Reduced expression of AP27 protein, the product of a growth factor-repressible gene, is associated with diminished adipocyte differentiation

(adipogenic differentiation/antisense RNA)

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ABSTRACT We have recently characterized an adipocyte cDNA (clone 5) that is enhanced in expression by environmental and hormonal conditions favoring adipogenic differentiation. Moreover, certain agents including fibroblast growth factor and phorbol 12-myristate 13-acetate (but not epidermal growth factor) markedly inhibit clone 5 gene expression and prevent TA1 cell differentiation. These results led us to propose that a threshold level of the clone 5 gene product (AP27 protein) is required for triggering adipocyte differentiation. We have constructed vectors that direct the synthesis of clone 5 antisense RNA to reduce the levels of AP27 in adipogenic cell lines TA1 and 3T3-L1. We show here that when these cells express clone 5 antisense RNA, they fail to undergo morphological differentiation, whereas adipogenesis is unaffected in cells expressing antisense β-actin or ferritin heavy-chain RNA. We further show that cells expressing clone 5 antisense RNA (but not the other antisense RNAs) are unable to induce the expression of characteristic "adipocyte-specific" mRNAs. The level of inhibition of differentiation by clone 5 antisense RNA correlates with decreased levels of AP27 protein. These results provide strong evidence that expression of AP27 is linked to adipogenic differentiation and that AP27 may be a component of an as-yet-uncharacterized signal-transduction pathway required for the triggering of adipocyte differentiation.

Adipogenic stem cells, such as 3T3-L1 (1) and TA1 (2), are widely used to study the mechanisms by which hormonal and environmental signals control the switch from a progenitor cell to its fully differentiated counterpart. Such cell lines resemble fibroblasts while growing at low density, but upon reaching confluence the cells stop growing and in time take on the morphological and biochemical characteristics of mature adipocytes (3). Although recent advances have produced insights into the transcriptional mechanisms mediating differentiation-specific gene expression (4, 5), there is still a paucity of data relating to the mechanisms by which hormonal and environmental signals are transduced to the elements mediating adipocyte-specific gene expression.

Many groups have presented studies in which growth factors were shown to inhibit the differentiation of determined (especially myogenic) stem cell lines (6–8). We have recently reported that the differentiation of TA1 cells is markedly suppressed by fibroblast growth factor (FGF) and the phorbol ester 12-myristate 13-acetate (PMA), in a growth-independent fashion, as reflected by the inability of the cells to undergo morphological differentiation or to accumulate adipocyte-specific RNAs. We also described the characteristics of a specific mRNA, clone 5, the expression of which is suppressed by these same agents in undifferentiated,

preconfluent cultures. This property, in addition to its induction by differentiation-promoting factors such as glucocorticoids and high cell density, suggested to us that the putative protein product of this mRNA (referred to as AP27) might play an important role in the decision of determined cells to commit themselves to the differentiated phenotype (9).

To test this hypothesis, we have developed vectors that direct the synthesis of clone 5 antisense RNA to reduce AP27 levels in target cells. We show here that cells expressing clone 5 antisense RNA fail to undergo morphological differentiation. In contrast, adipogenesis is not affected in cells expressing antisense RNAs directed against β-actin or ferritin heavy chain (FHC; an RNA expressed in TA1 cells). Moreover, cells expressing clone 5 antisense RNA (but not the other antisense RNAs) were unable to induce the expression of characteristic mRNAs normally detected at abundant levels in all adipogenic cells lines. Finally, we demonstrate that in cells where AP27 levels are low, differentiation is impeded on a morphological as well as on a molecular level. These results provide strong evidence that AP27 may be a component of an as-yet-uncharacterized signal-transduction pathway required for triggering adipocyte differentiation.

METHODS

Vector Construction. The unique BamHI site of the retroviral vector pSVX (10) was used to insert the 758-base-pair (bp) EcoRI–Sac II fragment of the clone 5 cDNA (9), which includes the start and the stop codons. This fragment was blunt-ended, and Bgl II linkers were added. pSVX-bACT(as) was constructed by inserting the 1.9-kilobase (kb) BamHI fragment from pHFβA-1 (11) into the unique BamHI site of pSVX. The plasmid pSVX-FHC(as) was provided by S. Torti (Veterans Administration Hospital, Palo Alto). All cDNAs were cloned into the expression vector so that their expression results in an RNA complementary to the corresponding mRNA (antisense RNA).

Cell Culture. TA1 and 3T3-L1 cells were grown as described (1, 9). Cells were transfected by lipofection (12) with 10 μg of plasmid DNA and 80 μg of Lipofectin (Syntex Research). Cells growing in selective medium (containing Geneticin at 400 μg/ml; GIBCO) were pooled and used for further analysis. Geneticin at 400 μg/ml was always present while the cells were growing. Dexamethasone and indomethacin (Sigma) were used at 1 μM and 125 μM, respectively.

RNA Isolation. Total cellular RNA was isolated from cells and analyzed in 1.4% agarose/formaldehyde gels as described (9). RNA was then transferred to Zeta-Probe membranes (Bio-Rad) and hybridized with ^{32}P-labeled probes: double-stranded DNA probes were made by using the random hexanucleotide priming method with the large fragment of DNA polymerase I (13), and RNA probes were produced by using the RNA probe system (14). The probes used were β-actin (15), lipoprotein lipase (16), adipocyte clones 47 (17) and 28/aP2 (18), and murine FHC (19).

Antibody Preparation and Immunoblot Analysis. Recombinant AP27 was expressed in *Escherichia coli* by inserting the clone 5 cDNA into the EcoRI site of pUC12. This insertion resulted in the production of a protein containing 6 amino acids of β-galactosidase fused to the NH$_2$ terminus of the predicted AP27 open reading frame. The protein was partially purified by passing a bacterial lysate over a DE-52 column (Whatman). The flow-through fraction, containing the fusion protein, was subjected to electrophoresis in polyacrylamide gels containing SDS. The predominant band was excised, the protein was electroeluted, and this protein was used to immunize rabbits. Antibody specificity was confirmed by comparing the *Staphylococcus aureus* protease V8 maps of protein immunoprecipitated from differentiated TA1 cells and from an *in vitro* translation reaction. To determine the relative levels of AP27 in clone 5 antisense and control cells, the anti-AP27 antiserum was used to immunoprecipitate protein from cell extracts. The immunoprecipitated AP27 was detected after gel electrophoresis by immunoblotting with the same polyclonal antibody followed by probing with ^{125}I-labeled protein A.

RESULTS AND DISCUSSION

The retroviral expression vector pZIP-NeoSV(X) [in this paper called pSVX (10)] was used to express antisense (as) RNA (20, 21) complementary to clone 5 (c15) mRNA in the adipogenic cell lines TA1 (17) and 3T3-L1 (1). As a control for potential nonspecific effects of double-stranded RNA on adipogenic differentiation we also generated cell lines expressing β-actin (βACT) and FHC antisense RNA. FHC was chosen because, like clone 5, it is expressed in both undifferentiated and differentiated adipocytes (19). No significant differences were seen in the number of colonies or in the overall appearance of either cell line transfected with these three vectors, except that for 3T3-L1 cells transfected with pSVX-βACT(as) we saw formation of giant cells. These cells, which were not used for further experiments, may have arisen from disruption of cytoskeletal structures. We note that decreased colony formation was observed by Izant and Weintraub (21) in LTK⁻ cells transfected with β-actin antisense vectors.

Analysis of RNAs from pools of transfected cells with a probe that detects common vector sequences verified that antisense RNAs were being expressed (Fig. 1A). Differences in sizes of RNAs, in particular those shorter than the control pSVX vector-derived RNA, may be from generation of aberrantly spliced transcripts, as discussed by Korman et al. (22). We used labeled DNA probes specific for the inserted sequences to document that the antisense RNAs expressed contain sequences complementary to the inserted DNA (data not shown).

We have also evaluated the relative concentrations of clone 5 antisense RNA to clone 5 mRNA by densitometric scanning of RNA blots like that shown in Fig. 1B. No antisense clone 5 RNA is detectable in 3T3-L1 cells transfected with the control vector pSVX alone (Fig. 1B, lanes a and b), whereas in cells transfected with pSVX-Cl5(as) (Fig. 1B, lanes c and d), the ratio of clone 5 antisense to endogenous mRNA is ≈30/1 on day 0 and ≈6/1 on day +1. In a pool of TA1 cells
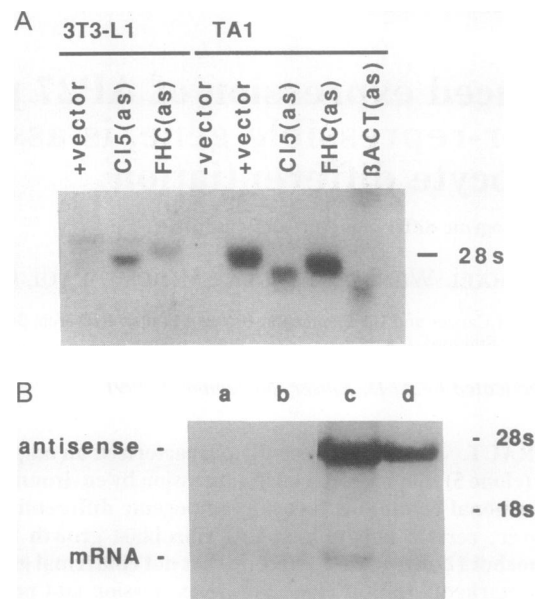

FIG. 1. Expression of antisense (as) RNAs in TA1 and 3T3-L1 cells. (A) Total RNA (4 μg per lane), isolated from 3T3-L1 and TA1 cells, either not transfected (−vector) or transfected (+vector) with pSVX, pSVX-Cl5(as) [Cl5(as)], pSVX-FHC(as) [FHC(as)], or pSVX-βACT(as) [βACT(as)] was electrophoresed through a formaldehyde/agarose gel, transferred to a membrane, and probed with a radiolabeled pSVX DNA probe. (B) Ten micrograms of whole-cell RNA isolated from 3T3-L1 cells transfected with pSVX (B, lanes a and b) or with pSVX-Cl5(as) (B, lanes c and d) was analyzed as above. RNAs were isolated on day 0 (lanes a and c) and day +1 (lanes b and d). Day 0 refers to the time at which cells reach confluence. The filter was probed with ^{32}P-labeled clone 5-specific DNA probe. The expected positions of clone 5 mRNA and antisense RNA, as well as those of 18s and 28s RNAs, are indicated.

expressing Cl5 antisense RNA, the ratio of antisense to endogenous mRNA ranges from ≈2/1 to 0.5/1 (data not shown). The increased ratio of antisense to sense RNA in 3T3-L1 cells is from an 8- to 9-fold lower expression of endogenous Cl5 mRNA in 3T3-L1 vs. TA1 cells (H.-M.W., unpublished observations).

We could not detect any significant difference in the amount of clone 5 mRNA in pools of cells transfected with either pSVX-Cl5(as) or the control vectors. Consistent with this result, Amini et al. (23) found that the expression of c-src antisense RNA does not alter c-src mRNA abundance, whereas the corresponding protein level is significantly decreased.

The effect of antisense RNAs on the morphological differentiation of 3T3-L1 cells was assessed by culturing the transformants in differentiation-inducing medium and then staining these cells with the lipid-specific dye oil red O (24). Compared to cells transfected with control vector pSVX (Fig. 2A, + vector), clone 5 antisense-expressing 3T3-L1 cells show a significantly decreased number of cells containing fat droplets, detected as dark-staining areas on the dishes (Fig. 2A, Cl5as). The few cells that still differentiate may result from very low expression of clone 5 antisense RNA or loss of the clone 5 DNA sequences within the vector. Lipid droplet-containing cells were counted from different pools of TA1 transformants and, as found for 3T3-L1 cells, there is a distinct reduction in the number of differentiated adipocytes in clone 5 antisense-expressing TA1 cells (Fig. 2B). This suppression of differentiation by clone 5 antisense RNA in TA1 cells was evident in cells that differentiated spontaneously and in cells in which the differentiation was accelerated by the synthetic glucocorticoid dexamethasone. The average difference in the number of mature adipocytes between

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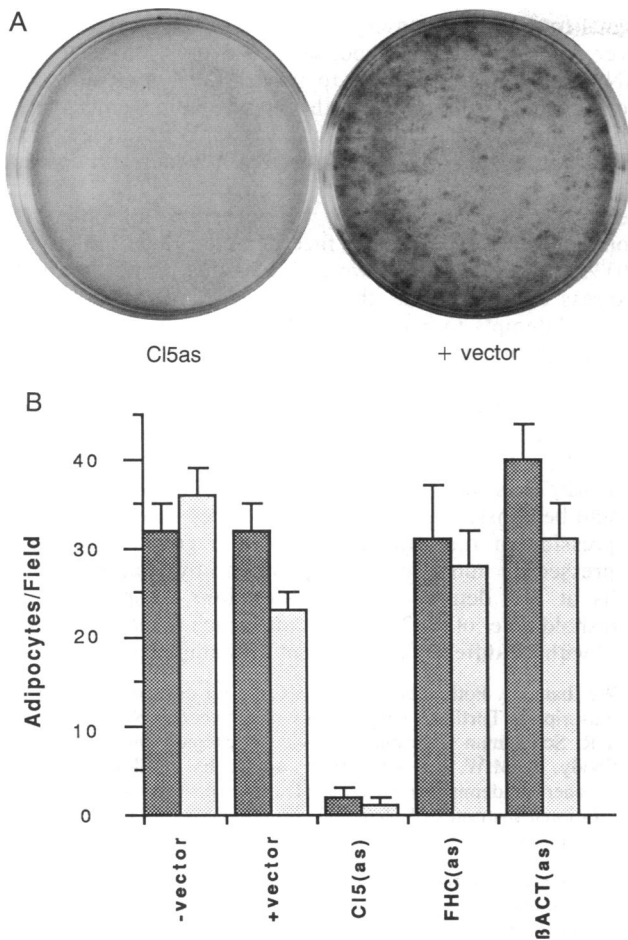
A

Cl5as   + vector

B

FIG. 2. Effect of antisense (as) RNA expression on differentiation of 3T3-L1 and TA1 cells. (A) 3T3-L1 cells, grown for 6 days in differentiation medium, were stained with oil red O (10) and photographed. Cells were transfected with either pSVX (+vector) or pSVX-Cl5(as) (Cl5as) as described in the legend to Fig. 1. (B) TA1 cells, transfected with the indicated vectors (see legend to Fig. 1), were monitored on day +12 (light bars) or day +6 (accelerated by dexamethasone, dark bars) for cells expressing the differentiated adipogenic phenotype. Ten randomly chosen fields under the microscope were screened for lipid droplet-containing adipocytes. These cells were counted, and the average of differentiated adipocytes was plotted. The SEM is indicated.

control cells and cells expressing clone 5 antisense RNA ranged from 13- to 18-fold in dexamethasone-treated cells and from 14- to 28-fold in untreated TA1 cells.

Although the results described above clearly indicate that the expression of clone 5 antisense RNA blocks the ability of adipogenic cells to accumulate lipid, they do not distinguish the mechanism by which the inhibition occurs. As an example of this, Spiegelman and Green (25) showed that when they blocked fatty acid synthesis (and thus lipid accumulation) in 3T3-F442A cells by depleting biotin from the culture medium, the increase in some key adipocyte RNAs, such as glycerophosphate dehydrogenase or fatty acid synthetase, was unaffected by the treatment (25). If clone 5 has a regulatory function (rather than simply participating in lipid synthesis or storage), then cells expressing antisense clone 5 RNA should not only fail to accumulate lipid droplets but should also fail to express adipocyte-specific RNAs. To determine this, RNAs were prepared from cells exposed to differentiation-inducing medium and were tested for the presence of adipocyte-specific RNAs. Fig. 3A shows the expression of two adipocyte genes, clone 28 [which is identical to aP2 (18)] and clone 47 (17) in 3T3-L1 cells transfected with pSVX, pSVX-
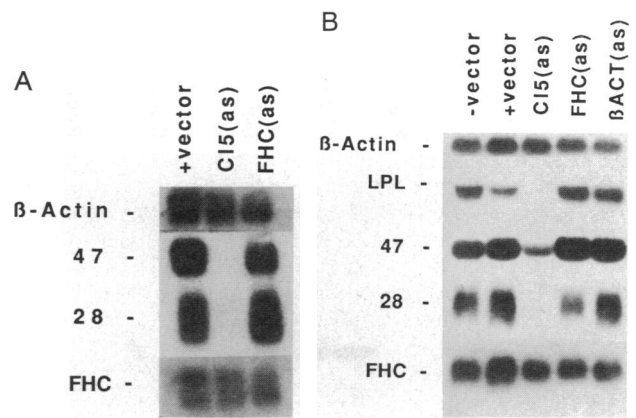
A   B

FIG. 3. Effect of antisense (as) RNA on expression of adipocyte-specific mRNAs. Four micrograms of whole-cell RNA isolated from 3T3-L1 (A) or 6 μg of RNA from TA1 (B) cells was electrophoresed and blotted as in Fig. 1. RNAs from 3T3-L1 cells were prepared on day +6 and from TA1 cells on day +3 after indomethacin treatment. Filters were probed with ^{32}P-labeled DNA probes specific for β-actin, lipoprotein lipase (LPL), adipocyte clones 47 and 28 (aP2), and murine FHC RNA.

Cl5(as), or pSVX-FHC(as). Only in those cells expressing clone 5 antisense RNA is the steady-state level of these two markers of differentiation dramatically reduced. Comparable results were obtained with TA1 cells (Fig. 3B), in which we also monitored the expression of a third adipocyte gene, that for lipoprotein lipase (16). The expression of β-actin RNA (which is expressed relatively independently of the differentiated state) and FHC RNA, which is only modestly induced during adipogenesis (19), is not affected by clone 5 antisense RNA expression in TA1 or 3T3-L1 cells. Based on normalization to β-actin expression, the expression of the three marker genes in pSVX-Cl5(as)-transfected TA1 cells is ≈15-fold lower than in cells transfected with pSVX. Similar results were obtained for other adipocyte marker genes, clone 10 (26) and glycerophosphate dehydrogenase (27) (data not shown). We have also isolated individual clones from pSVX and pSVX-Cl5(as)-transfected TA1 and 3T3-L1 cells. Each of five clones from TA1 and two clones from 3T3-L1 cells, transfected with pSVX or pSVX-Cl5(as), were isolated and analyzed for expression of clone 28 and clone 47 mRNA. Again, the expression of both marker genes was reduced in clone 5 antisense-expressing cells (data not shown). The Northern (RNA) blot experiments show that the failure of Cl5(as)-expressing cells to differentiate morphologically is reflected by the inability of these cells to express a class of genes normally induced during adipocyte differentiation (2, 28). The normal differentiation of cells expressing antisense RNAs directed against β-actin and FHC mRNA clearly shows the specificity of clone 5 antisense RNA in inhibiting the adipogenic differentiation. This result strongly suggests that the clone 5 gene product AP27 is a component of a pathway that initiates expression of this class of genes after the proper hormonal and environmental conditions are met.

If the level of AP27 were linked to the potential for adipogenic differentiation, we would expect that reduction in its concentration should result in impaired differentiation. However, because AP27 may only be one of many components of a differentiation switch, it is conceivable that in these experiments, inability to express adipogenic functions may result from a block at some other stage of the pathway in the face of normal AP27 levels. To investigate the extent of reduction in AP27 level resulting from expression of clone 5 antisense mRNA, a polyclonal antibody was generated and used to analyze a number of transfectants. The result of clone 5 antisense expression is not always reflected by a concom-
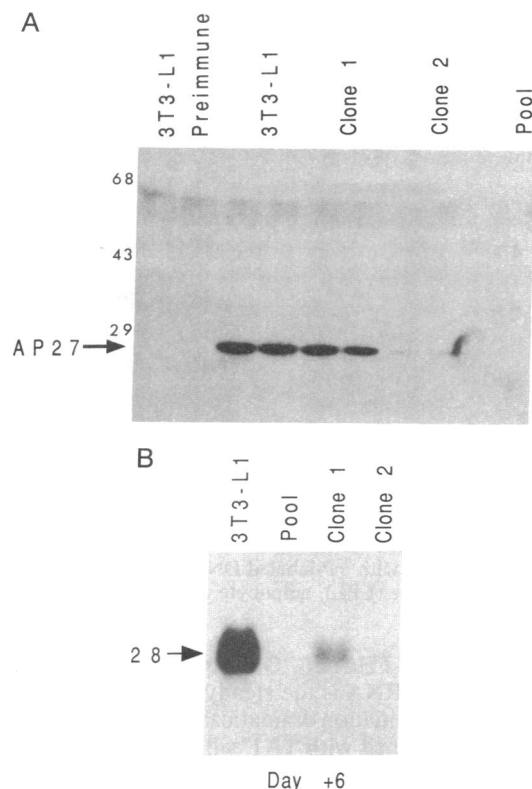
FIG. 4. Correlation between levels of AP27 and extent of differentiation. (A) AP27 levels. A rabbit polyclonal antiserum raised against clone 5 protein was used to immunoprecipitate protein in duplicate from extracts of preconfluent 3T3-L1-derived cell lines. These consisted of parental untransfected cells, a pool, and two independently derived clones transfected with pSVX-Cl5(as). As control, preimmune serum was also used to immunoprecipitate proteins from 3T3-L1 cells. The immunoprecipitated AP27 (arrow) was detected after gel electrophoresis by immunoblotting with the same polyclonal antibody followed by ^{125}I-labeled protein A. Numbers ($\times 10^{-3}$) indicate position of molecular weight markers. (B) RNA analysis. Extent of differentiation was investigated by analyzing 10 μg of total RNA from cells differentiated for 6 days with an RNA probe to clone 28 (aP2; arrow).

itant reduction in AP27 level. Fig. 4 illustrates the results from a pool and two independently isolated clones of stably transfected 3T3-L1 cells. The pool and clone 2 show greatly reduced levels of AP27 (Fig. 4A), fail to express clone 28 mRNA (Fig. 4B), and fail to accumulate lipid (data not shown). In contrast clone 1 exhibits modestly reduced levels of AP27 (Fig. 4A) and only some reduction of clone 28 mRNA expression. We note that in all clone 5 antisense RNA-expressing pools ($n = 3$) and clones ($n = 8$) in which the levels of AP27 are lower than in wild type, differentiation is impeded as assessed by the failure to express normal levels of the adipocyte-specific mRNA, clone 28, and the failure to accumulate a high density of lipid droplets.

In the aggregate these results lead us to propose that AP27, the product of the clone 5 gene, which is regulated by many environmental factors, must at a certain point reach a threshold level for differentiation to occur. From our observations, however, the absolute level required in any given cell (e.g., 3T3-L1 vs. TA1) may vary according to the levels of other as-yet-undefined components of the "commitment switch." The biochemical function of the protein is still unknown. The translated amino acid sequence, which most closely resembles a family of polyol dehydrogenases (29–31), suggests that it is unlikely to be a nuclear transcription factor but more likely to be a cytosolic or membrane-associated component (M.N., unpublished results) of an as-yet-uncharacterized

signal-transducing pathway. It should be emphasized, however, that we are not proposing that expression of clone 5 RNA is in itself sufficient to trigger the conversion of a nondetermined stem cell to the mature adipogenic phenotype. This result contrasts with the recently described genes MyoD, MRF4, Myf5, and for myogenin, which can convert C3H 10T½ cells into determined myoblasts (32–35), or a gene(s) recently reported to be necessary for adipocyte conversion (11). We have, in fact, detected clone 5 mRNA in 10T½ and 3T3-C2 cells, two cell lines that do not normally express the adipogenic phenotype (H.-M.W., unpublished data). Attempts to generate AP27-overexpressing cell lines by transfection with the pSVX-Cl5(sense) vector have failed to produce any stable transformants. Recent results indicate that AP27 is responsible for the absence of colony formation, because a frame-shift mutant gave rise to stable transformants (P.C., unpublished data). This observation may suggest that AP27 has an antiproliferative effect in these cells, which would be consistent with previous studies showing that the expression of the endogenous clone 5 gene is markedly depressed by mitogenic stimulation and by maintenance of cells at low density (9). We, therefore, propose that a threshold level of AP27 at cell confluence is closely associated with the differentiation potential of adipogenic cell lines.

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