Temporal regulation of gene expression in adipocyte differentiation

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Abstract Cells usually become 'committed' to differentiate long before any actual morphological change is apparent. In one model commitment is a decision which corresponds to the expression of a control gene, while differentiation is the ultimate consequence of that decision. We have been studying adipocyte commitment and differentiation at the molecular level. Earlier we showed that the introduction of a specific DNA sequence into 'uncommitted' cells renders those cells committed to differentiate into adipocytes. Here we report temporal regulation of the expression of this DNA sequence; furthermore, we show that this RNA is in the non-polyA$^+$ fraction of total cellular RNA. These data suggest that coordinate regulation of this and other genes is important in promoting differentiation.

Key words: Adipocyte differentiation; RNA expression; Temporal regulation

1. Introduction

The molecular mechanisms by which a single cell gives rise to an entire organism with its multitude of cell types, tissues, and organs remain largely unknown. Expression of specific genes during differentiation is under spatial and temporal controls. When a cell is to become a specific cell type, it must become determined or committed to a particular differentiation pathway [1,2]. In many cases commitment has been proposed to begin with the activation, or possibly the creation by DNA rearrangement, of one or a small number of genes, whose gene-product(s) commit a cell and its descendants to carry out a specific differentiation upon induction. Genes responsible for early differentiative decisions have been isolated in *Drosophila* and vertebrates [3–7]. Often, these initiating genes encode protein domains that are highly conserved in nature. For example, the 'homeo' domain found in genes whose products are necessary for proper segmentation of insects, is found in a range of organisms from fly to human [4,8,9]. Some of the most extensively characterized determining genes at the molecular level are those for muscle cell determination and differentiation [10,11]. In vertebrate embryos, control by the MyoD family of muscle-specific basic-helix-loop-helix proteins, which includes MyoD, myogenin, myf5, and MRF4, is critical to skeletal muscle formation [11–16]. The MyoD protein seems to activate myogenesis by directly binding to the control regions of muscle-specific genes [17–19]. Thus, myoD is considered to be a 'master regulatory gene'. In contrast to homeotic genes studied in *Drosophila* or *C. elegans*, myoD seems to affect the identity of a single cell type, not that of many types of cells in pattern formation.

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We are using adipocyte differentiation as our model system to study 'determination' or 'commitment' genes. We have been characterizing a group of genes from mouse and human cells that determine adipocyte commitment (AC). Our system depends on a cell-culture bioassay of gene activity. The first studies were based on the Swiss 3T3 cells [20,21]. When their growth is arrested in culture, certain subclones of 3T3 mouse fibroblasts such as 3T3-F442A or 3T3-L1 will express the adipocyte phenotype at nearly 100% efficiency under appropriate conditions [20–23]. Clonal null sublines of 3T3, such as 3T3-C2, which have a very low frequency of spontaneous fat conversion were isolated [20–24]. We had shown earlier that cotransfection of a selective marker (neomycin) with high molecular weight DNA isolated from uninduced mouse preadipocyte fibroblast cells (3T3-F442A) or human fat tissue into 3T3-C2 recipient cells empowers a significant number of cells surviving the drug selection to display the adipocyte phenotype. DNA from 3T3-C2 did not convert 3T3-C2 recipients into adipocytes upon transfection [24]. Through genomic DNA cosmid cloning and subsequent plasmid subcloning, we isolated two short DNA sequences (clones A and B). Each one alone is capable of committing a variety of mouse and rat cells to differentiate into adipocytes [25]. Despite the small size of clone A (1.2 kb), it acts functionally as a gene in our bioassay. The vector (pUC8) used for subcloning from the cosmid library does not provide eukaryotic enhancers, promoters, splice or polyadenylation signals. Both strands of clone A have been sequenced, and fail to show an assembly of splice sites, enhancer, promoter, CAAT, TATA, polyadenylation, and other recognition sequences consistent with a protein encoding region. A scan of all possible frames did not reveal a preferred open reading frame. The longest open reading frame of the genomic clone A is 60 amino acids. Since clone A is a genomic clone, the possibility remains that through splicing a functional protein may emerge. In this communication we report the detection of clone A specific RNA at 1 day before cells reach confluence; at such time there are no detectable cellular or morphological changes.

2. Materials and methods

2.1. Cell culture and DNA transfection

All cells were maintained in DME medium plus 10% calf serum (Gibco BRL). Recombinant plasmid DNAs were prepared as described [27]. DNAs were transfected into recipient cells together with pKoNeo as a selectable marker. DNA transfections were performed by the calcium phosphate method modified as previously described [24,25]. 60 mm plates were seeded with recipient cells at a density of 4×10^5. 10–20 μg of DNA were transfected or co-transfected with pKoNeo. Cells taking in exogenous DNA were selected in medium containing G418 (Gibco) at the lowest concentration that kills untransfected cells in a 2-week period as experimentally determined (400 μg/ml for C57L-MSF, mouse precrisis skin fibroblast cells). Individual G418^r colonies were isolated, expanded, and frozen for future analysis.

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2.2. Adipose conversion

All G418^r colonies were tested for conversion to adipocytes. After approx. 7 days of growth to confluence, 5 μg/ml of insulin are added. Visible adipocyte foci can be seen against a background of undifferentiated cells after ≈7–14 days. 3 weeks after the first adipocytes are seen under phase contrast, plates were fixed with formaldehyde and stained with oil-red-O. Fat foci were scanned under a dissecting microscope at ×16 or ×40 after staining and counted as positive if containing at least 50 fat cells [24,25].

2.3. RNA

Total RNA is prepared by TRI REAGENT (Molecular Research Center) according to the manufacturer's directions. Using these methods we have routinely obtained about 100 μg of total RNA/10 cm plate of confluent cells. Total RNA was quantified by ethidium bromide staining of ribosomal RNA bands. Poly A$^+$ RNA is obtained using the PolyAtract mRNA isolation system (Promega) or MPG direct mRNA purification kit (CPG). Both mRNA isolation systems gave the same results. Northern blots were performed as described earlier [27], using 20–40 μg of total RNA per lane. Human fat cDNA library was purchased from Clontech. Screening of the cDNA library was carried out as described by the manufacturer.

3. Results

3.1. Isolation of cell lines with stable integrated clone A DNA

Clone A can commit mouse and rat fibroblasts to adipogenesis. The ability of clone A to induce fat conversion in precrisis mouse cells was tested using C57L-MSF precrisis mouse skin fibroblasts [25]. About one-third of the neomycin-resistant C57L-MSF transfectant clones isolated were positive for adipocyte differentiation. Since exogenous DNA was taken up by co-transfection, we conclude that approximately one-third of the neomycin-resistant clones integrated a functional clone A DNA, as judged by the abilities of these clones to differentiate into adipocytes. The number of fat foci (each focus containing more than 50 fat cells) ranged from 50 to >10^3 per 60 mm plate (>10^3 means >90% of the plate). Two of the clones that gave >10^3 clones were used for RNA analysis (MSFA-1 and MSFA-2). One of the neomycin-resistant but adipocyte-differentiation negative clones was used as a negative control for RNA analysis (MSFA-3). The background of spontaneous fat-cell conversion exhibited by C57L-MSF cells is low (between 1 and 5 per 60 mm plate).

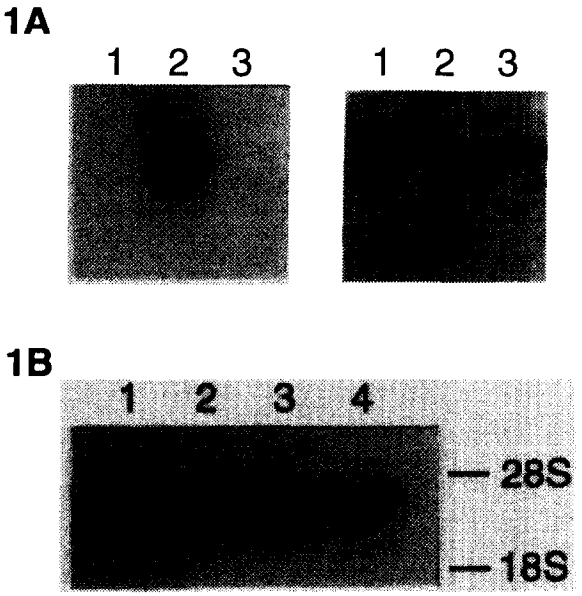

Fig. 1. Northern blot hybridization of clone A DNA to total RNA isolated from (A). (Left) MSFA-1 cells at different time points: lane 1, 2 days prior to confluence; lane 2, 1 day prior to confluence; lane 3, at confluence. (Right) The same blot reprobed with actin. (B) Independent isolations of total RNA from cells at 1 day prior to confluence. Lanes 1,2, MSFA-1 cells; lanes 3,4, MSFA-2 cells. Note the positive signal consistently appears as a band just below the 28S RNA marker.

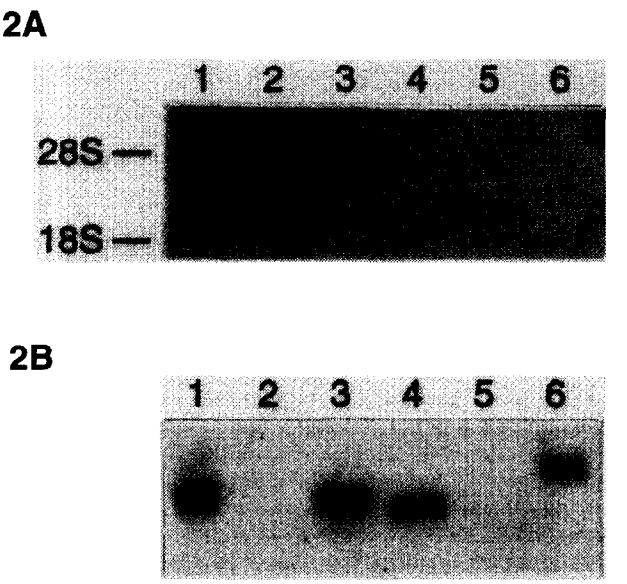

Fig. 2. Northern blot analysis using clone A DNA probe. MSFA-1 RNA lanes 1–3: total, non-polyA$^+$, polyA$^+$; MSFA-3 RNA lanes 4–6: total, non-polyA$^+$, polyA$^+$. Clone A specific band was only detected in total RNA and non-polyA$^+$ RNA of the adipocyte-conversion positive cells, MSFA-1, and not in the adipocyte-conversion negative cells, MSFA-3. (B) Same blot reprobed with GAPDH as a control to demonstrate integrity of RNA samples.

3.2. Detection of clone A-specific RNA

We screened a commercially available human-fat cDNA library for clone A specific RNA by using radiolabeled clone A DNA as probe. No positive clone was identified. We next reasoned that if any RNA product is transcribed in transfected preadipocytes, it only may appear briefly, only in the committed but not differentiated state, or only in low abundance. To test these possibilities, MSFA-1 and MSFA-3 RNAs were collected each day from all stages of growth through differentiation. We were able to detect the presence of clone A RNA from MSFA-1 cells only at 1 day prior to confluence (Fig. 1A, lane 2), not in RNA from other time points (Fig. 1A, lanes 1,3). The presence of clone A specific RNA in MSFA-1 cells was confirmed with several different RNA preparations (Fig. 1B). No positive clone A signal was detected in MSFA-3 RNA. Since we did not want to discriminate between polyA$^+$ and non-polyA$^+$ RNA, total RNA was used in these Northern blot experiments. We then separated the RNA into non-polyA$^+$ and polyA$^+$ fractions, and again analyzed by Northern blots. A positive signal, seen just below the 28S ribosomal RNA band, was only detected in the total RNA and non-polyA+ fraction of total RNA from MSFA-1 cells (Fig. 2A, lanes 1,2). No positive signal was observed with RNA from MSFA-3 cells (Fig. 2A, lanes 4–6). The same blots were then probed with GAPDH to confirm the adenylation nature and the integrity of the separated RNA fractions (Fig. 2B).

4. Discussion

The establishment of stable clonal cell lines from mouse 3T3 cells suggested an in vitro model system to examine adipocyte differentiation. We had demonstrated earlier that transfection of genomic DNA from tissue presumably with active sequences is able to commit the null cell clone 3T3-C2 to undergo adipogenesis. Furthermore, we were able to show that the activities resided in two small nonhomologous sequences. In this communication we present evidence that the expression of one of the sequences is under temporal regulation.

There are several models of developmental decision and determination. Examples of known mechanisms that would allow stable expression include inherited modifications of chromatin such as methylation, demethylation or the creation of a new gene by DNA rearrangement (as takes place in the immune system or in the antigen switching of certain trypanosomes). Another way to modulate expression of sets of genes would be by the formation of a stable transcription complex resulting from the combination of necessary factors within a particular cell. Recent studies, for example, suggest that different promoters may require different basal transcription factors [28]. Yet another mechanism recently observed to regulate cell growth and differentiation was shown to be mediated by a 3′-untranslated RNA (UTR). Using a genetic complementation assay based on function, 3′-UTRs of three muscle structural genes were found to act as trans-acting regulators that inhibited cell proliferation and promoted differentiation [29–32]. These or other unknown mechanisms may be used by the adipocyte differentiation pathway.

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