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Biochemistry

CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program

(3T3-L1 preadipocyte/422 adipose P2 protein/glucose transporter 4)

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ABSTRACT **Previous studies showed that CCAAT/enhancer binding protein α (C/EBPα) is required for differentiation of 3T3-L1 preadipocytes induced by exogenous hormonal agents. It was not possible to ascertain, however, whether C/EBPα alone is sufficient to induce differentiation because its antimitogenic activity precluded propagating 3T3-L1 cell lines that constitutively express C/EBPα at high levels. This problem was circumvented by using 3T3-L1 preadipocytes stably transfected with an isopropyl β-D-thiogalactoside (IPTG)-inducible p42 C/EBPα expression vector system. IPTG-induced expression of the 42-kDa isoform of C/EBPα in preadipocytes caused expression of several endogenous adipocyte-specific genes (genes encoding the 422 adipose P2 protein, glucose transporter 4, and C/EBPα) and the accumulation of cytoplasmic triglyceride. Thus, C/EBPα is not only necessary but also is sufficient to trigger differentiation of growth-arrested 3T3-L1 preadipocytes without use of exogenous hormonal agents.**

When appropriately stimulated with exogenous hormonal agents, 3T3-L1 preadipocytes differentiate into cells possessing the biochemical and morphological phenotype of adipocytes (1–9). Following induction using this standard differentiation protocol there is coordinate transcriptional activation of adipocyte-specific genes and massive accumulation of cytoplasmic triglyceride (10–12). Considerable evidence has implicated CCAAT/enhancer binding protein α (C/EBPα) as a transcriptional activator in the differentiation process (1). C/EBPα has been shown to bind to the promoters of a number of adipocyte genes [e.g., genes encoding 422 adipose P2 protein 422(aP2), stearoyl-CoA desaturase 1 (SCD1), and glucose transporter 4 (GLUT4)] and to transactivate reporter gene expression driven by these promoters (12–15). Moreover, the C/EBPα gene itself is transcriptionally activated just prior to the coordinate expression of these adipocyte genes (13, 16); mutation of the C/EBP binding sites within the promoters of these genes prevents transactivation (13–15). Proof that expression of C/EBPα is required for preadipocyte differentiation was provided by using the antisense RNA approach (17, 18). Constitutive expression of antisense C/EBPα RNA not only blocked expression of C/EBPα but also the expression of 422(aP2) protein, SCD1, and GLUT4 and the accumulation of cytoplasmic triglyceride (18). Furthermore, rescue of the adipocyte phenotype was accomplished by transfecting the stable antisense cell lines with a complementary sense C/EBPα RNA expression vector (18).

While these findings demonstrated that C/EBPα was required for preadipocyte differentiation, it was uncertain whether its expression was sufficient to initiate differentiation. Attempts to assess the effect of C/EBPα on differentiation by constitutive expression of C/EBPα in preadipocytes have been hampered by the fact that C/EBPα is antimitogenic and therefore, such stable cell lines cannot be propagated (refs. 19 and 20; also unpublished results). Nevertheless, Umek *et al.* (19) obtained a transfected 3T3-L1 cell line that constitutively expressed a conditionally active p42 C/EBPα-estrogen receptor fusion protein. It was observed that activation of the fusion protein by exposure of the cells to estrogen blocked mitosis but did not induce adipocyte differentiation. However, when induced by using a standard differentiation protocol supplemented with estrogen, expression of 422(aP2) mRNA and the accumulation of cytoplasmic triglyceride were accelerated.

Constitutive expression of a 30-kDa isoform of C/EBPα had a similar effect on preadipocyte differentiation (21). Thus, expression of the 30-kDa isoform accelerated the differentiation program initiated by exogenous inducers but was itself unable to initiate differentiation. It should be noted that both the 42-kDa and 30-kDa isoforms of C/EBPα are normally expressed by 3T3-L1 adipocytes, adipose tissue, and liver (16, 21, 22). The two isoforms are translation products of a single species of C/EBPα mRNA (21, 22). Mutational analysis revealed (21) that the full-length 42-kDa and 30-kDa proteins are alternative translation products initiated at the first and third methionine codons, respectively. The two isoforms appear to have different functions—e.g., the 42-kDa isoform is antimitotic, while the 30-kDa isoform is not (21). However, both isoforms transactivate specific adipocyte gene promoters (21).

In contrast to the findings that neither the 42-kDa nor the 30-kDa isoform of C/EBPα was sufficient itself to induce differentiation, Freytag and Geddes (20) discovered conditions under which p42 C/EBPα appeared to activate adipocyte conversion, albeit at low frequency. They showed that after transfection of 3T3-L1 preadipocytes with a C/EBPα expression vector, foci (with up to 200 cells per focus) could be selected that expressed C/EBPα. Although these foci could not be further propagated, presumably because of the antimitotic activity of C/EBPα, some cells appeared to undergo differentiation spontaneously.

To resolve the discrepancy regarding the ability of C/EBPα to initiate differentiation, we sought to express the 42-kDa isoform of C/EBPα in 3T3-L1 preadipocytes, using the LacSwitch inducible expression vector system. Expression vectors for the Lac repressor and p42 C/EBPα, the latter under the control of a viral promoter and several Lac repressor regulatory elements, were cotransfected into 3T3-L1 preadipocytes. As reported in this paper, exposure to isopropyl β-D-thiogalactoside (IPTG) led to expression of p42 C/EBPα and acquisition of the adipocyte phenotype at high frequency without use of exogenous inducers of preadipocyte differentiation. Our findings indicate that p42 C/EBPα is

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Abbreviations: C/EBPα, CCAAT/enhancer binding protein α; 422(aP2), 422 adipose P2 protein; GLUT4, glucose transporter 4; IPTG, isopropyl β-D-thiogalactoside.

sufficient to trigger the differentiation program of 3T3-L1 preadipocytes.

EXPERIMENTAL PROCEDURES

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum. Where indicated, a standard protocol was employed in which differentiation was induced by treating 2-day (designated day 0) postconfluent cells with DMEM containing 10% (vol/vol) fetal bovine serum (FBS), 1 μg of insulin per ml, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine until day 2 as described (8, 9). Cells were then fed every other day with DMEM supplemented with only insulin and 10% FBS. Cytoplasmic triglyceride was stained with Oil-Red-O (23).

The LacSwitch inducible system (from Stratagene) consists of two expression vectors: p3'SS, which contains the *Escherichia coli lacI* gene and a hygromycin-resistance gene, both under the control of animal viral promoters, and pOPI3CAT, which contains the chloramphenicol acetyltransferase (CAT) gene just 3' to an intron [from simian virus 40 (SV40)] containing three *lac* operator/repressor binding sites and a neomycin-resistance gene—both under the control of viral promoters. pOPI3/CEBPα42 was prepared by replacing the CAT gene in pOPI3CAT with a 2-kb segment of mouse C/EBPα cDNA excised from pMSV-TS[13]m42 (unpublished data) with *Eco*RI. This segment of DNA contains sequences from the translation start site to the *Hin*dIII site in the 3' untranslated region, modified so that the first translation initiation codon had been changed to an ideal Kozak sequence (CCCATGG→ACCATGG; ref. 24) and the third in-frame methionine codon had been mutated from ATG to TTG (21). This modified C/EBPα cDNA was inserted by blunt-end ligation into pOPI3 at the *Not* I site located just 3' to the SV40 intron sequence and was designated pOPI3/CEBPα42. Since the translation start site for p30 C/EBPα had been mutated, p42 C/EBPα was the only translation product.

The p3'SS and the pOPI3/CEBPα42 vectors were cotransfected into 30% confluent, low-passage 3T3-L1 preadipocytes in a 1:1 ratio by calcium phosphate coprecipitation. Double selection with 300 μg of G418 per ml and of hygromycine B 100 μg per ml for 2 weeks generated >50 foci, 12 of which were propagated for further study. To induce expression of p42 C/EBPα from the transgene, 5 mM IPTG was added to the culture medium.

For analysis of the expressed proteins, cells were lysed in buffered SDS, and the lysate was boiled immediately. One to two hundred micrograms of total protein from the whole-cell lysates were subjected to SDS/polyacrylamide gel electrophoresis. After transfer to Immobilon-P membranes, Ponceau-S staining was performed to ensure equal loading of each sample. C/EBPα and 422(aP2) proteins were detected with antibodies against the 14-amino acid N-terminal sequence (unpublished data), an internal sequence, or the C-terminal sequence of C/EBPα (18) and against the 14-amino acid C-terminal sequence of 422(aP2) protein (18), which were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham).

Total RNA was isolated by the method of Chirgwin *et al.* (25). For Northern blot analysis, 20 μg of total RNA was denatured with glyoxal and dimethyl sulfoxide and analyzed by electrophoresis on 1% agarose gels as described (18). After transfer to Hybond-N membranes and UV-crosslinking, blots were stained with methylene blue to ensure equal loading and to locate 28S and 18S RNAs. Blots were hybridized with probes labeled by random priming (26) corresponding to a *Sma* I fragment of an internal sequence of C/EBPα cDNA (18) and full-length 422(aP2) cDNA (27). In some experiments a random-labeled cDNA probe for GLUT4 (28) was employed.

RESULTS AND DISCUSSION

IPTG-Induced Expression of p42 C/EBPα in Preadipocytes Harboring a LacSwitch p42 C/EBPα Expression System. Attempts to establish 3T3-L1 preadipocyte cell lines that constitutively express high levels of C/EBPα have been unsuccessful because of the antimitotic activity of this transcription factor (refs. 19 and 20; also unpublished results). To circumvent this problem, an inducible p42 C/EBPα expression vector was constructed in which a modified mouse C/EBPα cDNA was inserted into pOPI3 just 3' to the Rous sarcoma viral promoter and an intron containing three *lac* operator/repressor binding sites (Fig. 1). Modifications of the C/EBPα cDNA insert included conversion of the first translational initiation sequence to an ideal Kozak sequence

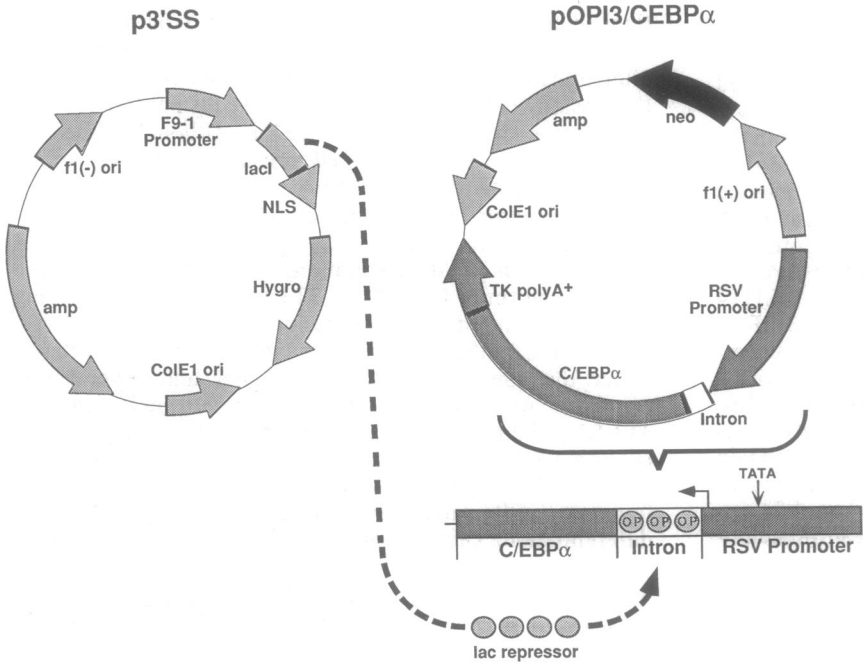

FIG. 1. The IPTG-inducible LacSwitch p42 C/EBPα expression system. p3'SS (Stratagene) contains the *Escherichia coli lacI* gene, which directs expression of the Lac repressor under the control of a mutated polyoma viral-based (F9-1) promoter; p3'SS also contains antibiotic resistance genes for selection with hygromycin (hygro) and ampicillin (amp). pOPI3/CEBPα42 contains a modified C/EBPα gene just 3' to a simian virus 40 intron with three *lac* operator/repressor binding sites and a Rous sarcoma virus (RSV) promoter. pOPI3/CEBPα42 also contains antibiotic resistance genes for selection with neomycin (neo) and ampicillin (amp). The dashed line and arrow indicate the sites of interaction of the Lac repressor with its binding sites in an expanded version of the p42 C/EBPα expression unit. NLS, nuclear localization signal; pOPI3/CEBPα, pOPI3/CEBPα42; TK polyA+, thymidine kinase polyadenylylation sequence; TATA, TATA box.

(CCCATGG→ACCATGG; ref. 24) and mutation of the third in-frame methionine codon from ATG to TTG (21). Thus, the pOPI3/CEBPα42 vector should only express the p42 isoform of C/EBPα. pOPI3/CEBPα42 and p3'SS (a *lac* repressor expression vector), both of which contain antibiotic resistance genes, were cotransfected into 3T3-L1 preadipocytes. Following selection with G418 and hygromycin, >50 foci were obtained. Of 12 clones selected for further study, 6 expressed the p42 isoform of C/EBPα when treated with inducer (i.e., IPTG), although the induced levels of expression of C/EBPα and the extent of induction by IPTG varied.

To assess the effect of p42 C/EBPα on cell proliferation, one of these cell lines (IP42-2), which expressed a relatively high level of p42 C/EBPα [albeit much lower than (i.e., ≤25%) of the level of expression by fully-differentiated 3T3-L1 adipocytes], was treated or not treated with IPTG when the cells were either actively proliferating or in the growth-arrested confluent state. Expression of C/EBPα was markedly induced by IPTG regardless of whether the preadipocytes were proliferating (i.e., at 30% and 70% confluent cell density) or were growth-arrested at confluence (Fig. 2). Induction of expression was rapid being detectable within a few hours of exposure to IPTG (results not shown).

Expression of p42 C/EBPα had no measurable effect on the rate of proliferation of 3T3-L1 preadipocytes. The doubling time of both untransfected 3T3-L1 cells and IP42-2 cells was 18 ± 1 hr during logarithmic growth regardless of whether IPTG was present. This result was unexpected, since Umek *et al.* (19) had found that activation by estrogen of a constitutively expressed, conditionally active C/EBPα-estrogen receptor fusion protein blocked mitosis of 3T3-L1 preadipocytes. It should be stressed, however, that in the present experiments the level of expression of C/EBPα by IPTG-treated IP42-2 cells was considerably lower (≤25%) than that of fully differentiated untransfected 3T3-L1 cells. Therefore, it appears that the maximal level of expression of C/EBPα achieved in IPTG-treated IP42-2 cells was insufficient to block cell proliferation.

IPTG-Induced Expression of p42 C/EBPα Triggers Adipocyte Differentiation in the Absence of Exogenous Hormone Inducers. Continuous exposure of confluent IP42-2 and IP42-8 cells to IPTG caused increased expression of both p42 C/EBPα and its mRNA that was sustained for at least 7 days (Fig. 3 *A* and *B*, respectively). During this period a large percentage of the cells acquired the adipocyte phenotype without having been subjected to the standard differentiation protocol. Thus, treatment of IP42-2 and IP42-8 cells with IPTG not only induced expression of p42 C/EBPα encoded

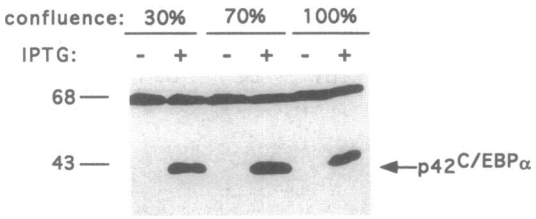

FIG. 2. IPTG-induced expression of p42 C/EBPα in proliferating and confluent 3T3-L1 preadipocytes harboring the LacSwitch C/EBPα expression system. The IP42-2 cell line, harboring a LacSwitch C/EBPα expression system, was plated at 10% confluent cell density in 10-cm dishes and then fed every other day with DMEM containing 10% calf serum with or without 5 mM IPTG. Cells were harvested every 24 hr after reaching 30%, 70%, and 100% confluent density. After cell lysis, 200 μg of whole-cell extract was subjected to SDS/polyacrylamide gel electrophoresis followed by Western blot analysis. The p42 C/EBPα protein was detected with antibody against an internal peptide of C/EBPα. The 68-kDa band that was generated by all cell lysates is due to nonspecific cross-reacting material and verified equal protein loading. Sizes are shown in kDa.

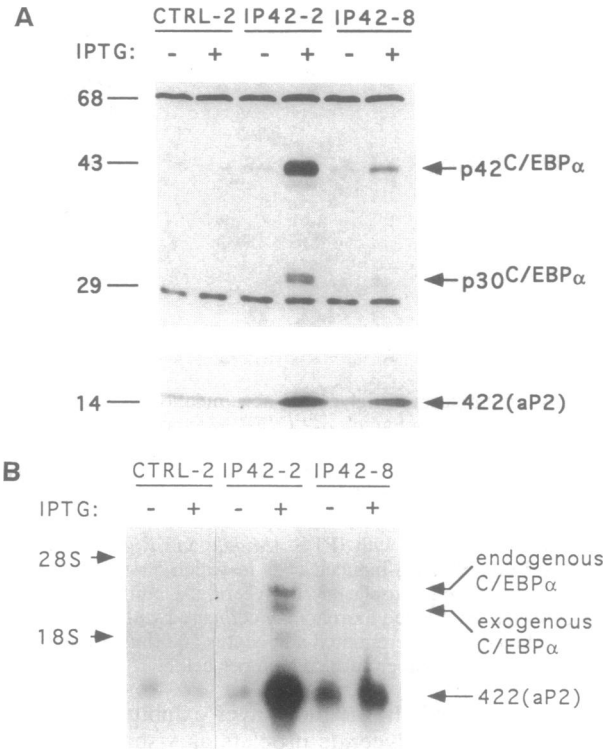

FIG. 3. Effect of IPTG on expression of C/EBPα and 422(aP2) proteins and mRNAs in 3T3-L1 cells harboring the LacSwitch p42 C/EBPα expression system. Cell lines harboring the LacSwitch p42 C/EBPα expression system (i.e., the IP42-2 and IP42-8 lines) or the LacSwitch expression system lacking a C/EBPα cDNA insert (i.e., the CTRL2 line) were treated or not treated with 5 mM of IPTG as the cells approached confluence. Cells were maintained in DMEM containing 10% calf serum with or without IPTG and were harvested for analysis 7 days later. (*A*) Cellular protein (100 μg) was subjected SDS/polyacrylamide gel electrophoresis and Western blot analysis with an antibody against an internal peptide of C/EBPα; the same blot was then rehybridized with an antibody against a C-terminal peptide of 422(aP2) protein. Sizes are shown in kDa. (*B*) Total RNA (20 μg) was subjected to Northern blot analysis on a 1% agarose gel. The blot was hybridized with probes specific for C/EBPα mRNA and 422(aP2) mRNA.

by the transgene but also activated expression of the endogenous C/EBPα, 422(aP2), and GLUT4 genes. Treatment with IPTG induced expression of the p30 isoform of C/EBPα (Fig. 3*A*). It was not possible to distinguish between p42 C/EBPα isoforms translated from the transgene and endogenous mRNAs, as both messages encode this protein. However, it was possible to distinguish the p30 isoform as the unique product of endogenous C/EBPα mRNA, since the translational start-site for the p30 isoform in the transgene message had been mutated (see above). Furthermore, the two C/EBPα messages were distinguishable on the basis of size—i.e., 2.7 kb for the endogenous mRNA and 2.4 kb for the transgene mRNA in IP42-2 cells (Fig. 3*B*). It was also shown that IPTG-induced expression of p42 C/EBPα caused expression of 422(aP2) protein and mRNA (Fig. 3 *A* and *B*, respectively). Other experiments revealed that expression of another differentiation-induced gene (i.e., GLUT4, the insulin-responsive glucose transporter) was similarly activated by IPTG in IP42-2 and IP42-8 cells (results not shown).

IPTG-induced expression of p42 C/EBPα also caused the accumulation of cytoplasmic triglyceride, another indicator of preadipocyte differentiation. When IP42-2 preadipocytes were treated with IPTG as they approached confluence, cytoplasmic triglyceride droplets became visible in many cells within 2–3 days (results not shown) and by day 7,

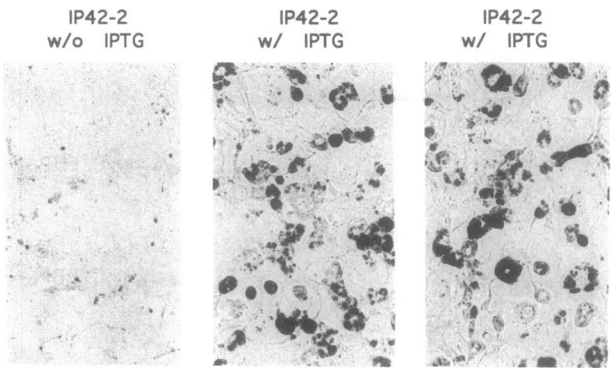

FIG. 4. Cytoplasmic triglyceride accumulation in IPTG-induced cells harboring the LacSwitch p42 C/EBPα expression system. The IP42-2 cell line was treated or not treated with 5 mM IPTG as the cells approached confluence. Seven days later, cell monolayers were washed with phosphate-buffered saline, fixed with formaldehyde, stained with Oil-Red-O, and then subjected to photomicroscopy. (*Left*) Cells not treated with IPTG. (*Middle* and *Right*) Two representative fields of IPTG-induced cells in which 50–60% of the cell population exhibited cytoplasmic triglyceride. Undifferentiated preadipocytes had a flattened morphology compared with the "rounded-up" morphology of differentiated adipocytes and thus occupied considerably more monolayer surface area per cell.

50–60% of the cells in the monolayer exhibited substantial accumulation of triglyceride droplets as visualized by Oil-Red-O staining (Fig. 4). As triglyceride accumulated, the preadipocytes lost their flattened fibroblastic morphology and "rounded-up" in a manner typical of differentiating 3T3-L1 preadipocytes subjected to the standard differentiation protocol. Like untransfected differentiated 3T3-L1 adipocytes, the IPTG-induced cells harboring the LacSwitch p42 C/EBPα expression system were dispersed evenly throughout the monolayer, although the frequency of adipose conversion (50–60%) was lower. Acquisition of the adipocyte phenotype was correlated with the level of IPTG-induced expression of p42 C/EBPα. For example, ≈30% and 50–60%, respectively, of the IP42-8 (not shown) and IP42-2 (Fig. 4) cells accumulated cytoplasmic triglyceride when treated with IPTG and expressed corresponding levels of C/EBPα (Fig. 3*A*).

The stage of growth at which expression of p42 C/EBPα is induced with IPTG does not appear to be critical for the induction of differentiation. It was observed (results not shown) that exposure of IP42-2 cells to IPTG as they approached confluence (Fig. 4) or continuous exposure from the time of plating or beginning after growth arrest (i.e., 2 days postconfluence; results not shown) led to differentiation. However, in all cases acquisition of the adipocyte phenotype did not occur until the cells were confluent. It appears that cell–cell contact at confluent cell density is a prerequisite for subsequent preadipocyte differentiation.

Control 3T3-L1 preadipocytes (untransfected or transfected with the expression vector lacking a C/EBPα cDNA insert) treated with IPTG neither accumulated cytoplasmic triglycerides (Fig. 4) nor exhibited increased expression of C/EBPα and 422(aP2) (Fig. 3) or GLUT4 (results not shown). While occasional clusters of triglyceride-containing cells were observed in control cell monolayers, this occurred at very low frequency relative to that of cells harboring the p42 C/EBPα expression vector and was independent of IPTG treatment.

The fact that IPTG-induced expression of p42 C/EBPα by IP42-2 cells (as well as other cell lines harboring the LacSwitch p42 C/EBPα expression system) was sufficient to trigger differentiation but was insufficient to block mitosis suggests that the threshold levels of C/EBPα for the two processes are different. It should be noted that once differentiation has been triggered, this process may become autocatalytic as C/EBPα is capable of autoactivating its own gene promoter (21, 29). The C/EBPα gene promoter possesses a C/EBP binding site through which C/EBPα can activate transcription of a reporter gene (29, 30). The level of C/EBPα generated in IPTG-treated cells harboring the LacSwitch p42 C/EBPα expression system may be sufficient to override the effect of transacting factors [e.g., the C/EBPα undifferentiated protein (CUP); ref. 31] which may maintain the endogenous C/EBPα gene in the repressed state in preadipocytes.

IPTG-Induced Expression of p42 C/EBPα Advances the Differentiation Program Initiated by Exogenous Differentiation Inducers. It has been shown (13, 16) that a combination of exogenous hormonal agents (e.g., isobutylmethylxanthine, dexamethasone, and insulin) used in the standard differentiation protocol are required to trigger expression of

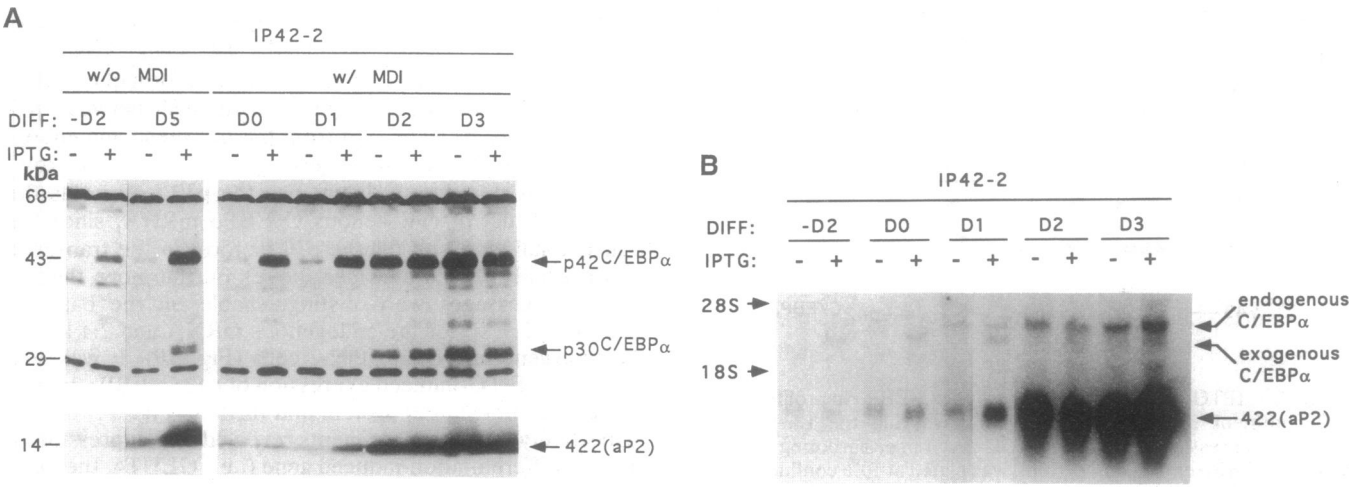

FIG. 5. Effect of IPTG on expression of C/EBPα and 422(aP2) proteins and mRNAs in cells harboring the LacSwitch p42 C/EBPα expression system subjected or not subjected to the standard differentiation protocol. As IP42-2 cells approached confluence (day −2), they were treated or not treated with 5 mM IPTG, and these treatments were continued every other day throughout the experiment. Two days after reaching confluence (day 0), the cells were shifted to DMEM containing 10% fetal bovine serum either without (w/o MDI) or with (w/MDI) the differentiation inducers methylisobutylxanthine (3-isobutyl-1-methylxanthine), dexamethasone, and insulin (MDI). Two days later (day 2) the MDI-treated cells were shifted to the same medium lacking isobutylmethylxanthine and dexamethasone. Cells were harvested on the days indicated (except that on day −2 cells were harvested after a 4-hr treatment or nontreatment with IPTG), and lysates were subjected to Western blot analysis (*A*), and total cellular RNA was subjected to Northern blot analysis (*B*).

C/EBPα and subsequent differentiation of growth-arrested 3T3-L1 preadipocytes. To assess the effect of premature expression of p42 C/EBPα on preadipocytes subjected to this differentiation protocol, IP42-2 cells were first exposed to IPTG (on day −2) and then induced with the combination of differentiation inducers (on day 0) described above. Western blot analysis revealed that expression of p30 C/EBPα (the unique product of the endogenous C/EBPα gene; see above) and 422(aP2) protein (Fig. 5*A*) as well as their corresponding mRNAs (Fig. 5*B*) were expressed prematurely on day 1 in IP42-2 cells induced with both IPTG and the exogenous differentiation inducers cited above. In the absence of IPTG, however, expression of these differentiation markers was delayed for a day until day 2. Likewise, the incipient cytoplasmic triglyceride droplets appeared about 1 day earlier in IP42-2 cells treated with IPTG (results not shown). Expression of these markers in wild-type 3T3-L1 and control cells (transfected with the LacSwitch vector lacking a C/EBPα cDNA insert) was unaffected by exposure to IPTG either in the presence or absence of the exogenous differentiation inducers (results not shown).

A large body of evidence (1) suggests that C/EBPα serves multiple functions in adipocyte differentiation including: (*i*) coordinate transcriptional activation of a group of adipocyte genes that gives rise to the adipocyte phenotype, (*ii*) termination of mitotic clonal expansion as cells enter the quiescent terminally differentiated state, and (*iii*) transcriptional autoactivation of the C/EBPα gene itself to maintain the quiescent terminally differentiated state. While antisense RNA experiments (17, 18) proved that C/EBPα is essential for differentiation, it remained uncertain whether C/EBPα alone could induce differentiation. The present study shows that IPTG-induced expression of the LacSwitch p42 C/EBPα expression vector can trigger differentiation at high (50–60%) frequency without use of exogenous hormonal agents. Nevertheless, the frequency was lower than that (90–100%) observed with wild-type 3T3-L1 preadipocytes subjected to exogenous hormonal inducers. The reason for the lower frequency of differentiation by IPTG-induced preadipocytes harboring the transgene may be that the level of C/EBPα was insufficient for maximal induction of differentiation or the relief of repression (31). This is suggested by the fact that the level of p42 C/EBPα expressed by IPTG-induction of the transgene is ≤25% of that observed with the endogenous gene with cells subjected to the standard differentiation protocol. This may also account for the fact that the level of IPTG-induced expression of C/EBPα during logarithmic growth of 3T3-L1 preadipocytes harboring the transgene was insufficient to block mitosis.

In addition to its role(s) in preadipocyte differentiation, C/EBPα may also serve to commit progenitor cells to the adipogenic lineage. The studies reported in this paper and recent findings of Freytag *et al.* (32) are reminiscent of the observation that transfection of 10T1/2 stem cells with a MyoD expression vector (and later other members of this family of transacting factors; refs. 33–36) commits the cells to the myogenic lineage.

Note. While this paper was being written, we learned that Freytag *et al.* (32), who used another approach, had also obtained results that indicate that expression of C/EBPα is sufficient to induce preadipocyte differentiation.

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