Induction of Bach1 and ARA70 gene expression at an early stage of adipocyte differentiation of mouse 3T3-L1 cells

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Using a subtraction method, we have isolated genes that are induced early in the differentiation of mouse 3T3-L1 preadipocyte cells into adipocytes. These include the genes encoding transcription factors and signalling proteins, as well as unknown genes. Bach1, a transcription factor, and ARA70, a cofactor, were rapidly induced during differentiation. The induction of these two genes was observed only in growth-arrested 3T3-L1 cells, and not in proliferating cells. In NIH-3T3 cells, no induction was observed under either set of conditions. These results strongly indicate that Bach1 and ARA70 have valuable roles at the onset of adipocyte differentiation.

Key words: diabetes, obesity, signalling factor, subtraction method, transcription factor.

INTRODUCTION

Obesity is a contributory factor for many diseases, such as hypertension, heart disease and diabetes [1]. Therefore further insight into the molecular basis of obesity is needed [2]. The differentiation of cells into adipocytes has been relatively well characterized, and several transcription factors have been identified as master regulators of the differentiation process. The peroxisome-proliferator-activated receptor γ (PPARγ) gene is expressed during adipogenesis and is known to activate adipocyte-specific genes, and members of the CCAAT/enhancer-binding protein (C/EBP) family have also been identified as master regulators [3–5].

Compared with the middle and late stages of adipogenesis, little is known about the earliest step in the differentiation process. Mouse 3T3-L1 preadipocyte cells, which are fibroblastic cells of adipocyte lineage, are widely used for studies on adipocyte differentiation [6]. Using this cell line, we previously isolated genes that are strongly induced at the beginning of adipocyte differentiation [7]. These genes encode transcription factors and signalling factors. Interestingly, almost all of the clones isolated are independent genes, and have been newly identified as genes that are expressed at the beginning of differentiation. Since we have not characterized all of the clones in this cDNA pool, we conducted further analysis and report here the identification of Bach1 [8] and ARA70 [9] as genes that may have a key role during adipocyte differentiation.

MATERIALS AND METHODS

Cell culture

Mouse 3T3-L1 preadipocytes (A.T.C.C. CL173) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) calf serum. For differentiation, the medium was changed to DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM dexamethasone (Dex) at 2 days post-confluence, as described previously [7]. Mouse NIH-3T3 fibroblasts (clone 5611; JCRB 0615) were maintained in DMEM supplemented with 10% (v/v) calf serum. Mouse C3H10T1/2 cells (clone 8) were maintained in basal Eagle's medium containing 10% (v/v) FBS. For differentiation, the cells were cultured to confluence. At 2 days post-confluence, the medium was changed to the same Eagle's medium supplemented with 10% (v/v) FBS and 1 μM BRL49653; cells were re-fed with this medium every 3 days.

RNA isolation and Northern blot analysis

3T3-L1, NIH-3T3 and C3H10T1/2 cells were harvested at given times after the addition of inducers, and total RNA was isolated using TRIzol (Gibco BRL Life Technologies, Gaithersburg, MD, U.S.A.). For Northern blot analysis, 20 μg of total RNA was electrophoresed on a 1.0% (w/v) agarose gel containing 2% (v/v) formaldehyde, and then transferred to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech). The filter was hybridized with a probe for Bach1 or ARA70 labelled with [α-^{32}P]dCTP using a random labelling kit (Takara Biomedicals, Kusatsu, Japan). The radioactivity corresponding to each band was measured using a bioimage analyser (BAS 2000; Fuji Film, Tokyo, Japan).

RESULTS

Isolation of genes induced at an early stage of adipocyte differentiation

Using a cDNA subtraction system, we previously isolated genes that were expressed at 3 h after the addition of inducers of adipocyte differentiation [7]. In that report, we chose 157 colonies for sequencing. Among these, 131 clones (83%) were independent, and finally 58 clones were isolated as representing genes whose expression was increased after induction. Since we did not characterize the entire cDNA pool, it is possible that other independent and uncharacterized clones still exist. Therefore, we analysed the cDNA pool further.

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IBMX, 3-isobutyl-1-methylxanthine; NF-E2, nuclear factor-E2; PPARγ, peroxisome-proliferator-activated receptor γ; SREBP-1, sterol regulatory element-binding protein-1.

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Table 1 Genes induced during the early stages of adipocyte differentiation of mouse 3T3-L1 cells

Expression level is defined as: + + +, highly induced; + +, moderately induced; +, slightly induced. The sequences of isolated clones were run through DNA databases, and the names are shown when identical with mouse clones. For the clones that show high sequence similarity to proteins from other species, the name of the source species and the percentage similarity are shown in parentheses. Clones isolated in the present study are indicated by *. †Rho (TC10) isolated in a previous study [7] was recently identified as a new clone, TCL (TC10-like protein) [17]. Abbreviations: TAF, TATA box-binding protein (TBP)-associated factor; TCP, T-complex polypeptide.

Level of expression	Protein
(a) Signalling proteins and transcription factors	
+	ARA70 (human; 80%)
+ + +	Bach1*
+	Grb2- and Fyn-binding protein*
+ +	GTP-binding protein (SARa)*
+ +	Kuzbanian*
+ + +	Na^+/K^+-ATPase α2 subunits (rat; 95%)
+ + +	Hypoxia-inducible factor-1α (HIF-1α)
+ + +	Heat-shock protein 105 (HSP105)
+ + +	Oncostatin M specific receptor β subunit (human; 60%)
+ +	p66 Mot1*
+	Phosphatase 1 nuclear targeting subunit (rat; 97%)
+ + +	Phosphoglycerate kinase*
+ + +	Protein phosphatase 2A (rat; 97%)
+ + +	Regulator of G-protein signalling 2 (RGS2)
+	Ran-GTP-binding protein 5* (human; 88%)
+	TAFII 105* (human; 84%)
+ + +	TCL†
+ +	Thyroid hormone-binding protein p55, cellular
+	Transforming growth factor-β receptor, type III (rat; 92%)
+ + +	Vitamin D receptor (VDR)
(b) Cytoskeletal and extracellular structures	
+ +	Actin-binding protein (human; 88%)
+	Annexin IV
+	Collagen α1, type IV*
+	Collagen α2, type VI
+	Collagen α3, type VI (human; 82%)
+ +	Golgi 4-transmembrane spanning transporter
+ +	Osteonectin
+ +	Proteoglycan PG-M
+	Transmembrane protein
(c) Other genes	
+ + +	*N*-Acetylglucosamine galactosyltransferase
+ + +	Agx-1 antigen (human; 68%)
+ +	63 kDa antigen (*Brugia*; 54%)
+ + +	Antioxidant protein 2
+	Calregulin
+	Chaperonin containing β TCP-1 (CCtb)*
+ + +	Chaperonin containing η TCP-1 (CCth)*
+	Connexin 43
+ +	Dihydrolipoamide dehydrogenase*
+ +	Early T-lymphocyte activation 1 protein
+ + +	ECA39
+	Fat facets homologue
+ + +	Fibroblast growth factor-inducible gene (FIN14)*
+ + +	Gal β1 (3GalNAc α2)
+ + +	Glutathione peroxidase*
+ + +	Monocarboxylate transporter*
+ + +	Nucleolar phosphoprotein of 140 kDa (rat; 88%)
+ + +	Spi2 protease inhibitor
+	Suilisol
+ + +	T1 protein
+ +	Thrombospodin 1
+ + +	Tropomyosin 4* (rat; 88%)
+ + +	UDP-glucose dehydrogenase (*Drosophila*; 57%)
+ + +	Xanthine dehydrogenase
(d) Unknown genes	
+ + +	14 clones (7 clones*)
+ +	17 clones (9 clones*)
+	15 clones (11 clones*)

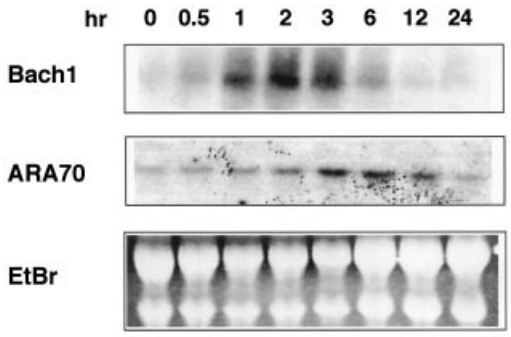

Figure 1 Time courses of Bach1 and ARA70 mRNA expression in the early stages of differentiation of mouse 3T3-L1 cells into adipocytes

Northern blot analyses were performed using 20 μg portions of total RNA prepared from 3T3-L1 cells at various times after the addition of inducers. The filter was hybridized with each probe as indicated and exposed to X-ray film. For confirmation that RNA was not degraded and that the amounts of RNA loaded in each lane were similar, ethidium bromide (EtBr)-stained RNA was included as a control.

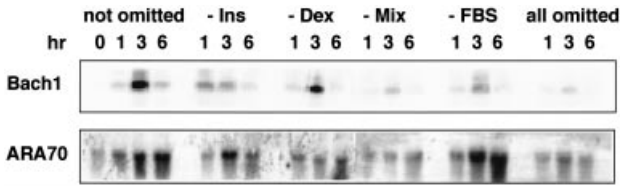

Figure 2 Profiles of Bach1 and ARA70 gene expression in deprivation induction media

Northern blot analyses were performed using 20 μg portions of total RNA prepared from 3T3-L1 cells at various times after the addition of inducers. In each panel showing -, the indicated inducer was omitted (Ins, insulin; Mix, IBMX); 'not omitted' indicates that all inducers were added. The filter was hybridized with each probe as indicated and exposed to X-ray film. It was confirmed that RNA was not degraded and that the amounts of RNA loaded in each lane were similar (results not shown).

For the present study, we chose another 143 clones from the cDNA pool, sequenced them, and finally obtained 111 that were independent. However, 14 of these clones were identified as types isolated previously [7]. Therefore we further analysed 97 clones by Northern blotting. As a result, 44 clones were newly identified as genes that are inducible during the early stages of adipocyte differentiation. As a summary of the cloning, the combined results (102 clones) from previous (58 clones) and present (44 clones) studies are shown in Table 1.

As in our previous study, genes encoding signalling proteins as well as unknown genes were identified in the present study. Interestingly, we identified 27 unknown genes that were not in the database; in total, 46 of 102 clones were identified as unknown genes. Since only 300–500 bp fragments were obtained by these subtraction methods, it is possible that the fragments are derived from the 3′-untranslated region. Therefore we are now cloning full-length cDNAs of these clones by library screening and 5′- and 3′-rapid amplification of cDNA ends.

Expression profiles of Bach1 and ARA70

Bach1 was newly identified in the present study (Table 1). Bach1 is a transcription factor which has a basic leucine zipper motif for DNA binding, and is a partner for heterodimerization with the small Maf family [8,10]. Since it was strongly induced during adipogenesis, Bach1 was characterized further in terms of its expression pattern.

ARA70 was isolated previously as a ligand-dependent androgen-receptor-associated protein, which functions as an activator to enhance androgen-receptor-mediated transcriptional activity [9]. ARA70 has been identified previously as a ligand-enhanced co-activator for PPARγ [11]. Therefore, although the level of induction of ARA70 was low (Table 1), ARA70 was also characterized further.

The time courses of the expression of Bach1 and ARA70 are shown in Figure 1. The expression of Bach1 was induced within 30 min, reached a peak at 2 h, and then decreased gradually. On the other hand, the level of ARA70 expression was increased 2 h after induction, and peaked at 3 h. The expression of both genes was undetectable after 24 h. As described previously, the levels of expression of β-actin [7] and glyceraldehyde-3-phosphate dehydrogenase [6] mRNAs change during differentiation. Therefore we confirmed by ethidium bromide staining that rRNAs were not degraded, and that the amounts of RNAs loaded were similar (Figure 1). The Northern blotting experiments were repeated at least three times using at least two different of lots of RNA, and reproducibility was confirmed throughout the study. The Figures show typical blots.

Effects of inducers on Bach1 and ARA70 expression

For the differentiation of mouse 3T3-L1 cells into adipocytes, four inducers (IBMX, Dex, insulin and FBS) were added to the differentiation medium. To characterize the effects of these inducers, the expression profiles of Bach1 and ARA70 were determined in deprivation medium from which only one inducer was omitted. The results of Northern blot analysis are shown in Figure 2. Dex had no effect on Bach1 expression, but the other three inducers were needed for full induction. In contrast, FBS had no effect on the induction of ARA70, and insulin had only a small effect. IBMX and Dex seemed to be major inducers of ARA70.

Expression profiles of Bach1 and ARA70 in non-differentiated cells

In order to differentiate into adipocytes, 3T3-L1 cells must first be grown to confluence and then kept for 2 days. After that, the inducers are added to the medium. Differentiation occurred only under these conditions, and proliferating cells did not differentiate into adipocytes even in the presence of inducers. Thus a state of growth arrest was required for the differentiation of 3T3-L1 cells. The mouse fibroblastic cell line NIH-3T3 did not differentiate under either set of conditions.

Next we compared the expression profiles in the two cell lines under two sets of conditions: growth arrest (post-confluence) and proliferation. As shown in Figure 3, Bach1 was strongly induced in the growth-arrested 3T3-L1 cells 3 h after induction. On the other hand, no induction was observed in proliferating 3T3-L1 cells; rather, the expression level decreased slightly. In NIH-3T3 cells, Bach1 expression also decreased under both sets of conditions.

These results strongly suggest that the expression of Bach1 was specific for adipocyte differentiation. Although the expression of the ARA70 gene was relatively weak, it was increased in the growth-arrested 3T3-L1 cells, indicating that ARA70 also seems to have some functional role in adipocyte differentiation.

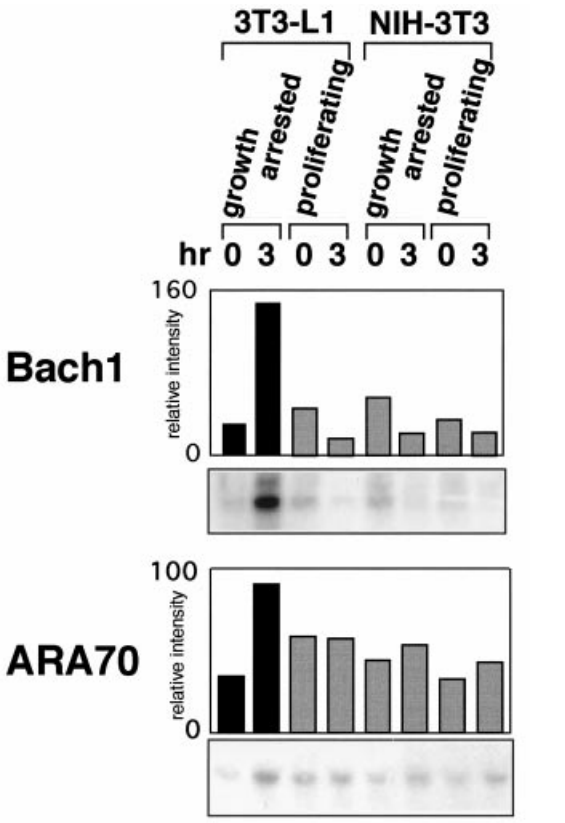

Figure 3 Expression profiles of Bach1 and ARA70 mRNAs in 3T3-L1 and NIH-3T3 cells treated with inducers at different stages of differentiation

Total RNA was prepared from 3T3-L1 and NIH-3T3 cells before and 3 h after treatment with inducers, and 20 μg portions of total RNA were analysed by Northern blotting. The filter was hybridized with each probe. Cells were treated with the inducers after growth arrest (growth arrested; the typical condition for adipocyte differentiation), or at the mid-exponential phase of growth (proliferating; not the typical condition for adipocyte differentiation). For the experiments with growth-arrested cells, both 3T3-L1 and NIH-3T3 cells were maintained in DMEM supplemented with 10% (v/v) calf serum for 2 days after confluence, and then the medium was changed to DMEM supplemented with 10% (v/v) FBS, insulin, IBMX and Dex. The radioactivities of corresponding bands were measured by an image analyser and are indicated as imaging units in each panel. It was confirmed that RNA was not degraded and that the amounts of RNA loaded in each lane were similar (results not shown).

Expression profiles of Bach1 and ARA70 in C3H10T1/2 cells

The conditions under which 3T3-L1 cells are induced to differentiate into adipocytes are remarkable, because the induction medium contains a physiologically non-relevant cocktail that includes Dex, IBMX and insulin. Therefore it is important to determine whether the same patterns of expression of Bach1 and ARA70 are also obtained in other cells that can differentiate into adipocytes. Mouse C3H10T1/2 cells represent a multipotent stem cell line, and can differentiate into adipocytes [6]. For example, treatment of these cells with BRL49653, which is a high-affinity ligand for PPARγ, results in their efficient differentiation into adipocytes [12]. By using this cell line with BRL49653 as an inducer of differentiation, we performed Northern blot analysis (Figure 4). Interestingly, the expression of both Bach1 and ARA70 was elevated at the beginning of the differentiation process. Thus it seems that the gene expression of Bach1 and ARA70 is closely related to the early stages of adipogenesis.

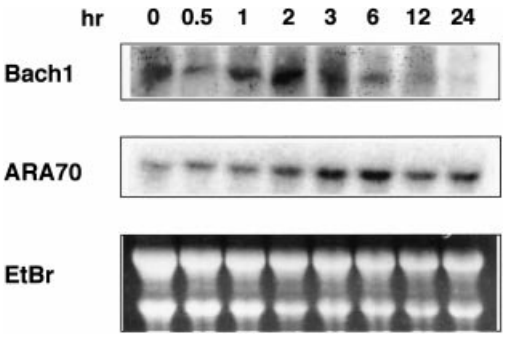

Figure 4 Time courses of mRNA expression of Bach1 and ARA70 in the early stages of differentiation of mouse C3H10T1/2 cells into adipocytes

Northern blot analyses were performed using 20 μg portions of total RNA prepared from C3H10T1/2 cells at various times after the addition of inducer, BRL49653. The filter was hybridized with each probe as indicated, and exposed to X-ray film. For confirmation that RNA was not degraded and that the amounts of RNA loaded in each lane were similar, ethidium bromide (EtBr)-stained RNA was included as a control.

DISCUSSION

During adipocyte differentiation, three kinds of transcription factors act as master regulators: PPARγ, the C/EBP family (C/EBPα, C/EBPβ and C/EBPδ) and SREBP-1 (sterol regulatory element-binding protein-1) [3–5]. The ectopic expression of PPARγ in the fibroblastic cell line NIH-3T3 caused adipogenesis, indicating a central role for this transcription factor in the development of adipocytes [13]. It is reported that the ectopic expression of C/EBPα also promotes the adipogenic programme in fibroblastic cells [14]. The double knockout of C/EBPβ and C/EBPδ impaired the synthesis of fat in mice [15]. The dominant-negative form of SREBP-1, also called ADD1 (adipocyte determination and differentiation-dependent factor 1) has been shown to repress adipocyte differentiation [16].

These findings suggest that these three transcription factors have important roles in adipocyte differentiation. However, all were expressed at the mid-stage of differentiation, and the events that occur in the early stages are not well characterized. In our previous report [7], genes induced in the early stages of adipocyte differentiation were identified. These include the genes for transcription factors and signalling factors, such as the vitamin D receptor and RGS2 (regulator of G-protein signalling 2) [7]. In the present study, we analysed further the rest of the isolated cDNA pool, and identified Bach1 as a novel induced gene. Northern blot analyses using total RNA from various cells revealed the possibility that Bach1 and ARA70 each have an important role in the process of differentiation into adipocytes.

Bach1 heterodimerizes with the small Maf family of proteins, and binds to the nuclear factor-E2 (NF-E2) binding site. NF-E2 is a haematopoietic transcription factor and may contribute to the differentiation of erythroid cells [8,10]. ARA70 was originally identified as a co-activator for the androgen receptor, and was subsequently found to be a co-activator for PPARγ as well [9,11]. These two factors were rapidly induced in the early stages of adipocyte differentiation, and had expression patterns that were differentiation-specific. Notably, Bach1 was negatively regulated in proliferating 3T3-L1 cells and in proliferating and growth-arrested NIH-3T3 cells, but was rapidly induced in growth-arrested 3T3-L1 cells. This result strongly suggests that Bach1 expression is closely related to the initiation of differentiation into adipocytes. Elevated expression of both Bach1 and

ARA70 was also observed in another cell line, C3H10T1/2, further supporting a relationship of these genes with adipogenesis. Since the functions of Bach1 and ARA70 *in vivo* are, however, still unknown, antisense and sense experiments for the inhibition and ectopic expression of Bach1 and ARA70 are required, and these are now under way.

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