A Growth Factor-repressible Gene Associated with Protein Kinase C-mediated Inhibition of Adipocyte Differentiation

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Abstract. The conversion of determined adipoblasts to fully differentiated adipocytes requires appropriate environmental conditions. A strict dependence on cell confluence and a facilitation by glucocorticoid hormones have previously been described. We have found that agents that are capable of activating protein kinase C, such as basic fibroblast growth factor and phorbol esters, inhibit the differentiation of the adipogenic cell line TA1 without stimulating cell growth. Here we describe the sequence and characterization of a cDNA (clone 5) that detects an RNA, the expression of which is enhanced by glucocorticoids and increasing cell density. In contrast, activators of protein kinase C including basic fibroblast growth factor, phorbol esters, and synthetic diacylglycerols inhibit clone 5 gene expression. It appears that clone 5 expression is closely linked to environmental and hormonal factors that promote the differentiation of adipogenic cells.

The hormonal environment in which a stem cell finds itself is likely to be a major determinant controlling its decision to continue replicating or to express its terminally differentiated phenotype. The process of differentiation is generally reflected by the de novo synthesis of gene products that define the specific functions of that cell type. Thus erythroid cells will express globins, B lymphocytes will express immunoglobulins, and myocytes will express novel contractile proteins. Although the mechanisms by which the respective genes are activated remain uncertain, it seems that, as a rule, progenitor cells do not express functions characteristic of the differentiated phenotype while actively growing. It is clear that environmental or hormonal signals must participate in directing the cell to withdraw from the cell cycle and to progress towards the differentiated state. For example, addition of mitogenic stimuli such as serum to myoblasts stimulates these cells to continue cycling and at the same time represses all morphological and biochemical markers of myogenic differentiation (Buckingham, 1977; Devlin and Konigsberg, 1983; Nguyen et al., 1983; Olson et al., 1983). Removal of mitogens is followed by the expression of such markers. Despite the apparent clarity of such phenomena, both the nature of the molecular switch that controls the decision to differentiate and the mechanisms by which the cell senses and communicates its environmental status to that switch remain obscure (Levenson and Housman, 1981).

We have been particularly interested in understanding the mechanisms by which hormonal cues dictate the ability (or inability) of adipogenic stem cells to differentiate into mature adipocytes. TA1 adipoblasts were isolated from the mesenchymal stem cell line, 10I/2, after treatment with 5-azacytidine (Chapman et al., 1984); Taylor and Jones (1979) first reported that 10I/2 cells have the potential to differentiate into myocytes, chondrocytes, and adipocytes after exposure to this drug. When maintained at low density, TA1 cells resemble fibroblasts and do not express gene products characteristic of the mature adipocyte. However, when maintained at confluence spontaneous differentiation occurs, a process that can be markedly accelerated by treatment with glucocorticoid hormones or the nonsteroidal antiinflammatory drug, indomethacin (Chapman et al., 1985; Knight et al., 1987).

In contrast, if cells are maintained at low density, even in the presence of an inducing agent such as indomethacin, differentiation-specific genes are not activated (Knight et al., 1987). Moreover, continual refeeding drives TA1 cells to proliferate but even at high density prevents them from expressing differentiated functions (Chapman, 1986; see below). Thus it appears that only when the cells are at high density and not under continual mitogenic stimulation will they undergo the transition to the differentiated state.

In this manuscript we describe the isolation and sequence of a cDNA (clone 5) that detects a glucocorticoid-inducible RNA in TA1 cells, the abundance of which increases with increasing cell density and decreases dramatically after treatment with FCS or basic fibroblast growth factor (bFGF).1

1. Abbreviations used in this paper: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; diC1, synthetic diacylglycerol, in 1,2-dioctanoylglycerol; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C; SSPE, 0.18 M NaCl, 10 mM Na2HPO4, pH 7.7, 1 mM EDTA.
We find that bFGF, as well as other agents that activate protein kinase C (PKC) prevents TA1 cells from differentiating and simultaneously inhibits the expression of clone 5 RNA. Suppression of clone 5 RNA expression by FGF or phorbol esters occurs in actively growing cells indicating that this effect is likely to be independent of the mitogenic activity of these factors. These studies suggest that activation of PKC prevents conversion of TA1 cells to the differentiated phenotype without inducing cell growth. Finally, although the following hypothesis is not directly tested by this work, the pattern of regulation of clone 5 gene expression leads us to speculate that the clone 5 protein may be required, though not sufficient, for the triggering of adipocyte differentiation.

Materials and Methods

Cell Culture

TA1 cells (Chapman et al., 1984) were grown in Eagle's basal medium (BME) (Gibco, Grand Island, NY) supplemented with 10% FCS (lot No. 507858, Irvine Scientific, Santa Ana, CA). All serum used was heat inactivated at 55°C for 30 min. Cultures were grown in either 35-mm wells (6-well plate) or 100-mm dishes at 37°C in a humidified incubator at 5% CO₂ atmosphere.

Acidic fibroblast growth factor (aFGF) and bFGF were a gift of Dr. D. Gospodorowicz (University of California, San Francisco). Crude platelet-derived growth factor (PDGF) was a gift of Dr. L. T. Williams (University of California, San Francisco). Epidermal growth factor (EGF) and partially purified bFGF were from Collaborative Research, Inc. (Bedford, MA). The phorbol esters tetradecanoylphorbol-13-acetate (TPA) and phorbidibutyrate were from Sigma Chemical Co. (St. Louis, MO); they were dissolved in DMSO at 1,000× concentrations so that the final DMSO concentration was never >0.1%. The synthetic diacylglycerol, sn-1,2-diacylglycerol (diC₂) was from Avanti Polar Lipids (Birmingham, AL). diC₂ was stored at 5,000× concentrations in DMSO. Dexamethasone and indomethacin (Sigma Chemical Co.) were made up in 95% ethanol and used at final concentrations of 1 μM and 125 μM, respectively.

RNA Isolation

Two methods were used for the isolation of RNA from TA1 cells. Total cellular RNA was isolated using the method of Chirgwin et al. (1979). Briefly, cells were suspended in 4 M guanidinium-thiocyanate, 25 mM sodium citrate, 0.2% N-laurylsarcosine, 0.2 mM 2-mercaptoethanol. The resulting cell lysate was layered on a 5.7 M cesium chloride solution and centrifuged at 80,000 g for 19 h. The RNA pellet was collected and its concentration was determined by reading absorbance at 260 nm. Cytoplasmic RNA was isolated as follows: cells were collected by trypsinization, washed once with PBS, and pelleted in a microfuge tube. The cell pellet was taken up in 250 μl of chilled lysis buffer (25 mM Tris, pH 7.4, 130 mM NaCl, 50 mM KCl, 3 mM MgCl₂, 1% NP-40) and vortexed for 30 s. The nuclei were pelleted by centrifugation for 1 min. The supernatant solution was brought to 0.2% SDS and 10 mM EDTA, and extracted with phenol/chloroform, then chloroform, and once more with phenol/chloroform. RNA was precipitated with NaCl and ethanol.

Analysis of RNA

5-10 μg of total RNA was treated with 2.2 M formaldehyde in 50% formamide, 10 mM NaH₂PO₄, pH 7.0, at 55°C for 15 min. Samples were then subjected to electrophoresis in a 1.4% agarose-formaldehyde gel containing 2 M formaldehyde and 10 mM NaH₂PO₄, pH 7.0, at 55°C for 15 min. The gel was then transferred to a nitrocellulose filter (Schleicher & Schuell) and subjected to fivefold in the adipocyte, and in both adipoblasts and adipocytes is induced two- to threefold by dexamethasone. The initial isolation of the clone 5 cDNA was from a TA1 library, prepared from dexamethasone-treated cells, which was differentially screened with radioactive probes from low-density adipoblasts (in which clone 5 RNA is extremely scarce; see below) and dexamethasone-treated adipocytes.

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Clone 5 RNA Levels Are Regulated by Cell Density and Serum Factors

The abundance of clone 5 RNA in TA1 adipoblasts increases as cells become more dense. For example, cells harvested 5 d before confluence (day -5) contain approximately four- to sixfold less clone 5 RNA than do cells at day 0 (Fig. 2 A). Similarly, when TA1 cells are plated at various dilutions and harvested 48 h later, the lower density cells contain less clone 5 RNA (Fig. 2 B). This could reflect the secretion of an autocrine factor that induces clone 5 RNA and/or the depletion of a component in the growth medium that suppresses clone 5 RNA expression. A strong suggestion that the latter is true is afforded by the observation that when cells are fed fresh medium, the levels of clone 5 RNA decrease fivefold within the subsequent 24-h period. Maximal suppression occurs ~16-18 h after feeding and a return to the prefed levels of clone 5 RNA is seen within 48-72 h (not shown).

To test the role of various components of the growth medium on clone 5 RNA expression we established a standardized experimental protocol: TA1 cells were plated at day -5 or -4 and 3 d later the cells were either refed (removal of old medium followed by replacement with fresh medium containing BME + 10% FCS) or treated by addition of test substances directly to the 3-d-old medium. Control dishes were either left undisturbed or refed with 3-d-old medium. Total RNA was harvested 24 h later and analyzed for clone 5 RNA levels. As seen in Fig. 3 A, the effect of refueling could be duplicated completely by addition of fresh serum.

To determine what factor(s) in serum might inhibit clone 5 expression, either modified serum or purified serum components were added to TA1 cells in the standardized assay described above. Initial experiments (not shown) indicated that whereas dialyzed serum suppresses clone 5 RNA expression, charcoal-extracted serum and insulin (at 1 μM) do not. Fig. 3 A also summarizes the various combinations of components and purified factors that we tested. Both bFGF and aFGF as well as PDGF maximally (approximately fivefold) suppress clone 5 RNA levels whereas EGF either in the presence or absence of insulin does not. As noted above, the suppression of clone 5 RNA by serum is transient; therefore, we tested whether the recovery of clone 5 RNA could be attributed to inactivation of FGF- or PDGF-like material in serum. Fresh medium (BME + 10% FCS) was incubated in tissue culture dishes (at 37°C, 5% CO₂) without cells for 24 or 48 h. As seen in Fig. 3 B preincubated medium loses its ability to suppress clone 5 RNA expression, thereby substantiating the notion that it is a labile factor in serum that is responsible for the refueling effect we have described.

In light of these results, one might question conclusions derived from the density experiment described earlier (Fig. 2 B). The higher levels of clone 5 RNA in the denser cultures could be attributed to the more rapid consumption of growth factors by these cells, followed by an earlier recovery from the inhibitory effect on clone 5 expression. To dissociate the effect of serum factors on clone 5 expression from the effects of cell density, we performed the following experiment: TA1 adipoblasts at day -1 from two dishes were seeded into twenty dishes, half of which received fresh medium and the other half “inactivated” medium which had been incubated for 48 h as described above. As shown in Fig. 4, the level of clone 5 RNA in these cells is reduced fivefold in both cases after seeding. For the cells seeded into fresh medium, this reduction in clone 5 RNA is not recovered. For the cells seeded into aged medium, the effect must be attributed to the effects of change in cell density due to trypsinization, since the medium alone is incapable of affecting clone 5 levels. Moreover, we see that the level of clone 5 RNA increases as the cells become more dense (at 48 and 72 h). Once again, this cannot be attributed to a recovery from inhibition by serum. By 72 h, the cells have
A Role for PKC in Control of Clone 5 Gene Expression

Both bFGF and PDGF have been shown to induce turnover of phosphatidylinositol thereby generating diacylglycerol and inositol triphosphate (Habenicht et al., 1981; Tauda et al., 1985). Since many of the actions of these hormones seem to be mediated by activation of PKC with diacylglycerol (Berridge, 1984; Nishizuka, 1984) we tested whether this might also be true of clone 5 RNA expression. Addition of exogenous activators of PKC, such as the phorbol esters TPA or phorbol dibutyrate to TA1 cells led to a dramatic reduction of clone 5 RNA levels (Fig. 3 C). The concentration of TPA required to suppress clone 5 RNA expression is in the range reported to be sufficient to activate PKC (Nishizuka, 1984). Finally, the use of diCs, when applied at dosages known to activate PKC in other systems (Ebeling et al., 1985, Ganong et al., 1986) also reduces clone 5 expression to the same extent as serum (Fig. 3 C). Thus we surmise that the ability of FGF and PDGF to inhibit clone 5 gene expression is most likely due to the activation of PKC, though other signal transduction pathways may also play a role.

Growth Factor Inhibition of TA1 Cell Differentiation

TA1 adipoblasts will not differentiate if maintained at low density (Knight et al., 1987) or if chronically stimulated with fresh complete medium (Chapman, 1986). Under the latter situation the cells continue to proliferate and do not express any markers of the differentiated phenotype. To test whether any of the growth factors that suppress clone 5 gene expression interfere with differentiation, TA1 cells were induced to differentiate by addition of indomethacin at day 0. In the absence of any further additions >95% of the cells differentiated within 3 d as determined by detection of accumulated lipids with oil red O (not shown) and induced active expression of clone 28 RNA (Fig. 5 A). However, addition of 10% FCS, bFGF, or TPA every 24 h suppresses the induction of clone 28 RNA at day 3 (Fig. 5 A). However, addition of 10% FCS, bFGF, or TPA every 24 h suppresses the induction of clone 28 RNA at day 3 (Fig. 5 A). and morphological differentiation of the cells (not shown). In contrast, neither EGF nor low concentrations of serum have much effect on the accumulation of clone 28 RNA. By slightly altering the differentiation assay, we can accentuate the inhibitory effects of bFGF and TPA on adipocyte differentiation. In the experiment shown in Fig. 5 B, the cells were reseeded 1 d before confluence. Indomethacin was added on day 0, along with bFGF or TPA. The daily addition of bFGF (1.0 ng/ml) or

Figure 3. Inhibition of clone 5 RNA accumulation by medium components and purified factors. TA1 cells were treated on day -2 or -1 with the indicated agents. 24 h later, cytoplasmic RNA was isolated from the cells and quantitatively analyzed for clone 5 RNA levels using the “slot blot” technique described in Materials and Methods. In all cases, cells were seeded on day -5 and not refed unless indicated below. (A) The added components were 10% FCS, aFGF at 10 ng/ml, bFGF at 1.0 or 0.1 ng/ml, PDGF at 10 ng/ml, 100 ng/ml EGF in the presence or absence of 1 μM insulin, or untreated cells (NT). (B) On day -2, medium was removed from TA1 cells, which were refed with medium pretreated for 24 or 48 h (see text), or with the same medium which was just removed from the dish. RNA was analyzed as above. (C) Inhibition by direct activators of PKC. On day -2 or -1, the indicated compound was added to the cells and RNA isolated 24 h later. The tested agents were 1 μM phorbol dibutyrate (PDB) or TPA at the concentrations shown. In the case of diCs, cells were treated initially with 50 μM (50/20) or 2 μM (2/2) diC8, and then treated six more times, every 2 h with 20 μM (50/20) or 2 μM (2/2) diC8. Cells were harvested 18 h after the initial treatment and analyzed for clone 5 RNA as described above.

It is particularly noteworthy that the effect of FGF or PDGF on clone 5 expression occurs in actively growing cells and that serum, depleted of clone 5 inhibiting activity by treatment with charcoal or preincubation, still supports the active growth of TA1 cells. Thus it appears that the suppression of clone 5 gene expression is not simply due to mitogenic stimulation of the cells.
TPA (100 nM) significantly suppressed the expression of the RNAs corresponding to clone 28 and clone 1 (another adipocyte-specific cDNA used for monitoring differentiation [Chapman et al., 1984]). When applied at concentrations that do not inhibit clone 5 expression (Fig. 3), bFGF (0.1 ng/ml) and TPA (1 nM) did not affect adipogenesis. Thus those agents that (at appropriate concentrations) inhibit clone 5 RNA expression are also those that suppress differentiation of TA1 cells.

The inhibition of differentiation by the various agents described above could be explained if the adipoblasts are stimulated to reenter the cell cycle and proliferate. In the case of daily additions of serum, this appears to be what happens. At the time of harvest, serum-treated cultures consistently have two- to threefold the number of cells per dish (2.6 × 10^6 cells/plate) as the untreated cells (1.1 × 10^6 cells/plate). In contrast, TA1 cultures treated with bFGF or TPA have the same number of cells as the untreated cultures (1.1 × 10^6 cells/plate). Thus, as is true for suppression of clone 5 RNA, inhibition of differentiation by growth factors or TPA cannot be attributed to mitogenic stimulation through the cell cycle.

**Sequence of the Clone 5 cDNA**

To characterize the clone 5 gene product, we rescreened the TA1 adipocyte cDNA library prepared by Chapman et al. (1984) and isolated a clone containing an insert of ~1,000 base pairs (the mRNA is ~1.1 kb in length). The inserts

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2. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number X07411.

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**Figure 5.** Inhibition of adipogenic differentiation by serum and growth factors. (A) Cultures of TA1 cells (not fed since seeding at day −5) were refed and treated with indomethacin on reaching confluence (day 0). In addition, the cells were treated with either 10% serum, 1% serum, bFGF at 10 ng/ml, 1 μM TPA, or no treatment (NT). These additions (but not indomethacin) were repeated on days 1 and 2. RNA was isolated on day 3, and the extent of differentiation determined by analyzing clone 28 levels (as in Fig. 1). (B) TA1 cells (not fed since seeding at day −5) were refed on day −1 and treated with indomethacin upon reaching confluence (day 0). Highly purified bFGF at 1 ng/ml or 0.1 ng/ml, or TPA at 100 or 1 nM were added along with the indomethacin, and repeated on days 1 and 2. RNA was isolated on day 3 and the extent of differentiation determined by analyzing the levels of clones 1 and 28.

**Figure 6.** Nucleotide sequence of clone 5 cDNA. The sequence is numbered from the 5′ end of the cDNA clone. Translation of the open reading proposed to be the clone 5 gene product is shown below the nucleotide sequence and encodes a peptide of 244 amino acids. The putative polyadenylation site (AAATAAA) is underlined. The inhibition of differentiation by the various agents described above could be explained if the adipoblasts are stimulated to reenter the cell cycle and proliferate. In the case of daily additions of serum, this appears to be what happens. At the time of harvest, serum-treated cultures consistently have two- to threefold the number of cells per dish (2.6 × 10^6 cells/plate) as the untreated cells (1.1 × 10^6 cells/plate). In contrast, TA1 cultures treated with bFGF or TPA have the same number of cells as the untreated cultures (1.1 × 10^6 cells/plate). Thus, as is true for suppression of clone 5 RNA, inhibition of differentiation by growth factors or TPA cannot be attributed to mitogenic stimulation through the cell cycle.

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been assumed to be true. It is only recently, however, that the ATP-binding domains of various protein kinases and the molecular and biochemical details associated with differentiation. In the case of steroid hormones, whose receptors act as regulators of gene transcription (Yamamoto, 1985), effects on differentiation are presumed to depend on activation (or inactivation) of a gene(s) whose product is intimately associated with the processes of adipocyte-specific gene transcription and high cell density increase the expression of clone 5 RNA. Moreover, factors that inhibit clone 5 expression such as bFGF or phorbol esters also inhibit adipogenic differentiation.

Some aspects of the control of clone 5 gene expression by growth factors deserve particular attention. First, the direct inhibition of gene expression by bFGF and PDGF (i.e., not separable from indirect effects on differentiation-dependent gene expression) has not, to the best of our knowledge, been actively studied. Our results suggest that, as is the case for PDGF-mediated induction of c-myc and c-fos (Coughlin et al., 1985; Kaibuchi et al., 1986), the repression of clone 5 expression by bFGF or PDGF is mediated by activation of PKC. Second, the effects of bFGF or PDGF on clone 5 expression appear to be separable from their mitogenic activity. Suppression of clone 5 gene expression by these factors (or TPA) occurs in TA1 cells already stimulated to grow by serum mitogens. Similarly, the inhibition of differentiation by these same agents occurs under conditions in which they do not stimulate cell growth. It therefore seems clear that the inhibition of TA1 cell differentiation by bFGF or TPA cannot simply be ascribed to the mitogenic activity of these agents.

The finding that known mitogens inhibit adipogenic differentiation without inducing cell proliferation is not unique to the TA1 cell type. Similar results have been observed with another adipogenic cell line, 3T3-L1. Hayashi et al. (1981) demonstrated that the addition of highly purified bFGF or PDGF to 3T3-L1 adipoblasts inhibited adipogenic conversion. On the other hand, EGF, even at high concentration, had no effect on 3T3-L1 differentiation. Moreover, the authors noted that bFGF and PDGF, both mitogens of 3T3 cells, also had little effect if any on cell multiplication under the assay conditions used. Similarly, Diamond et al. (1977) and Shimizu et al. (1983) have shown that TPA treatment of 3T3 adipocytes also inhibits adipogenesis. These authors also found that the addition of TPA to 3T3 preadipocytes did not force those cells to divide. Thus the mechanisms by which these factors inhibit differentiation appears to be independent of their effects on cell proliferation.

The effect of growth factors on differentiation is not limited to adipogenic lines. The differentiation of many myogenic cell lines has been shown to be sensitive to FGF. As an example, Clegg et al. (1987), while studying the FGF-mediated inhibition of MM14 myoblast differentiation, found that in the absence of serum, FGF would block myogenesis without inducing cell replication, causing the cells to stop cycling in

Figure 7. Amino acid sequence homology between the putative clone 5 protein and E. aerogenes ribitol dehydrogenase (Moore et al., 1978). The upper line shows the sequence of the clone 5 open reading frame; the lower line shows the ribitol dehydrogenase sequence. The numbering for each sequence is also shown. : indicates identical amino acid matches. The match was prepared using the BIONET program ALIGN.

12 amino acids later by lysine; this motif is characteristic of the ATP-binding domains of various protein kinases and ATPases (Kamps et al., 1984).

Discussion

That hormones control aspects of differentiation has long been assumed to be true. It is only recently, however, that well-defined systems have become available to study the molecular and biochemical details associated with differentiation. In the case of steroid hormones, whose receptors act as regulators of gene transcription (Yamamoto, 1985), effects on differentiation are presumed to depend on activation (or perhaps repression) of regulatory genes (Ringold, 1985) whose products control one or more critical steps in the differentiation process.

We have previously documented that glucocorticoid hormones accelerate the conversion of preadipocytes to adipocytes in the cell line TAI (Chapman et al., 1985, Knight et al., 1987). The hormonal effect is associated with the precocious activation of adipocyte-specific gene transcription whereas the absolute level of gene expression that is eventually achieved is equivalent in the presence or absence of hormone. Thus we have hypothesized that glucocorticoids regulate a gene(s) whose product is intimately associated with the triggering of the conversion from preadipocytes to adipocytes; such a gene product would need to reach a threshold level to initiate the biochemical processes associated with differentiation (Ringold et al., 1986). While glucocorticoids regulate the rate at which the TAI cells progress to the differentiated state, other environmental factors influence the decision to continue proliferation or to withdraw from the cell cycle in a manner permissive for differentiation. Knight et al. (1987) have for example demonstrated that preconfluent cultures of TAI cells show little ten-
G₁. In a parallel set of studies, Lathrop et al. (1985a, b) have shown aFGF can dedifferentiate the reversibly differentiated myogenic cell line, BC₃H₁I. As with the MM14 myoblasts, the addition of FGF to these cells does not induce them to traverse the S phase of the cell cycle when kept quiescent under low serum conditions. Indeed Lathrop et al. (1985b) have provided evidence that aFGF causes quiescent BC₃H₁I myoblasts to exit from G₀ (a point in the cell cycle permissive for differentiation) and to become restricted at a new point in G₁ where the cells are incapable of expressing the differentiated phenotype.

All of these results indicate that factors known to activate PKC are capable of suppressing the differentiated phenotype in several cell types, but without affecting cell proliferation. Thus it could be proposed that it is the growth factor receptor-mediated activation of PKC that inhibits differentiation. Currently, there is no well-defined role for PKC activity in cells which is activated by mitogenic factors. For example, it has been shown that while FGF and PDGF activate PKC in many fibroblast lines, depletion of the enzyme by phorbol ester pretreatment of the cells does not eliminate the mitogenic potential of the peptides (Coughlin et al., 1985; Kabiuchi et al., 1986). We would suggest that the role of PKC activation in these cells is to convey a signal that cell cycle withdrawal (entry into G₀) is not permissible. In the specific case of TA1 (and possibly 3T₃-LI) cells, this might be accomplished by the down-regulation of clone 5 RNA levels.

The data presented here provide a strong association between clone 5 RNA expression and the ability of TA1 cells to differentiate. However, direct tests will be required to assess whether the expression of the clone 5 gene product at a certain threshold level is required for the triggering of adipocyte differentiation. Identification of the clone 5 protein and determination of its intrinsic enzymatic or regulatory functions present avenues for further investigation.

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