts was determined. The results of these studies hold promise for future experiments towards the original objectives of this project.

G. Publications resulting from the research project

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6. Faerman, A., Goldhamer, D., Puzis, R., Emerson, C.P. and Shani, M. 1995. The distal human *MyoD* enhancer sequences direct a unique tissue-specific pattern of *lacZ* expression during mouse development. *Devel. Biol.* in press.