Terminal differentiation of stem cells is characterized by cessation of cell proliferation as well as changes in cell morphology associated with the differentiated state. For adipocyte differentiation, independent lines of evidence show that the transcription factors peroxisome proliferator activated receptor \( \gamma \) (PPAR\( \gamma \)) and CCAAT/enhancer-binding protein \( \alpha \) (C/EBP\( \alpha \)) as well as the tumor suppressor retinoblastoma (Rb) protein are essential. How these proteins promote adipocyte conversion and how they function cooperatively during the differentiation process remain unclear. We have used retinoic acid (RA) inhibition of adipogenesis to investigate these issues. RA blocked adipogenesis of 3T3-L1 cells induced to differentiate by ectopic expression of PPAR\( \gamma \) and C/EBP\( \alpha \) independently or together. However, under these circumstances RA was only effective at preventing adipogenesis when added prior to confluence, suggesting that factors involved in regulation of the cell cycle might play a role in establishing the commitment state of adipogenesis that is insensitive to RA. During differentiation of wild type 3T3 L1 preadipocytes, we found that Rb protein is hyperphosphorylated early in adipogenesis, corresponding to previously quiescent cells re-entering the cell cycle, and later becomes hypophosphorylated. The data suggest that, together with the coexpression of PPAR\( \gamma \) and C/EBP\( \alpha \), permanent exit from the cell cycle establishes the irreversible commitment to adipocyte differentiation.

The molecular mechanisms relating to cell proliferation and cell differentiation are inadequately understood. Adipocyte conversion provides an excellent model system to study terminal differentiation. In the case of 3T3-L1 cells, differentiation is induced upon exposure of cells to a mixture of hormonal stimuli, including dexamethasone, isobutylmethylxanthine, insulin, and fetal calf serum (1, 2). These pharmacological stimuli, or alternatives such as thiazolinediones or other activators of peroxisome proliferator activated receptors (PPARs)\(^1\) (3–6), are necessary for adipocyte differentiation of 3T3-L1 cells. During adipocyte conversion, a variety of transcription factors are induced, including C/EBP\( \beta \), PPAR\( \gamma \), and C/EBP\( \alpha \) (reviewed in Ref. 7). Enforced expression of PPAR\( \gamma \) (8), C/EBP\( \alpha \) (9–11), or C/EBP\( \beta \) (10, 12, 13) stimulates adipogenesis in NIH 3T3 fibroblasts, suggesting the essential roles of these transcription factors in regulating adipogenesis. Furthermore, combined expression of PPAR\( \gamma \) and C/EBP\( \alpha \) has synergistic effects on promoting fat cell conversion in myoblasts (14). Therefore, it is likely that PPAR\( \gamma \) and C/EBP\( \alpha \) function cooperatively to establish terminal adipocyte differentiation.

In addition to the expression of differentiated marker genes, terminal differentiation is characterized by permanent withdrawal of cells from the cell cycle. One protein that is involved in cell cycle progression is the retinoblastoma susceptibility gene product, Rb (15). Hypophosphorylation of Rb inhibits cell cycle progression, and this inhibitory effect of Rb is lost upon phosphorylation of the protein (16, 17). The involvement of Rb in adipocyte differentiation is suggested by the observation that ectopic expression of protein kinase C\( \gamma \) in quiescent NIH 3T3 cells induces hypophosphorylation of Rb and promotes adipogenesis (18), whereas Rb binding by SV40 large T antigen interferes with adipogenic differentiation (19). Moreover, Rb\(^{–/–}\) mouse embryonic lung fibroblasts failed to undergo adipocyte differentiation under appropriate conditions, and ectopic expression of Rb restored the adipogenic phenotype of the Rb\(^{–/–}\) cells (20). Together, these observations suggest an essential role of Rb in adipocyte differentiation.

To explore early events in adipogenesis, we have used retinoic acid (RA), which normally inhibits adipocyte differentiation of 3T3-L1 cells (21–23). Liganded RAR blocks C/EBP-stimulated gene transcription, and RA also prevents adipogenesis due to ectopic expression of C/EBP\( \alpha \) or \( \gamma \) (13). However, during normal adipogenesis RA exerts its inhibitory function only when added in the first 24–48 h after exposure to differentiating stimuli that are applied postconfluence. The inhibitory function of RA is mediated by RA receptors (RARs), which are down-regulated early in adipocyte differentiation (23). However, ectopic expression of RAR in 3T3 L1 cells only extends the period during which RA is effective in preventing adipogenesis by an additional 24–48 h. Interestingly, at this time, although PPAR\( \gamma \) is already induced, the level of PPAR\( \gamma \) protein is diminished upon RA treatment. This could be explained by the fact that RA/RAR inhibits C/EBP function, which may be responsible for maintaining the level of PPAR during differentiation (23).

We first examined the effect of RA on adipocyte differentiation due to incubation of wild type 3T3-L1 cells with the PPAR\( \gamma \) ligand BRL49653, as well as in 3T3-L1 cells that ectopically express PPAR\( \gamma \). RA inhibited PPAR\( \gamma \) activator-mediated fat cell conversion, suggesting that RA blocks adipogenesis due to endogenous PPAR\( \gamma \). The inhibitory effects of RA were also
dominant over ectopic co-expression of both PPARγ and C/EBPα when the cells were maintained in RA at the time of gene transduction and thereafter. However, if added after gene transduction but rather at the time of confluence, RA was no longer effective at blocking differentiation of 3T3-L1 cells that ectopically expressed these adipogenic transcription factors. Thus, co-expression of PPARγ and C/EBPα in cells was not sufficient to commit cells to undergo differentiation in the presence of RA until the cells became postconfluent. Since confluency is associated with withdrawal from the cell cycle, we hypothesized that the stage of irreversible commitment to adipocyte differentiation required exit from the cell cycle as well as the co-expression of both PPARγ and C/EBPα. Indeed, during adipocyte differentiation of wild type 3T3-L1 cells, confluent cells undergo clonal expansion followed by permanent withdrawal from the cell cycle that occurs at about the time RA loses effectiveness in preventing differentiation. During this process Rb protein shifts from a highly phosphorylated state to its hypophosphorylated form. We conclude that the state of RA-resistant commitment to adipocyte differentiation involves not only expression of PPARγ and C/EBPα but also hypophosphorylation of Rb and withdrawal from the cell cycle.

MATERIALS AND METHODS

Cell Culture and Differentiation—3T3-L1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in growth medium (GM) containing 10% iron-enriched fetal bovine serum in Dulbecco’s modified Eagle’s medium. For standard adipocyte differentiation, 2 days after cells reached confluency (referred as day 0), cells were exposed to differentiation medium (DM) containing 10% fetal bovine serum, 1 μM dexamethasone, and 0.5 μM isobutylmethylxanthine, for 48 h. Cells then were maintained in postdifferentiation medium containing 10% fetal bovine serum, and 10 μg/ml of insulin. RA was dissolved in ethanol and used at a concentration of 10 μM. For BRL49653-induced adipogenesis, cells were maintained in GM. 1 μM BRL49653 was added to cells on day 0. Cells were exposed to BRL49653 constantly for 7–10 days until fat cells were seen. For experiments involving RA, retrovirally infected cells were studied in the following two protocols: 1) cells were maintained in the constant presence of 10 μM RA from the time of infection and exposed to the presence or absence of 1 μM BRL49653 at day 0, or 2) cells were grown in the absence of RA until day 0 and then exposed to RA and various conditions. The first of these two conditions corresponds to that described previously (13).

Construction of Plasmids and Retroviral Infection—pLXSN-PPARγ was generated by insertion of a 1.26 kilobase pair HI fragment of C/EBPα into the BamHI site of pTS13 vector (HgmB). Standard calcium phosphate-DNA transfections were performed. To generate retrovirus-producing packaging cells, 293T cells were transfected with 7.5 μg of plasmid DNA and viral gag and pol plasmids (25). 48 h post-transfection, filtered viral supernatants from the retroviral packaging cell line were used to infect 3T3-L1 cells. Two days after infection, cells were selected in G418 (400 μg/ml; Life Technologies, Inc.) or hygromycin B (200 μg/ml) for 10–14 days. For double infection, pLXSN-PPARγ cells were infected with virus containing TS13-C/EBPα into the BamHI site of the TS13 vector (HgmBα). Standard calcium phosphate-DNA transfections were performed. To generate retrovirus-producing packaging cells, 293T cells were transfected with 7.5 μg of plasmid DNA and viral gag and pol plasmids (25). 48 h post-transfection, filtered viral supernatants from the retroviral packaging cell line were used to infect 3T3-L1 cells. Two days after infection, cells were selected in G418 (400 μg/ml; Life Technologies, Inc.) or hygromycin B (200 μg/ml) for 10–14 days. For double infection, pLXSN-PPARγ cells were infected with virus containing TS13-C/EBPα for 48 h. Cells then were selected in G418 plus hygromycin growth medium.

Oil Red O Staining—Dishes were washed three times with phosphate-buffered saline, fixed by 10% formalin in phosphate buffer for 1 h at room temperature. After fixation, cells were washed once with phosphate-buffered saline and stained with a filtered oil red O stock solution (0.5 g of oil red O (Sigma) in 100 ml of isopropyl alcohol) for 15 min at room temperature. Cells then were washed twice with water for 15 min each and visualized.

Western (Immunoblot) Analysis—Lysates from 3T3-L1 cells were lysed in cell lysis buffer (500 μl for the 10-cm dish and 150 μl for the 60-mm dish), and cells were incubated on ice for 30 min, followed by centrifugation at 17,000 rpm at 4 °C for 30 min. Supernatant was collected, and protein concentration was determined by Bio-Rad protein assay. 60–100 μg of protein was subjected to 10% polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane, and Ponceau-S (Sigma) staining was performed to verify equal loading/transfer. The membrane was incubated first with primary antibody (anti-PPARγ, 1:1500 (23); anti-C/EBPα, 1:300 (Santa Cruz Biotechnology, Inc.)) for 2 h, followed by secondary antibody (horseradish peroxidase-conjugated) for 1 h. Blots were developed using ECL chemiluminescence detection reagent (Amer sham Life Sciences, Inc.) and visualized by exposure to autoradiography film.

BrdUrd Labeling Assay—Cells were incubated with BrdUrd (Amer sham Corp.) for 24 h and then trypsinized, washed twice in phosphate-buffered saline, and fixed with 70% ethanol on ice for 30 min or longer. Fixed cells were incubated with anti-BrdUrd and then fluorescein isothiocyanate-conjugated secondary antibody. BrdUrd-positive cells were sorted by flow cytometry.

RESULTS

RA Inhibits PPARγ Ligand-mediated Adipogenesis in 3T3 L1 Cells—During DM-induced 3T3-L1 differentiation, RA is only effective in preventing adipogenesis when added during the first 24–48 h (day 0 and day 1), when PPARγ has not yet been induced (7). Indeed, under circumstances when PPARγ is already expressed, RA can lead to the loss of expression of PPARγ, consistent with its ability to block the transcriptional activity of C/EBPβ, which induces PPARγ during adipogenesis (12, 23). To further the use of RA as a tool for understanding the stages of adipocyte differentiation, we first tested the ability of RA to affect the adipogenesis of 3T3-L1 cells that is induced by the PPARγ activator, BRL49653. BRL49653 induces adipogenesis of post-confluent 3T3-L1 cells, typically causing 20–70% of cells to differentiate into adipocytes within 7–10 days (26). This is presumably caused by the activation of an endogenous low level of PPARγ in the preadipocytes (23) and subsequently the activation of the differentiation program and fat cell conversion. These results were confirmed in Fig. 1 (column 1, row 2), where fat cell differentiation is indicated by
oil red O staining. RA was able to inhibit BRL49653-induced differentiation (Fig. 1, column 1, row 3). Fig. 2 shows that BRL49653 treatment induced both PPARγ and C/EBPα and that, consistent with the oil red O staining, this induction was blocked by RA. Note that adipocytes express two forms of C/EBPα, referred to in this paper as C/EBPα-LAP and C/EBPα-LIP using nomenclature derived from similar alternative translation products for C/EBPβ (27).

**RA Inhibits PPARγ-induced Adipogenesis**—Since RA blocked PPARγ induction by BRL49653, we next examined whether expression of PPARγ would be sufficient to establish insensitivity to RA by testing the efficacy of RA on cells that are ectopically expressing PPARγ (designated as L1-PPARγ2). Fig. 3A shows that these cells constitutively express PPARγ2 protein. In a typical experiment, approximately 20–50% of L1-PPARγ2 cells spontaneously differentiated into fat cells in the absence of any adipogenic stimuli in 7–10 days postconfluence, due either to a low level of endogenous ligand or constitutive activity of the ectopic PPARγ (see Ref. 28 and Fig. 1, column 2, row 1). The addition of the PPARγ ligand BRL49653 accelerated and enhanced the differentiation process, with nearly 100% of cells differentiating into fat cells. This is shown by oil red O staining in Fig. 1 (column 2, row 2). In addition, Fig. 3 shows that the adipocyte markers C/EBPα-LAP and C/EBPα-LIP were markedly induced (lane 5). However, when maintained in the presence of RA, cells that were grown in GM with or without the PPARγ ligand BRL49653 did not convert into fat cells, documented both by oil red O staining (Fig. 1, column 2, row 4) and C/EBPα expression (Fig. 3, lanes 6 and 7). The presence of RA had no effect on the ectopic expression of PPARγ (Fig. 3, compare lanes 6 and 7 to lanes 4 and 5). These results suggest that RA is able to block adipogenesis induced by ectopic PPARγ protein expression, and expression of PPARγ is not able to override the inhibitory effect of RA on adipogenesis.

**RA Is Ineffective When Added to Postconfluent Cells That Ectopically Express PPARγ or C/EBPα**—Above we showed that RA blocks the function of PPARγ to induce fat cell conversion, suggesting that additional factor(s) may be necessary to be insensitive to RA. Previously we have shown that adipogenesis in cells that ectopically express either C/EBPα or C/EBPβ is also blocked when cells are maintained in the presence of RA from the onset of ectopic gene expression (Fig. 1 and Ref. 13). This was confirmed in Fig. 1 (column 3, row 3, where the ectopic C/EBPα-expressing cells are referred to as L1-C/EBPα cells). Interestingly, when RA was added not at the onset of ectopic expression of either PPARγ or C/EBPα, but rather at a later time when cells were confluent, it was no longer effective at blocking differentiation (Fig. 1, row 5). Constitutive expression of C/EBPα in the L1-C/EBPα cells is demonstrated in Fig. 4 (lanes 5 and 7). The vector used to ectopically express C/EBPα does not express the smaller translation product of C/EBPα, called C/EBPα-LIP (23). Thus, C/EBPα-LIP expression serves as a marker of adipogenesis in these experiments (29). Note that C/EBPα-LIP was undetectable in the L1-C/EBPα cells at day 0 (Fig. 4, lane 5), consistent with their lack of adipocyte phenotype. In contrast, continued incubation of the L1-C/EBPα cells for 9 days postconfluence did induce adipogenesis (Fig. 1 (column 3, row 1) and Fig. 4 (note C/EBP-LIP expression in lane 6)). Consistent with the cell morphology shown in Fig. 1, this adipose conversion of L1-C/EBPα cells was blocked when cells were grown in media containing RA (Fig. 1, column 3, row 3; Fig. 4, lane 8).

**PPARγ and C/EBPα Are Mutually Regulated**—Note that while allowing the cells to reach confluence prior to the addition of RA appeared to prevent the effects of RA on the ectopic C/EBPα- and PPARγ-expressing cells, RA normally prevents adipocyte differentiation of wild type 3T3-L1 cells. Those cells, however, do not express PPARγ or C/EBPα until they have begun to differentiate. Thus, the state of refractoriness to RA seems to require both confluency and expression of PPARγ and C/EBPα. Indeed, during normal 3T3-L1 differentiation, RA loses effectiveness at times when PPARγ and C/EBPα are expressed (23). Since C/EBPα (12, 13) and C/EBPβ induce PPARγ (12, 13), we considered the possibility that PPARγ and C/EBPα may induce each other, leading after some time to a state that is refractory to RA inhibition of adipogenesis. To determine whether PPARγ is able to activate C/EBPα gene expression. L1-PPARγ cells were collected prior to differentiation and subjected to Western analysis. Fig. 3 shows that a low but detectable level of C/EBPα protein was present in day 0 L1-PPARγ preadipocytes (lane 3). This is notably different from wild type 3T3-L1 cells, which do not express C/EBPα on day 0 (e.g. lane 1). Thus, ectopic PPARγ expression induced a low level of expression of C/EBPα. The C/EBPα expression was even greater on day 7, consistent with the adipocyte phenotype of these cells. Interestingly, RA blocked expression of C/EBPα despite continued expression of the ectopic PPARγ, suggesting that RA inhibits PPARγ induction of C/EBPα. This would be consistent with the results of others indicating that liganded RAR can interfere with PPAR-
mediated gene transcription (31).

In a reciprocal experiment we examined the expression of endogenous PPARγ in L1-C/EBPα cells. Fig. 4 shows that endogenous PPARγ was expressed in the day 0 L1-C/EBPα preadipocytes (lane 7), whereas expression of PPARγ was undetectable in day 0 control preadipocytes (lane 1). Expression of PPARγ in the L1-C/EBPα cells was abolished by treatment with RA from the time of C/EBPα expression (lanes 7 and 8), consistent with the ability of RA to inhibit transcriptional activation by C/EBPα (13).

RA Inhibits Adipogenesis of Preadipocytes Coexpressing Ectopic PPARγ and C/EBPα—The above results demonstrate that PPARγ and C/EBPα can activate each other’s expression. Therefore, it is likely that the inability of RA to prevent fat cell conversion in L1-PPARγ or C/EBPα cells when added postconfluency is due to the coexpression of PPARγ and C/EBPα. We next tested whether the requirement for confluence could be overcome by forcing cells to express both PPARγ and C/EBPα at higher levels. For these experiments, we doubly infected 3T3 L1 cells with LXSN-PPARγ (neo”) and TS13-C/EBPα (hygromycin”). These cells grew slowly, perhaps related to growth suppressive properties of C/EBPα in other cell lines (30); cells that express only ectopic C/EBPα grew somewhat slowly but closer to normal than those ectopically expressing both PPARγ and C/EBPα, suggesting that PPARγ provided a second growth-inhibitory signal in 3T3-L1 cells. In any case, the cells coexpressing C/EBPα and PPARγ spontaneously differentiated into adipocytes during 10 days of selection, even prior to confluence. However, when cells were selected and maintained in the presence of RA, they were able to grow to confluence at normal rates, and no fat cells were observed in the absence or presence of BRL49653 (Fig. 1, column 4, row f). The ability of RA to prevent differentiation of cells that ectopically express both PPARγ and C/EBPα suggested that concomitant expression of these two proteins was not sufficient to establish a commitment state to adipogenesis that is no longer responsive to RA.

PPARγ, C/EBPα, and Withdrawal from the Cell Cycle Are Necessary for Commitment to Adipogenesis—We demonstrated that RA is able to inhibit 3T3-L1 adipogenesis due to C/EBPα and PPARγ, alone or in combination, provided that RA is added at the time of gene transduction. In contrast, RA did not have an appreciable effect when added to cells postconfluency. Since confluence is associated with withdrawal of cells from the cell cycle and since cell proliferation and differentiation are often mutually exclusive events, we hypothesized that both cell cycle arrest and the expression of adipogenic genes are necessary for the commitment to fat cell differentiation. It is known that standard differentiation medium induces mitosis of quiescent 3T3-L1 cells prior to cell cycle withdrawal and completion of differentiation along adipogenic lineage. Although RA blocks differentiation, it does not prevent the mitosis and resultant increase in cell number due to the adipogenic stimulation (22). Fig. 5A shows the time course of the mitotic response to adipogenic stimulation in the absence and in the presence of RA, using BrdUrd incorporation as a measure of DNA synthesis. In both cases, a major increase in BrdUrd incorporation occurs on days 1 and 2 following adipogenic stimulation. Thus, the cells become postmitotic on day 3 and beyond. These data indicate that, although RA prevented adipocyte differentiation, it did not block the clonal expansion that occurs following adipogenic stimulation, although it appears to be quantitatively reduced. Note that in the presence or absence of RA, the adipogenically stimulated cells return to a quiescent state characterized by few mitotic events. However, these states are fundamentally different as shown in Fig. 5B. The RA-treated cells are quiescent due to contact inhibition, because reseeding them at low density allows them to re-enter the cell cycle. In contrast, Fig. 5B shows that adipocytes differentiated by the standard protocol in the absence of RA are permanently postmitotic and do not divide after reseeding at low density. These results suggest
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Fig. 6B shows that the presence of RA at the time of adipogenic stimulation altered the phosphorylation state of Rb. The degree of hyperphosphorylation on day 1 was reduced, consistent with the reduced percentage of cells that underwent clonal expansion (Fig. 5A). Furthermore, in the presence of RA, Rb was present in both hyper- and hypophosphorylated forms on all days after exposure to adipogenic stimulation. Consistent with the lack of adipogenesis, there was no induction of PPARγ or C/EBPα in the presence of RA (Fig. 6B). These data suggested a correlation between terminal differentiation and the hypophosphorylation of Rb protein. Together with our earlier demonstration of the role of confluency in determining RA insensitivity of cells that ectopically express both PPARγ and C/EBPα, we conclude that irreversible commitment to adipogenic differentiation requires both the expression of the adipogenic transcription factors and the cell cycle arrest associated with hypophosphorylation of Rb.

**DISCUSSION**

We have used the ability of RA to inhibit adipogenesis as a tool to explore the molecular events that occur during the adipogenic differentiation process. In particular, we were interested in pursuing the observation that at early times after exposure of confluent 3T3-L1 cells to adipogenic stimulation, the cells become committed to the differentiation pathway and no longer respond to RA. RA was able to block BRL49653-mediated differentiation in wild type 3T3-L1 cells, as well as adipogenesis due to ectopic expression of C/EBPα, PPARγ, or both, even in the presence of the PPARγ ligand BRL49653. These results strongly suggest that the insensitivity to RA inhibition that occurs during normal differentiation is not due solely to the concomitant expression of PPARγ and C/EBPα. However, we have noted that allowing C/EBPα- or PPARγ-expressing cells to reach confluence reproduces the committed, RA-irreversible state that occurs after about 48 h during wild type 3T3-L1 cell differentiation. Consistent with this notion, we found that during standard 3T3-L1 cell differentiation, at the point at which cells normally become refractory to the effects of RA, cells are not only expressing PPARγ and C/EBPα but are exiting the cell cycle following clonal expansion.

The ability of RA to block PPARγ-induced adipocyte differentiation suggests that liganded endogenous RAR can interfere with the function of PPARγ in addition to its ability to block transcriptional activity of C/EBPs (13). Indeed, the ability of liganded RAR to block PPAR transactivation can be demonstrated in transfected cells (Ref. 31 and data not shown). However, during normal adipogenesis, RA is effective at times prior to expression of PPARγ, suggesting that its natural target is C/EBPβ-mediated transcription (13).

We have now shown that RA blocks adipocyte differentiation of confluent 3T3-L1 cells when added prior to expression of adipogenic transcription factors and also prevents adipogenesis of cells that constitutively express C/EBPα and PPARγ when added prior to confluency. Thus, excluding the situation where RAR is limiting (23), preadipocytes enter a state of commitment to differentiation that is not reversible by RA when PPARγ and C/EBPα are expressed and growth cessation has occurred. In the absence of RA, cells that ectopically express PPARγ and C/EBPα undergo adipocyte differentiation before cells reach confluency, indicating that cell-cell contact may not be necessary for adipogenesis to occur. However, these cells grow very slowly, suggesting that expression of adipogenic genes may contribute to the cessation of cell growth that is also required for cells to undergo adipogenic differentiation.

Both PPARγ and C/EBPα activate several adipocyte-specific genes. Coexpression of PPARγ and C/EBPα has been shown to have a synergistic effect and strongly promote fat cell differen-
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tiation of fibroblastic (32) and myogenic cell lines (14). C/EBPα has known antimitotic effects, suggesting that its role in promoting differentiation is at least partially related to suppression of cell growth (30). Expression of C/EBPα in 3T3-L1 cells reduced their growth rate, and cells that ectopically expressed both PPARγ and C/EBPα grew extremely slowly and underwent adipocyte conversion even before they reached confluence. However, we found that co-expression of these two proteins was not sufficient to commit cells to undergo differentiation in a manner that was irreversibly by RA unless the cells were growth-arrested. These observations suggest that growth arrest played a positive role, along with the specific differentiation factors, in determining whether cells proliferate or differentiate.

During the normal differentiation process, we found that Rb protein is first hyperphosphorylated and then hypophosphorylated. This correlated well with the cell cycle status of the cells both during and after clonal expansion. While this work was in progress, another group also showed that Rb protein levels were stable throughout adipogenesis but reported that the Rb protein was hypophosphorylated throughout the differentiation process (33). The discrepancy appears to be due to the use of different Rb antibodies, and using the same antibody as we used, those investigators have more recently found changes in Rb phosphorylation similar to those reported here. The role of Rb in adipogenesis has been predicted by other studies. In addition to being required for the adipogenesis of mouse lung fibroblasts (20), Rb is necessary for myoblastic differentiation (32). C/EBPα has known antimitotic effects, suggesting that its role in promoting cell differentiation should be further pursued.

It is unlikely that cell cycle arrest is merely a downstream effect of adipogenic differentiation factors because expression of C/EBP and PPARγ complement but cannot replace the important effects of cell growth arrest on the ability of 3T3-L1 cells to irreversibly commit to adipocyte differentiation. Taken together, our results suggest that cell cycle arrest is as important as the expression of adipogenic genes in promoting cell differentiation and the irreversible commitment of preadipocytes to this process.

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REFERENCES

1. Green, H., and Meuth, M. (1974) Cell 3, 127–133
2. Green, H., and Kehinde, O. (1975) Cell 5, 19–27
3. Chawla, A., and Lazar, M. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1786–1790
4. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) Genes & Dev. 8, 1224–1234
5. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) J. Biol. Chem. 270, 12953–12956
6. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Cell 83, 803–812
7. Mandrup, S., and Lane, M. D. (1997) J. Biol. Chem. 272, 5367–5370
8. Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) Mol. Cell. Biol. 15, 351–357
9. Freytag, S. O., Paielli, D. L., and Gilbert, J. D. (1994) Genes & Dev. 8, 1654–1663
10. Yeh, W.-C., Cao, Z., Classon, M., and McKnight, S. L. (1995) Genes Dev. 9, 168–181
11. Lin, F.-T., and Lane, M. D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8757–8761
12. Wu, Z., Bucher, N. L. R., and Farmer, S. R. (1995) Genes & Dev. 9, 2359–2363
13. Schwarz, E. J., Regnato, M. J., Shao, D., Kreukow, S. L., and Lazar, M. A. (1997) Mol. Cell. Biol. 17, 1552–1561
14. Hu, E., Tontonoz, P., and Spiegelman, B. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9856–9860
15. Weinberg, R. A. (1996) Trends Biochem. Sci. 15, 199–202
16. Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y., and Lee, W. H. (1989) Cell 58, 1193–1198
17. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Pwnica-Worms, H., Huang, C. M., and Livingston, D. M. (1989) Cell 58, 1085–1095
18. Livneh, E., Shimon, T., Bechor, E., Doki, Y., Schieren, I., and Weinstein, I. B. (1995) Oncogene 12, 1545–1555
19. Higgins, C., Chatterjee, S., and Cherington, V. (1996) J. Virol. 70, 745–752
20. Chen, P.-L., Riley, D. J., Chen, Y., and Lee, W. H. (1996) Genes & Dev. 10, 2794–2804
21. Kuri-Harazhi, W., Wise, L. S., and Green, H. (1978) Cell 14, 53–59
22. Stone, R. L., and Bernlohr, D. A. (1990) Differentiation 45, 19–27
23. Xue, J.-C., Schwarz, E. J., Chawla, A., and Lazar, M. A. (1996) Mol. Cell. Biol. 16, 1567–1575
24. Miller, A. D., and Rosman, G. J. (1989) Biotechniques 7, 985–989
25. Landau, N. R., and Littman, D. R. (1992) J. Virol. 66, 5101–5113
26. Kallen, C. B., and Lazar, M. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5793–5796
27. Descombes, P., and Schibler, U. (1991) Cell 67, 569–579
28. Adams, M., Regnato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997) J. Biol. Chem. 272, 5128–5132
29. Lin, F.-T., and Lane, M. D. (1992) Genes & Dev. 6, 533–544
30. Tontonoz, P., Hu, E., Devine, J., Beale, E. G., and Spiegelman, B. M. (1995) J. Biol. Chem. 270, 803–812
31. Imakado, S., Bickenbach, J. R., Bundman, D. S., Rothnagel, J. A., Attar, P. S., Wang, X.-J., Walczak, V. R., Wisniewski, S., Pote, J., Gordon, J. S., Heyman, R. A., Evans, R. M., and Roop, D. R. (1995) Genes & Dev. 9, 317–329
32. Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994) Cell 79, 1147–1156
33. Richon, V. M., Lyle, R. E., and McGehee, R. E., Jr. (1997) J. Biol. Chem. 272, 10117–10124
34. Chen, P.-L., Riley, D. J., Chen-Kiang, S., and Lazar, M. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 465–469
35. Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993) Cell 72, 309–324

2 B. McGehee, personal communication.