# Regulation of adipose cell differentiation. II. Kinetics of induction of the aP2 gene by fatty acids and modulation by dexamethasone

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**Abstract** Fatty acids behave as activators of the aP2 gene expression in committed, lipid-free, non-terminally differentiated Ob1771 cells. Like fatty acids, dexamethasone provokes a dose-dependent accumulation of aP2 mRNA. However, fatty acids and dexamethasone act through different mechanisms to activate the aP2 gene expression since *i*) fatty acids and dexamethasone act in a synergistic manner; *ii*) the effect of dexamethasone is rapid and transient (maximal effect after 8 h), whereas that of fatty acids is slower, and maintained as long as the inducer is present and is fully reversible upon fatty acid removal; *iii*) the induction of the aP2 gene expression by dexamethasone does not require ongoing protein synthesis, while the response to fatty acids is completely prevented by cycloheximide; and *iv*) the induction of the aP2 gene expression by fatty acids but not by dexamethasone is confined to preadipocyte cell lines. This suggests that the process of activation by fatty acids, rather than the expression of the aP2 gene, is unique to adipose cells. Besides their effects on the aP2 gene, fatty acids activate the expression of the acyl CoA synthetase gene which encodes another protein involved in fatty acid metabolism. Activation of both genes by fatty acids appears not to be mediated by the CCAAT enhancer binding protein, a nuclear factor reported as transactivator of the aP2 promoter activity, since the enhancer binding protein mRNA is not expressed under these conditions. —**Amri, E. Z., G. Ailhaud, and P. Grimaldi**. Regulation of adipose cell differentiation. II. Kinetics of induction of the aP2 gene by fatty acids and modulation by dexamethasone. *J. Lipid Res.* 1991. **32:** 1457–1463.

**Supplementary key words** glucocorticoids • acyl CoA synthetase gene

The adipose conversion of preadipose cells is accompanied by the transcriptional activation of adipose-related genes (1, 2). Among the various adipose phenotypes, the emergence of aP2 is closely related to the induction of fatty acid- and triglyceride-synthesizing enzymes (3, 4); aP2, a protein sharing sequence homology to a class of fatty acid-binding proteins, binds in vitro fatty acids and retinoic acid (5), is rapidly phosphorylated on tyrosine 19 in response to insulin (6), and appears involved in the uptake of fatty acids by adipose cells (7). We have shown (8) that exposure of committed, lipid-free, nonterminally differentiated cells to fatty acids provokes an induction of the aP2 gene expression with no effect on other adipose-related genes, i.e., glycerophosphate dehydrogenase (GPDH) and adipsin, which remain silent. Thus the aP2 gene can be differentially expressed in cells devoid of other key phenotypes. Clearly, several agents, described as inducers of the adipose conversion process, behave as potent and rapid activators of the transcription of the aP2 gene. Treatment by glucocorticoids and cyclic AMP (cAMP) of confluent 3T3-L1 cells leads within hours to an increase up to 100-fold of the aP2 mRNA abundance (9). These effects appear mediated by different elements of the aP2 promoter region (9, 10). Analysis of the interaction of nuclear proteins with these DNA regions, showed that the products of protooncogenes Fos and Jun interact with the element located at – 120 to control promoter function (11, 12). In addition, it has been demonstrated that the interaction of the CCAAT enhancer binding protein (C/EBP) (13) with the DNA element located at – 140 is critical for the aP2 promoter activity (14, 15). Since the expression of C/EBP mRNA also appeared to be a differentiation-dependent event, it was concluded that the accumulation of this nuclear factor triggers the activation of the aP2 gene during the adipose conversion process (14, 15).

In the present study, we have characterized further the activation of the aP2 gene by fatty acids with respect to

Abbreviations: ACS, long-chain acyl-CoA synthetase; cAMP, adenosine 3':5'-cyclic monophosphate; C/EBP, CCAAT/enhancer binding protein; 8-CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GH, growth hormone; GPDH, glycerol-3-phosphate dehydrogenase; H7, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine; $T_3$, triiodothyronine.

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differentiation-promoting agents and cell specificity. The effect of fatty acids on the expression of the acyl-CoA synthetase (ACS) gene and on that of the C/EBP gene were also investigated. Acyl-CoA synthetase catalyzes the first reaction in the metabolism of fatty acid leading to the formation of acyl-CoA and it has been recently shown that the abundance of ACS mRNA in rat liver is modulated by dietary manipulation such as carbohydrate-rich or lipid-rich diet (16).

## MATERIALS AND METHODS

### Cell culture

Ob1771 (17), Ob1754 (18), 3T3-F442A (19), 3T3-C2 (20), and $C_2C_{12}$ (21) cells were plated at a density of $2 \times 10^3$ per $cm^2$ and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% adult bovine serum, 200 units/ml of penicillin, 50 μg/ml of streptomycin, 33 μM biotin, and 17 nM pantothenate. This medium is termed standard medium. Confluence was reached within 5 days. Differentiation of Ob1771 and 3T3-F442A cells was obtained by shifting the cells, after confluence, in DMEM containing 8% fetal bovine serum, 17 nM insulin, and 2 nM $T_3$ (differentiation medium). Fusion of $C_2C_{12}$ cells was obtained by shifting the cells, at confluence, in DMEM containing 5% horse serum. Media were changed every other day. α-Linolenate was dissolved in ethanol at a concentration of 50 mM and aliquots were immediately added to standard medium in order to obtain the final fatty acid concentration as indicated. This medium was pre-warmed at 37°C for 45 min and then added to the cells after removal of the previous culture medium.

### RNA analysis

RNA were prepared according to Chomczynski and Sacchi (22) and analyzed as previously described (8, 18).

### Materials

Culture media were obtained from Gibco (Cergy-Pontoise, France). Recombinant human growth hormone was a kind gift from KabiVitrum (Stockholm, Sweden). Radioactive materials, random priming kit, and Hybond membranes were from Amersham (France). Bovine and horse sera, fatty acids, and other chemical products were purchased from Sigma Chimie (France).

## RESULTS

### Relationships between fatty acids and promoting agents of adipose conversion in the activation of aP2 gene in Ob1771 cells.

The effects of agents known to promote the adipose conversion on the aP2 mRNA abundance were examined in 1-day post-confluent Ob1771 cells exposed or not to various concentrations of α-linolenate for 24 h. As reported in **Table 1A**, the very weak aP2 mRNA signal obtained for cells maintained in standard medium increased as a function of the α-linolenate concentration to reach a 12-fold increase at 300 μM, which was the maximal concentration at which the cells remained viable. Addition of 1.2 nM GH or 17 nM insulin did not change the basal expression of the aP2 mRNA and did not affect the response of this gene to fatty acids (not shown). Exposure of the cells to a stable analog of cAMP neither induced an accumulation of aP2 mRNA nor modulated the

TABLE 1. Combined effects of α-linolenate and various agents on aP2 mRNA expression

| Treatment | α-Linolenate Concentration | | | | |
|---|---|---|---|---|---|
| | 0 | 10 μM | 30 μM | 100 μM | 300 μM |
| **A** | | | | | |
| Control | 1.0 ± 0.1 | N.D. | 4.0 ± 0.3 | 8.2 ± 0.5 | 12.0 ± 0.7 |
| 8-CPT-cAMP (100 μM) | 1.1 ± 0.1 | N.D. | 3.3 ± 0.3 | 8.5 ± 0.9 | 11.5 ± 0.8 |
| H7 (50 μM) | 1.1 ± 0.1 | N.D. | 4.3 ± 0.4 | 8.0 ± 0.7 | 12.7 ± 0.9 |
| Dexamethasone (1 μM) | 3.8 ± 0.3 | N.D. | 18.1 ± 0.8 | 32.9 ± 0.9 | 60.8 ± 4.7 |
| **B** | | | | | |
| Control | 0.25 ± 0.03 | 0.29 ± 0.03 | 0.85 ± 0.09 | | |
| Dexamethasone (1 μM) | 0.86 ± 0.08 | 0.88 ± 0.09 | 3.80 ± 0.40 | | |
| Serum (0.1%) | 0.13 ± 0.02 | 1.02 ± 0.11 | 1.09 ± 0.12 | | |
| Serum (0.1%) + Dexamethasone (1 μM) | 1.09 ± 0.12 | 7.52 ± 0.60 | 9.56 ± 0.72 | | |

(A) One-day post-confluent Ob1771 cells maintained in standard medium were exposed for 24 h to various concentrations of α-linolenate in the absence or the presence of the indicated agent. (B) One-day post-confluent Ob1771 cells maintained in standard medium were washed twice with serum-free medium and then incubated for 24 h in medium containing 0.1% or 8% serum (control) and supplemented with the indicated concentration of α-linolenate, in the absence or the presence of 1 μM dexamethasone. RNA were analyzed by dot-blot as described in Materials and Methods. The results were normalized to β-actin signals and are expressed by dividing each value by that obtained with cells maintained in standard medium. The results are the mean ± SD of five (A) and three (B) independent experiments.

fatty acid-induced accumulation. The use of another cAMP analog, such as 8-Br cAMP, or other cAMP-elevating agents, such as methyl isobutyl xanthine or forskolin, was also ineffective (not shown). It can be concluded that cAMP did not mimic the effect of fatty acids on the induction of the aP2 gene expression. This was further supported by the lack of inhibitory effect of H7, an inhibitor of kinase C and partially that of protein kinase A, on the induction of the aP2 gene expression in response to α-linolenate supplementation (Table 1A).

In contrast to other agents, dexamethasone provoked an accumulation of aP2 mRNA. The treatment of the cells by a combination of α-linolenate and dexamethasone led to a striking induction of the aP2 mRNA. It is noteworthy that, at any α-linolenate concentration, the effects of both inducers were synergistic. This observation is also well illustrated by the dose-response curves for dexamethasone on the aP2 mRNA content in cells maintained either in the absence or in the presence of α-linolenate (**Fig. 1**). The half-maximal accumulation of aP2 mRNA was obtained with approximately 20 nM dexamethasone. In addition to their potent effect on the aP2 gene expression, fatty acids provoked a significant increase in the sensitivity of the response to dexamethasone, as demonstrated with a shift by one order of magnitude, the half-maximal accumulation of aP2 mRNA now taking place at a concentration of about 2 nM. Taken together, these results indicate that fatty acids and dexamethasone act in a concerted and rather complex manner to induce the aP2 gene expression in committed preadipose cells.

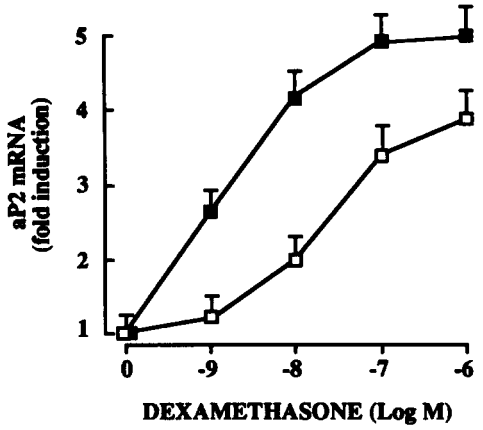


**Fig. 1.** Stimulation of the aP2 gene expression by dexamethasone in Ob1771 cells exposed or not to α-linolenate. One-day post-confluent cells maintained in standard medium were exposed for 24 h to various concentrations of dexamethasone in the absence (□) or the presence (■) of 250 μM α-linolenate. RNA were prepared and analyzed by dot-blot as described in Materials and Methods. The results were normalized to β-actin signals and are presented by dividing each value by that obtained in cells untreated with dexamethasone (0.23 ± 0.03 and 2.66 ± 0.23 integrator units for cells maintained in the absence or the presence of α-linolenate, respectively). The results are the mean ± SD of three independent experiments.

From the experiments reported above, it cannot be excluded that the induction of the aP2 mRNA could require some serum component(s) or could be mediated by some undefined molecules present under a masked form and released from serum proteins by fatty acids. As shown in Table 1B, after a 24-h incubation in medium containing 0.1% serum, a basal expression of aP2 mRNA was observed but this was lowered approximately 2-fold as compared to cells maintained in standard medium. This expression might be due to residual fatty acids present in serum and/or to the low but still detectable endogenous fatty acid synthesis from glucose. When maintained in medium containing low serum concentration, the cells remained fully responsive to both dexamethasone and α-linolenate. Moreover, reducing the serum concentration to 0.1% led to a significant improvement of the dexamethasone induction of the aP2 gene expression (8.4- and 3.4-fold induction in cells maintained in 0.1% and 8% serum, respectively) and to a significant decrease in the effective concentration of α-linolenate. For instance, a concentration of 10 μM α-linolenate, which failed to provoke any response in cells maintained in standard medium, induced a near maximal response under these conditions. The improvement of the response to both inducers at low serum concentrations could be interpreted by the actual increase in the concentrations of unbound dexamethasone and fatty acids. It can be concluded that the stimulatory effect of dexamethasone and fatty acids on aP2 gene expression does not involve additional components from serum.

### Kinetics of aP2 mRNA accumulation in the presence of fatty acids and dexamethasone

Kinetics of aP2 mRNA accumulation in response to fatty acid or dexamethasone alone or in combination were next investigated by treatment of 1-day post-confluent Ob1771 cells with optimal concentrations of each inducer. As illustrated in **Fig. 2A**, when present alone each inducer showed different kinetics. The accumulation of aP2 mRNA in response to dexamethasone was rapid, peaked after 8 h, and declined thereafter. By contrast, the effect of α-linolenate was slower than that of dexamethasone, and appeared continuously increasing. This effect was reproducible but weak after 4 h and increased in a linear manner for longer periods of time, reaching a 25-fold increase after 48 h of treatment. When both inducers were used in combination, their effects were additive during the first 8 h of treatment and became synergistic for longer periods of time, reaching a 120-fold stimulation after 48 h (Fig. 2B).

The effects of fatty acids on the aP2 gene expression were rapidly and fully reversible, since after a 24-h exposure to α-linolenate alone (Fig. 2A) or in combination

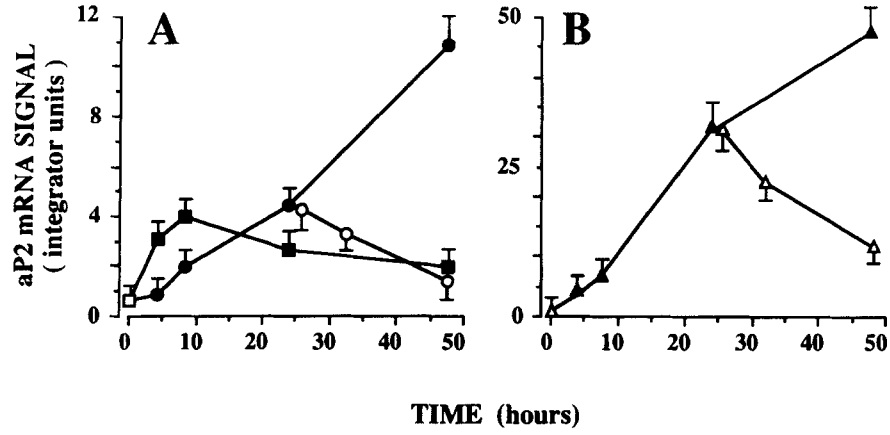


**Fig. 2.** Time course of dexamethasone and α-linolenate effects on aP2 mRNA expression in committed, lipid-free Ob1771 cells. One-day post-confluent cells maintained in standard medium were treated in A with 1 μM dexamethasone (■) or 250 μM α-linolenate (●) and in B with 1 μM dexamethasone and 250 μM α-linolenate (▲). Removal of α-linolenate was performed by two washes and incubation in standard medium (○) or in standard medium containing 1 μM dexamethasone (△). RNA were prepared and analyzed as in Fig. 1. The results are presented as the mean integrator units ± SD of three independent experiments.

with dexamethasone (Fig. 2B), removal of fatty acids from medium led to a decrease of the aP2 mRNA abundance. In both cases, the half-time of decay of the signals was approximately 14–18 h, a value consistent with the turnover rate of the aP2 mRNA in fully differentiated 3T3-F442A cells reported to be 12 h (1).

### Differential requirement of protein synthesis for the induction of the aP2 mRNA in response to fatty acid and glucocorticoid treatment

To determine whether or not the induction of the aP2 gene in response to dexamethasone and to fatty acids was dependent upon de novo protein synthesis, cells were exposed to cycloheximide 15 min before the addition of the inducers. Control experiments had shown that a 97% decrease of [$^3$H]leucine incorporation into proteins occurred within 10 min in cells treated with cycloheximide, without any significant change in cell viability for the next 15 h. As shown in **Fig. 3**, cycloheximide treatment for 4 and 8 h did not affect the basal expression of aP2 mRNA (columns a). The effects of dexamethasone on the aP2 mRNA induction (4.7- and 6-fold increases after 4 and 8 h, respectively) were unaffected by inhibition of protein synthesis (columns b). By contrast, the inducing effects of fatty acids (1.4- and 2.75-fold after 4 and 8 h, respectively) were completely prevented by cycloheximide treatment

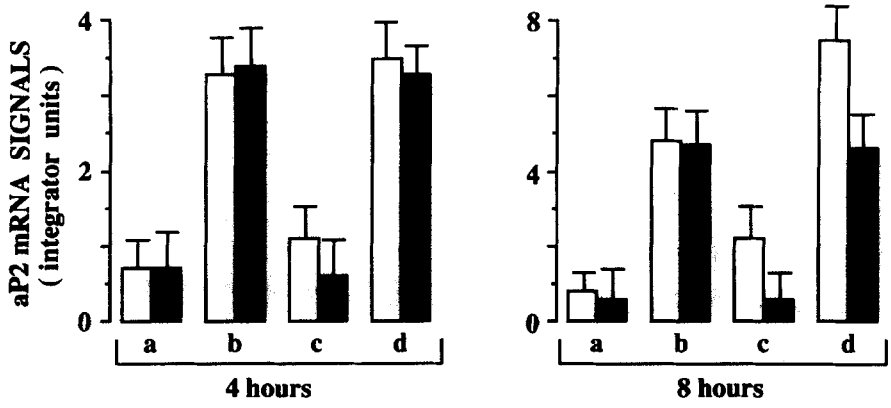


**Fig. 3.** Differential effects of cycloheximide on the induction of aP2 mRNA by fatty acids and dexamethasone in Ob1771 cells. One-day post-confluent cells maintained in standard medium were pre-treated (filled columns) or not (open columns) with 10 μM cycloheximide for 15 min. Cells were then maintained, in the absence or presence of 10 μM cycloheximide, either in the absence of any addition (a) or in the presence of 1 μM dexamethasone (b), 300 μM α-linolenate (c), or 1 μM dexamethasone plus 300 μM α-linolenate (d). RNA were prepared at the indicated times and analyzed as in Fig. 1. The results are the mean integrator units ± SD of four independent experiments.

(columns c). In cells treated with dexamethasone and fatty acids and exposed to cycloheximide, the content of the aP2 mRNA was similar to that observed in cells treated with dexamethasone alone (columns d).

These observations clearly demonstrated that the induction of the aP2 gene by dexamethasone is a primary response independent from ongoing protein synthesis, while the response to fatty acids appears to be indirect and require the synthesis of regulatory protein(s).

### Induction of the aP2 gene by fatty acids, but not by glucocorticoids, is unique to adipose cells

The effect of fatty acids and dexamethasone on the aP2 gene expression was next examined in other preadipose cell lines, such as 3T3-F442A and Ob1754 cells, and in cells that do not express the adipose phenotype, such as fibroblastic 3T3-C2 and myogenic $C_2C_{12}$ cells. As reported in **Table 2**, the regulation of the aP2 gene by fatty acids and dexamethasone appeared to be quite similar in 3T3-F442A and Ob1754 cells to that described in Ob1771 cells. By contrast, this regulation was different between adipose and nonadipose cells with respect to fatty acids but not to dexamethasone: confluent 3T3-C2 fibroblasts maintained in standard medium did not contain a detectable amount of aP2 mRNA, and treatment with α-linolenate for 24 h failed to induce any expression of the aP2 gene. However, dexamethasone treatment led to a weak, but easily detectable aP2 mRNA signal in this nonpreadipose cell line. Fatty acids were unable to provoke any effect additional to that of dexamethasone. A similar pattern of regulation of the aP2 gene expression was observed in differentiated cells of the myogenic $C_2C_{12}$ cell line.

Taken together, the results of Table 2 indicate that the expression of aP2 mRNA can be induced in nonpreadipose cells by glucocorticoid treatment, and that the activation of aP2 gene by fatty acids is an event so far unique to adipose cells.

### Effects of fatty acids on the expression of long-chain acyl-CoA synthease and C/EBP genes in adipose cells

The effects of exposure of confluent Ob1771 cells to α-linolenate and dexamethasone were next investigated on ACS and C/EBP mRNAs. As shown in **Fig. 4**, the cDNA probes derived from rat for ACS and C/EBP were efficient in detecting specific mRNA signals of approximately 3.8 kb (for ACS) and 2.7 kb (for C/EBP), in both fully differentiated Ob1771 (lane e) and 3T3-F442A cells (lane f), whereas both mRNAs remained undetectable in 1-day post-confluent Ob1771 cells (lane a). When compared to the expression of aP2 mRNA, dexamethasone and fatty acids, used separately (lanes b and c) or in combination (lane d), were ineffective in inducing any detectable expression of C/EBP mRNA, albeit overexposure of the autoradiogram or use of higher amounts of analyzed RNA (not shown). By contrast, fatty acid treatment led to an activation of the ACS gene expression (lane c), wherease dexamethasone failed to induce this expression (lane b). When used in combination with α-linolenate, the glucocorticoid analog did not provoke any further additional effect on the ACS mRNA abundance (lane d versus lane c). The lack of effect of dexamethasone on the regulation of ACS gene expression was clearly at variance with that of the aP2 gene expression. Moreover, it is worthy to note that more accurate determinations, performed by dot-blot assays, have shown that, when cells are exposed to fatty acids for longer periods of time than reported here, the abundance of ACS mRNA remains weak as compared to that observed in differentiated cells (up to 20-fold difference); this is again at variance with the pattern observed for the aP2 mRNA.

These observations suggest that fatty acids are able to activate in preadipose cells the expression of two genes encoding key proteins, i.e., aP2 and ACS, involved in fatty acid metabolism, and that C/EBP does not appear to play a role in this activation process.

## DISCUSSION

The results of this study demonstrate that fatty acids act in adipose cells as potent activators of the expression of genes encoding proteins directly involved in their metabolism. The effect of fatty acids appears in preadipose cells to be confined to genes encoding proteins playing key roles in fatty acid metabolism, but does not affect the expression of other genes, excluding some unspecific effect

TABLE 2. Induction of the aP2 gene by fatty acids is confined to adipose cells

| Treatment | 3T3-F442A | Ob1754 | 3T3-C2 | $C_2C_{12}$ |
|---|---|---|---|---|
| None | 0.30 ± 0.04 | 0.13 ± 0.03 | undetectable | undetectable |
| Dexamethasone | 1.06 ± 0.10 | 0.48 ± 0.06 | 0.53 ± 0.07 | 0.22 ± 0.03 |
| α-Linolenate | 3.60 ± 0.25 | 1.82 ± 0.12 | undetectable | undetectable |
| Dexamethasone + α-linolenate | 21.52 ± 1.55 | 8.15 ± 0.40 | 0.61 ± 0.10 | 0.21 ± 0.03 |

One-day post-confluent 3T3-F442A, Ob1754, and 3T3-C2 cells maintained in standard medium and $C_2C_{12}$ cells maintained for 5 days in DMEM containing 5% horse serum were exposed for 24 h to 1 μM dexamethasone, 300 μM α-linolenate, or a combination of the two agents. RNA were analyzed as in Table 1. The results are the mean of integrator units ± SD of three independent experiments.

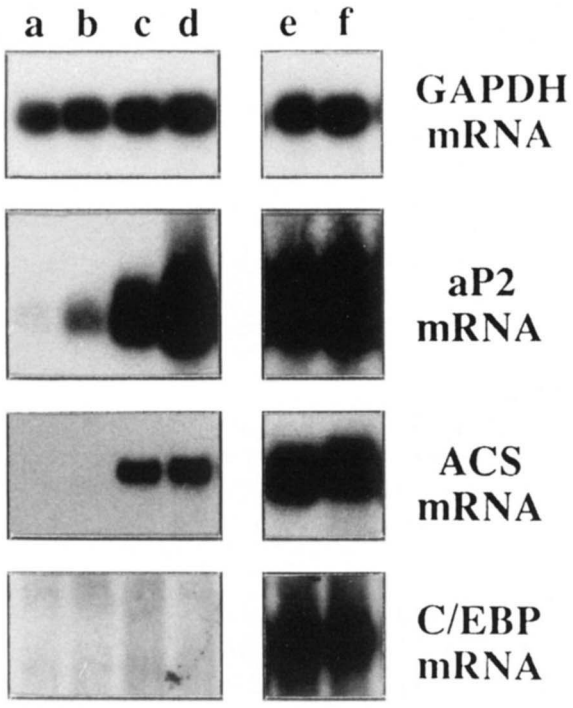


**Fig. 4.** Effect of fatty acids and dexamethasone on ACS and C/EBP mRNA expression in committed, lipid-free Ob1771 cells. RNA were prepared and analyzed by Northern-blot (20 μg/lane) as described in Materials and Methods. Lanes a–d: 1-day post-confluent Ob1771 cells were maintained in standard medium. Cells were maintained for 24 h either in the absence of any addition (a) or in the presence of 1 μM dexamethasone (b) 250 μM α-linolenate (c), or 1 μM dexamethasone plus 250 μM α-linolenate (d). Lanes e and f: Ob1771 (e) and 3T3-F442A (f) cells were maintained in differentiation medium for 15 and 11 days, respectively. The autoradiogram shown is representative of four independent experiments that gave very similar results.

of fatty acids on the overall differentiation-dependent genes.

Clearly, the mechanisms involved in the induction of aP2 gene expression by fatty acids are different from those of other inducers of the gene in preadipose cells. Moreover, the induction of aP2 gene expression by fatty acids seems to be confined to adipose cells, whereas dexamethasone appears to be able to induce the expression of the aP2 gene in both adipose and nonadipose cells. This expression of aP2 gene in nonadipose cells is not surprising since significant amounts of aP2 mRNA (3 to 9% that determined in adipose cells) have been detected in heart, kidney, and muscle from adult mice (23). Likewise, it has been reported that the expression of aP2 mRNA could be induced in 3T3-fibroblasts exposed to dexamethasone despite the absence of morphological differentiation (24).

An attractive hypothesis to explain the acquisition of a cell-type response to fatty acids which requires the synthesis of regulatory protein(s) should be the involvement of a mediator specifically induced in preadipocytes. For that reason, C/EBP, which is expressed in a differentiation dependent-manner and described as a transactivator of the aP2 gene promoter (14, 15), could be the candidate protein acting as the mediator. However, since the C/EBP mRNA remains unexpressed in cells exposed to fatty acids, it can be concluded that C/EBP appears not to be involved and that other trans-acting factor(s) should play a role in this regulation. It has been demonstrated recently that the binding sites for AP-1 and C/EBP are not sufficient or necessary to drive adipose-specific expression of the aP2 gene in vivo and that the tissue-specific expression of the gene requires only a DNA element located between 4.9 to 5.4 kb upstream the transcription initiation site (25). It is hypothesized that this DNA element may be involved in the induction of aP2 gene by fatty acids in a fat-tissue-specific manner. In any event, the involvement of fatty acids in the regulation of aP2 and ACS gene expression raises the interesting possibility that these amphipathic molecules or one of their metabolites could trigger, in a rapid and fully reversible manner, the synthesis of trans-acting factor(s) at a transcriptional or translational level.

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