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## Cloning and Regulation of a mRNA Specifically Expressed in the Preadipose State\*

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Christian Dani†§, Alain Doglio¶, Ez-Zoubir Amri‡, Sylvie Bardon||, Philippe Fort\*\*,  
Bénédicte Bertrand‡, Paul Grimaldi‡, and Gérard Ailhaud‡

From the ‡Centre de Biochimie du Centre National de la Recherche Scientifique (UPR 7300), Faculté des Sciences, Parc Valrose, 06034 Nice cédex, the ¶Service de Bactériologie-Virologie, Faculté de Médecine, Chemin de Vallombrose, 06034 Nice cédex, the ||Station de Recherches de Nutrition, Institute National de la Recherche Agronomique, Domaine de Vilvert, 78350 Jouy-en-Josas, and the \*\*Laboratoire de Biologie Moléculaire, Université des Sciences et Techniques du Languedoc, Place E. Bataillon, 34060 Montpellier, France

A cDNA library of Ob1771 preadipocytes was constructed, and a cDNA clone designated pOb24 was isolated by differential screening. The pOb24 mRNA, 6 kilobases in length, rose sharply in early differentiating Ob1771 and 3T3-F442A cells and decreased thereafter. In mouse adipose tissue, it was present at a high level in stromal-vascular cells (containing adipose precursor cells) and at a low level in mature adipocytes. Thus, pOb24 mRNA appears to be both *in vitro* and *in vivo* a unique marker of the preadipose state, *i.e.* of cell commitment during adipose cell differentiation. In contrast to glycerol-3-phosphate dehydrogenase mRNA, the emergence of pOb24 mRNA in Ob1771 cells required neither growth hormone or triiodothyronine as obligatory hormones nor insulin as a modulating hormone.

Comparative studies of the expression of pOb24 and dihydrofolate reductase genes during the cell cycle suggest that arrest at the G<sub>1</sub>/S boundary was critical for the entry into the preadipose state. Tumor necrosis factor and transforming growth factor- $\beta$  were able to induce a large decrease of pOb24 mRNA level in growth-arrested Ob1771 cells. This decrease was shown to be only confined to early differentiating, glycerol-3-phosphate dehydrogenase negative cells as no decrease of pOb24 mRNA level was observed in glycerol-3-phosphate dehydrogenase positive cells. This result suggests that signals generated by tumor necrosis factor and transforming growth factor- $\beta$  have no effect on a commitment-related gene in late differentiated cells.

Adipose conversion of preadipocytes from established cell lines involves both morphological changes and the acquisition of phenotypic markers. These changes take place in 3T3, TA1, and Ob17 cells through corresponding changes in mRNA content detected by translation and/or by hybridization using cDNA probes. Changes in mRNA concentrations have been reported for mRNAs encoding for (i) glycerol-3-phosphate dehydrogenase (ii) a myelin P<sub>2</sub>-like protein of 13 kDa which

appears to be specific to adipose tissue, (iii) a homologue of serine proteases of 44 and 37 kDa only detected in adipose tissue, sciatic nerve, and skeletal muscle, and (iv) unidentified proteins (1-4). The induction of these various mRNAs occurs during the adipose conversion process at a late stage during which the cells accumulate triacylglycerol. This stage, giving rise to terminally differentiated cells, is regulated by growth hormone and triiodothyronine as obligatory hormones as well as by insulin and glucocorticoids as modulating hormones (5-9). In contrast to terminal differentiation, the early stage of the adipose conversion process is characterized by the emergence of lipoprotein lipase and does not require the above hormones (10). It has been shown that this early stage in the differentiation of preadipocyte cell lines, giving rise to non-terminally differentiated cells, is coupled to growth arrest (10, 11), but the characteristics of this state, the molecular events linked to growth arrest, and the effectors regulating gene expression at that stage of the cell cycle, remain poorly documented.

In this paper, we describe the molecular cloning of a cDNA probe and the early emergence of the corresponding mRNA in differentiating Ob1771 and 3T3-F442A cells. This mRNA, namely pOb24 mRNA, appears in post-natal mice to be confined in adipose tissue to the stromal-vascular fraction containing adipose precursor cells. Thus, pOb24 mRNA appears to be an early marker of adipose cell differentiation *in vitro* and *in vivo* and also to offer the opportunity to study cell commitment at the molecular level. The regulation of pOb24 gene expression has been investigated in the present work as a function of the cell cycle with respect to its hormonal requirements. Since it has been reported in 3T3-L1 cells that the tumor necrosis factor (TNF)<sup>1</sup> regulates the lipoprotein lipase mRNA content (12, 13) and that exposure to transforming growth factor- $\beta$  (TGF- $\beta$ ) prevents the expression of glycerol-3-phosphate dehydrogenase activity and the acquisition of the adipocyte phenotype (14), the effects of TNF and TGF- $\beta$  on pOb24 gene expression have been also investigated.

### EXPERIMENTAL PROCEDURES

**Animals**—Four-week-old male OF-1 mice were obtained from Iffa-Credo (Lyon, France).

**Cell Culture**—The characterization of Ob1771 cells has been previously described (5). Clonal lines 3T3-F442A and 3T3-C2 were previously described (15) and obtained through the courtesy of Dr. H. Green (Boston). Cells were plated at  $1.5 \times 10^3$  cells/cm<sup>2</sup> in 100-

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§ To whom correspondence should be addressed.

<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; GH, growth hormone; T<sub>3</sub>, triiodothyronine; LPL, lipoprotein lipase; TGF- $\beta$ , transforming growth factor- $\beta$ ; kb, kilobase; DHFR, dihydrofolate reductase.

mm diameter dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 units/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, 33  $\mu\text{M}$  biotin, and 17  $\mu\text{M}$  pantothenate (defined as the standard medium). After confluence, 2 nM triiodothyronine and 17 nM insulin (Ob1771 cells) or 800 nM insulin (3T3-F442A and 3T3-C2 cells) were added to the standard medium (defined as differentiation media). In order to study the hormonal regulation of pOb24 gene expression in Ob1771 cells, growth hormone-depleted bovine serum (5) or triiodothyronine-depleted fetal bovine serum (6) were used. Growth arrest experiments were performed by adding 5 mM thymidine to Ob1771 cells (100-mm culture dishes) inoculated at  $10^4$  cells/cm<sup>2</sup> and actively growing in the standard medium. Growth-arrested cells were then maintained in the same medium for 24 h. Control experiments showed that DNA synthesis resumed immediately after block removal (detected within 10 min) and that the cell number almost doubled within 24 h (determined with a Coulter counter). In the experiments to investigate the role of TNF and TGF- $\beta$  on the regulation of pOb24 gene expression, 3-day post-confluent Ob1771 cells (100-mm culture dishes) maintained in the differentiation medium were exposed with suitable controls to 1.5 nM TNF or 80 pM TGF- $\beta$  for 24 h, in the absence or presence of 2  $\mu\text{g}/\text{ml}$  aphidicolin. Poly(A)<sup>+</sup> RNA was then isolated as described above. Control experiments of thymidine incorporation into DNA were performed in parallel in 35-mm culture dishes: 3-day post-confluent Ob1771 cells were maintained 24 h in 2 ml of the differentiation medium containing [<sup>3</sup>H] thymidine (6  $\mu\text{M}$ ; 1  $\mu\text{Ci}/\text{dish}$ ) in the absence or in the presence of 2  $\mu\text{g}/\text{ml}$  aphidicolin, with or without 1.5 nM TNF or 80 pM TGF- $\beta$ . After homogenization, cell extracts were processed for counting the radioactivity incorporated into DNA (10).

**Construction and Screening of a cDNA Library from Ob1771 Cells Containing Early Markers**—Poly(A)<sup>+</sup> RNA isolated from 4-day post-confluent (early differentiating) Ob1771 cells was used to synthesize double-stranded cDNA using oligo(dT) as the first strand primer. The second strand was synthesized by the procedure using RNase H, DNA polymerase I, and DNA ligase from *Escherichia coli* as described by Gubler and Hoffman (16). The cDNAs were dC-tailed with terminal transferase and annealed to oligo(dG)-tailed pBr322 vector as described by Land *et al.* (17) and used to transform *E. coli* RR1.

Plasmids in bacterial colonies (approximately  $10^3$  recombinants) were amplified, transferred to nitrocellulose filters, and lysed according to Maniatis *et al.* (18). Filters were hybridized for 24 h at 42 °C to <sup>32</sup>P-labeled single-stranded DNA complementary to poly(A)<sup>+</sup> RNA from exponentially growing (undifferentiated) Ob1771 cells. After autoradiography, the filters were washed free of probe and then hybridized under the same conditions to <sup>32</sup>P-labeled single-stranded cDNA synthesized from poly(A)<sup>+</sup> RNA from early differentiating Ob1771 cells. Hybridization and washing procedures were identical to those used for Northern blot analyses described in Ref. 7 with the exception that dextran sulfate was not included in the hybridization buffer. One positive clone (designated pOb24) containing a *PstI/PstI* 1200-base pair insert size was isolated and prepared according to Maniatis *et al.* (19).

**Northern Blot Analysis**—Poly(A)<sup>+</sup> RNA from 3T3-C2, 3T3-F442A, Ob1771 cells, and various mouse tissues was prepared as previously described (7), with slight modifications when isolating RNAs from white adipose tissue. After lysis of the tissue in the thiocyanate-buffered solution, two lipid extractions were performed in the presence of an equal volume of ethyldioxide. Poly(A)<sup>+</sup> RNA was electrophoresed through a formaldehyde/agarose gel, transferred onto an Amersham nylon membrane filter, and hybridized with various nick-translated probes as previously described (20).

**Nuclear Transcription Assays**—Nuclei from Ob1771 cells and nuclear transcription assays were performed as described previously (5). The results were quantitated by densitometry using a LKB laser densitometer scanning. Nuclei from stromal vascular cells and adipocytes were prepared as follows. After collagenase digestion of the epididymal fat pads and sedimentation to separate the stromal-vascular and adipocyte fractions (21), the stromal-vascular cells were homogenized in a tight-fitting Dounce homogenizer in the presence of 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl and 3 mM MgCl<sub>2</sub> (buffer A) at a ratio of 0.3 ml of buffer A/pad. Nonidet P-40 was added to a final concentration of 0.5% (v/v). After 5 min at 4 °C, nuclei were pelleted by centrifugation at 500 g for 5 min, washed once in 20 ml of buffer A containing 0.5% Nonidet P-40, and repelleted by centrifugation at 500 g for 5 min.

Nuclei from 60 pads were resuspended in 1 ml of buffer A containing 0.5% Nonidet P-40 and pelleted at 500 g for 5 min. They were then resuspended in 0.2 ml of 50 mM Tris-HCl buffer, pH 8.3,

containing 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, and 40% (v/v) glycerol. In the case of the adipocyte fraction, adipocytes were homogenized in buffer A as above and then stored for 10 min at 4 °C. After removing the fat cake with a spatula, Nonidet P-40 was added to the fat-free homogenate as described above. The following steps were then identical to those described above for the stromal-vascular cells.

**Materials**—[*methyl*-1',2'-<sup>3</sup>H]Thymidine, [ $\alpha$ -<sup>32</sup>P]dCTP, [ $\alpha$ -<sup>32</sup>P]UTP, and nick translation kit were purchased from Amersham International (Buckinghamshire, United Kingdom). Restriction enzymes, DNA ligase, and DNA polymerase were from Boehringer Mannheim. Culture media and fetal bovine serum were from GIBCO (Cergy-Pontoise, France). Guanidinium monothiocyanate was a product of Fluka (Bucks, Switzerland). Porcine TGF- $\beta$  (batch GF 1471) was a product of R&D Systems, Inc. (Minneapolis, MN). Recombinant mouse TNF ( $4 \times 10^8$  units/mg; purity > 99%) was a kind gift of Dr. Jan Tavernier (Biogent, Gent, Belgium).

## RESULTS

**Expression of pOb24 mRNA during Adipose Cell Differentiation and in Different Mouse Tissues**—A cDNA library was screened by sequential hybridization using <sup>32</sup>P-labeled cDNAs made from poly(A)<sup>+</sup> RNA isolated from exponentially growing (undifferentiated) and early confluent (differentiating) Ob1771 cells. Using this approach, we selected a cDNA clone (named pOb24) corresponding to a mRNA specifically induced during adipose conversion. The pOb24 cDNA hybridized to a 6–6.5 kb RNA which appeared within 1 day after confluence in Ob1771 and 3T3-F442A cells as assayed by RNA blot analysis. This 6-kb transcript was absent from growing cells and also from growing and confluent non-preadipocyte 3T3-C2 cells (Fig. 1). The low content of pOb24 mRNA observed in growing 3T3-F442A cells was likely due to the small percentage of confluent, already differentiated, lipid-containing cells present at that stage. In all cases a single band of 2.2 kb was observed when probed for mouse  $\beta$ -actin mRNA. Since no significant change in the  $\beta$ -actin mRNA content was observed, this signal was subsequently used to correct for possible differences in the amounts of poly(A)<sup>+</sup> RNA loaded and in transfer efficiency. The pOb24 mRNA was present in adipose tissue of 4-week old mice<sup>2</sup> but could not be detected in liver and kidney (Fig. 1), skeletal muscle, brain, heart, and spleen (not shown). This lack of detection in tissues other than adipose tissue was not due to a degradation of the various messenger RNAs since, in all cases, a single well-developed band of 2.2 kb was observed for  $\beta$ -actin mRNA.

Taken together, these results suggest that pOb24 mRNA, which is confined to adipose tissue, can be considered as a marker of adipose cell differentiation. This point was further investigated both *in vitro* and *in vivo*.

**Early Expression of pOb24 mRNA during Adipose Conversion of Preadipose Cells and in Adipose Precursor Cells**—The emergence of pOb24 mRNA was examined during adipose conversion of Ob1771 and 3T3-F442A cells and compared with that of mRNAs encoding for glycerol-3-phosphate dehydrogenase and adipin. After analysis by densitometry, the results were quantitated and normalized to signals generated by probing with the cDNA probe for mouse  $\beta$ -actin mRNA. As shown in Fig. 2A, the pOb24 mRNA content of differentiating Ob1771 cells clearly attained a maximal level and then decreased rapidly at a time when mRNAs encoding for adipin and glycerol-3-phosphate dehydrogenase were still increasing. The maximal increase in glycerol-3-phosphate dehydrogenase mRNA occurred 4 days later and that in adipin mRNA was further delayed by at least 1 week.

The study of the developmental expression of the above

<sup>2</sup> pOb24 mRNA is also detected in the stromal-vascular fraction of adipose tissue of younger (2-week-old) and older (3-month-old) mice.

markers has been extended to 3T3-F442A preadipocyte cells, and the chronology of events was found to be similar to that described for Ob1771 cells (Fig. 2B). Transcription analyses using nuclei isolated from growing and early confluent Ob1771 cells revealed that the transcription of the pOb24 gene was nil in growing cells but was taking place in early confluent cells (not shown). These results strongly suggest that the activation of pOb24 gene transcription is primarily responsible for the accumulation of pOb24 mRNA at the time of confluence, as has been demonstrated for other genes induced during adipose conversion (22, 23).

The results so far indicate that pOb24 mRNA was only detected in adipose tissue albeit at a low level, whereas *in vitro* it was only expressed at high levels for a short period of time during differentiation of Ob1771 cells. Therefore, the possibility that pOb24 mRNA was only present in nonterminally differentiated adipose cells was next investigated. For that purpose stromal-vascular cells and adipocytes were isolated from mouse epididymal fat pads after cell dissociation by collagenase treatment and centrifugation. Under these conditions some cross-contamination of each fraction by other cell types was unavoidable. Nevertheless, the Northern blot analysis presented in Fig. 2C indicates clearly that pOb24 mRNA was present at a high level in cells of the stromal-vascular fraction and at a low level in the adipocyte fraction. It is very likely that the pOb24 mRNA detected in the stromal-vascular fraction was only due to preadipocytes and not to other cell types (fibroblasts, endothelial cells, and mastocytes), since the latter were also present in various tissues in which pOb24 mRNA was not detected (Fig. 1). In contrast to pOb24 mRNA, the mRNA for glycerol-3-phosphate dehydrogenase and even more so the mRNA for adipsin were present at higher proportions in the adipocyte fraction than in the stromal-vascular fraction. These observations were not unique to the periepididymal fat tissue, as almost identical results were found for the inguinal fat tissue of the same mice (not shown).

The relative transcription rates of  $\beta$ -actin, pOb24, and glycerol-3-phosphate dehydrogenase genes were measured by using a nuclear transcription assay. Nuclei were isolated from cells of the stromal-vascular and adipocyte fractions of 4-week-old mice. Dot-blot analysis showed no difference for the transcription of the actin gene in nuclei of stromal-vascular cells and adipocytes (Fig. 3). In contrast to the actin gene, the transcription of the pOb24 gene was higher in nuclei of stromal-vascular cells, whereas that of the glycerol-3-phosphate dehydrogenase gene was higher in nuclei of adipocytes. Nuclei from adipocytes preincubated with  $\alpha$ -amanitin at 2  $\mu$ g/ml for 10 min before addition of labeled UTP and ribonucleotide triphosphates did not transcribe the genes corre-

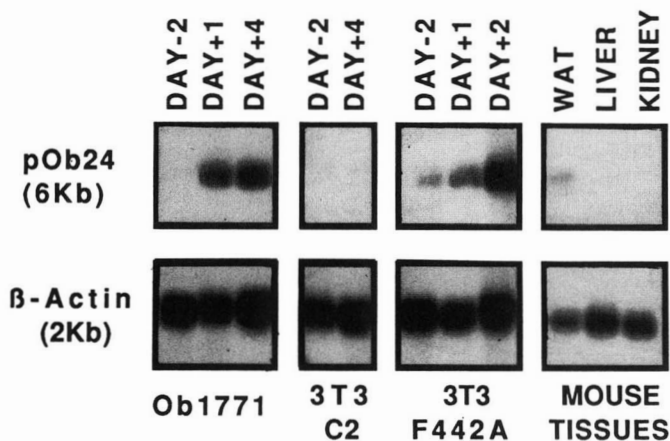


FIG. 1. pOb24 mRNA distribution in preadipocyte and non-preadipocyte cell lines and in various mouse tissues. 6 and 12  $\mu$ g of poly(A)<sup>+</sup> RNA, prepared from cells taken at the indicated times relative to confluence (day 0) and from mouse tissues, were fractionated by electrophoresis on 1.2% denaturing agarose gels containing 1 M formaldehyde and transferred onto nylon membranes (Hybond N, Amersham International, U.K.). The blot was successively hybridized with pOb24 and  $\beta$ -actin cDNA probes labeled by nick translation. After washing, the membrane was exposed to XAR-5 Kodak films at -70 °C with intensifying screen during 12 and 24 h for  $\beta$ -actin and pOb24 mRNA, respectively. The size of the mRNA bands were determined from standards of ribosomal RNAs and 0.24–9.5-kb RNA ladder (GIBCO/BRL, France). Poly(A)<sup>+</sup> RNA of various tissues was prepared from 4-week-old OF1 mice. WAT, white adipose tissue.

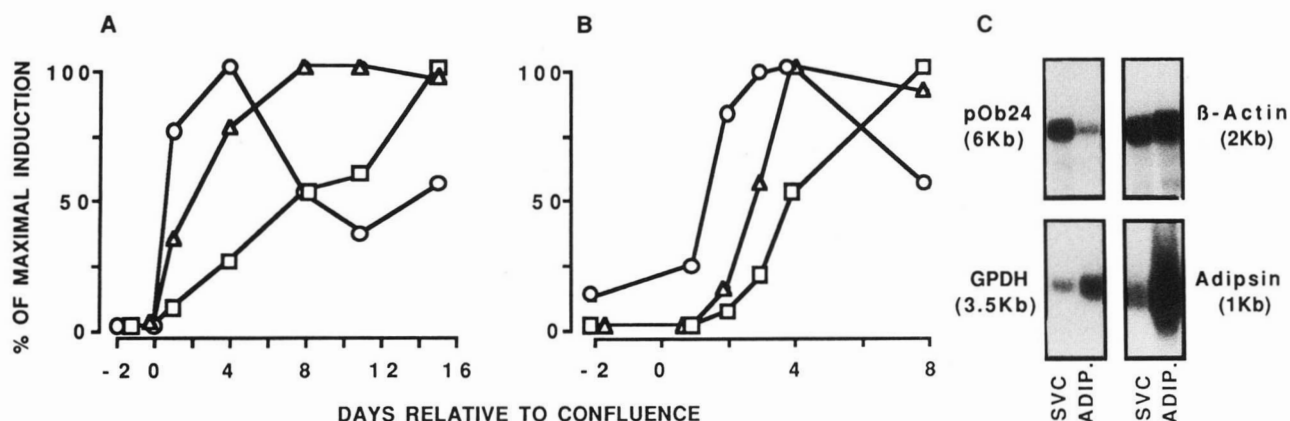


FIG. 2. Expression of pOb24, glycerol-3-phosphate dehydrogenase, and adipsin mRNAs during differentiation of Ob1771 cells (A) and 3T3-F442A cells (B) and in mouse white adipose tissue (C). Cells were maintained since confluence in the differentiation medium. Poly(A)<sup>+</sup> RNA was prepared at the indicated days and analyzed as described in Fig. 1. The autoradiograms were scanned by densitometry with a laser densitometer (Ultrascan LKB). Values were normalized to  $\beta$ -actin signals and expressed by taking as 100% the maximal signal obtained for each probe:  $\circ$ , pOb24 mRNA;  $\Delta$ , glycerol-3-phosphate dehydrogenase mRNA;  $\square$ , adipsin mRNA. To assay for the differential expression of pOb24, glycerol-3-phosphate dehydrogenase, and adipsin mRNAs in stromal-vascular cells and adipocytes from mouse white adipose tissue, epididymal fat tissue of 4-week-old OF1 mice was minced and digested with collagenase (21). 5  $\mu$ g of poly(A)<sup>+</sup> RNA from stromal-vascular cells (SVC) and adipocytes (*adip.*) was analyzed as described in Fig. 1. The  $\beta$ -actin mRNA signal was used as internal control.

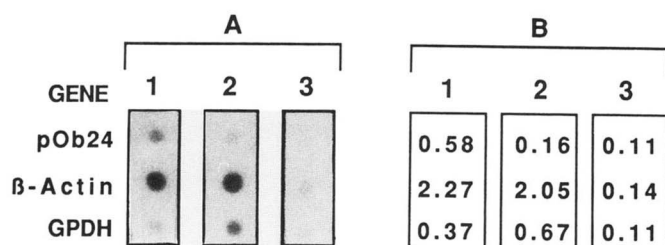


FIG. 3. Transcription of pOb24, glycerol-3-phosphate dehydrogenase, and  $\beta$ -actin genes in isolated nuclei from stromal-vascular cells and adipocytes.  $6 \times 10^6$  cpm of purified RNAs transcribed in nuclei from stromal-vascular cells (1) and adipocytes (2), obtained from 60 epididymal fat pads of OF1 mice, were hybridized to the immobilized DNAs. Adipocyte nuclei were treated in parallel with 1  $\mu$ g/ml of  $\alpha$ -amanitin to determine the basal value of transcription catalyzed by RNA polymerases I and III (3). After hybridization, the filters were treated with RNase A (2.5  $\mu$ g/ml) and RNase T1 (20 units/ml), washed, and exposed for 4 days to Kodak x-ray film (A). The values obtained by densitometry for each dot are given in B.

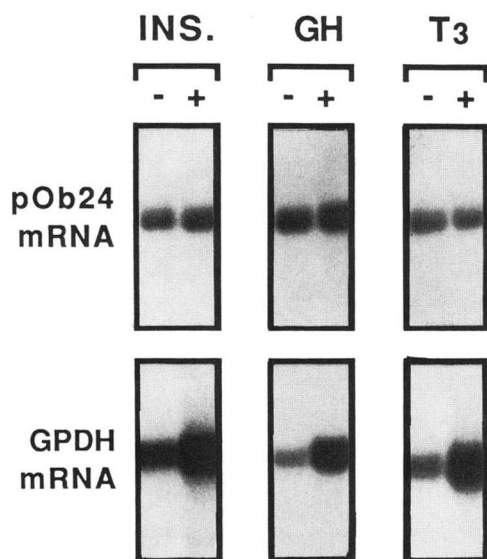


FIG. 4. Differential regulation of pOb24 and glycerol-3-phosphate dehydrogenase gene expression by growth hormone, triiodothyronine, and insulin. Ob1771 cells were grown in the presence of 10% fetal bovine serum or 10% bovine serum. At confluence, these culture media were further supplemented (+) or not (-) with 17 nM insulin, 1.2 nM GH, or 2 nM T<sub>3</sub>, respectively. Eleven days after confluence, poly(A)<sup>+</sup> RNA was prepared and analyzed for the occurrence of pOb24 and glycerol-3-phosphate dehydrogenase mRNAs.

sponding to  $\beta$ -actin, pOb24, and glycerol-3-phosphate dehydrogenase. The results of Fig. 3 indicate that the transcriptional signal observed for the pOb24 gene in nuclei of adipocytes was similar to that observed in  $\alpha$ -amanitin-treated nuclei, strongly suggesting that the transcription rate of the pOb24 gene was low or absent in nuclei of adipocytes. Thus, both *in vitro* in early differentiated cells and *in vivo* in stromal-vascular cells and adipocytes, the regulation of pOb24 mRNA accumulation appeared to be primarily at a transcriptional level.

**Expression of pOb24 mRNA and Hormonal Requirements**—To ascertain whether the same events were regulating early and late differentiation-related genes, we studied the hormonal regulation of pOb24 and glycerol-3-phosphate dehydrogenase gene expression. Clearly, as shown in Fig. 4, the expression of pOb24 mRNA in confluent Ob1771 cells was unimpaired in T<sub>3</sub>-depleted medium or in GH-depleted me-

dium. Insulin, which acts only as a modulating hormone (8), was also not required. Since these various mRNAs were detected after long-term exposure of confluent cells to hormones, it could be argued that a transient effect of any of these hormones could have escaped detection. This is not the case as identical results were obtained by exposing confluent cells (from day 1 to day 14) to these hormones for time periods ranging from 1 to 24 h (not shown). In agreement with previous data (5–8), the expression of glycerol-3-phosphate dehydrogenase mRNA required GH and T<sub>3</sub> whereas insulin was acting as a mere modulator.

**Expression of pOb24 mRNA in G<sub>1</sub> and S Phases of the Cell Cycle**—We had previously shown that induction of pOb24 mRNA and LPL activity in confluent Ob1771 cells can be triggered by growth arrest of exponentially growing cells exposed to a high concentration of thymidine (10). To define more precisely this cell cycle-specific expression, a comparative study with the expression of the dihydrofolate reductase (DHFR) gene was performed. As shown in Fig. 5A, after a single thymidine block of actively growing cells, *i.e.* in the absence of intercellular contacts, the emergence of pOb24 mRNA is rapid (24 h) whereas that of glycerol-3-phosphate dehydrogenase mRNA remains undetectable (not shown). The expression of pOb24 mRNA can be induced by other blocks. It occurred in a similar way in the presence of aphi-

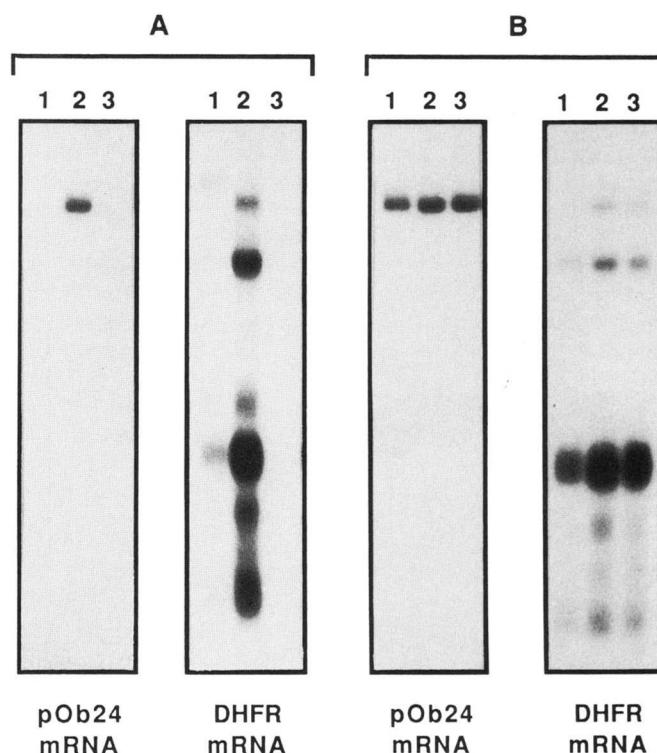


FIG. 5. Expression of pOb24 and DHFR mRNAs in exponentially growing and growth-arrested Ob1771 cells. A, 6- $\mu$ g samples of poly(A)<sup>+</sup> RNA were analyzed as described in Fig. 1. The blot was successively hybridized with pOb24 and DHFR cDNA probes. The blot was exposed during 24 and 48 h for the detection of pOb24 and DHFR mRNA, respectively. 1, exponentially growing cells; 2, same cells after growth arrest in the presence of 5 mM thymidine for 24 h; 3, same cells as in 2 and removal of the block for 24 h. B, cells were cultured during the exponential phase in 10% (lane 1), 3% (lane 2), and 1% (lane 3) fetal bovine serum. RNA from 1-day post-confluent cells was prepared and 6- $\mu$ g samples of poly(A)<sup>+</sup> RNA were analyzed as in A. The size of the various species of DHFR mRNA is identical to that reported by other investigators for 3T6 cells (27). Under all conditions (A and B), no glycerol-3-phosphate dehydrogenase mRNA could be detected.

dicolin, a specific inhibitor of DNA polymerase- $\alpha$  (24), and after  $\text{Ca}^{2+}$ -deprivation. These various treatments are known to block the cells near or at the  $G_1/S$  stage of the cell cycle (25, 26). This interpretation of the block at the  $G_1/S$  boundary is supported by control experiments showing an incorporation of [ $^3\text{H}$ ]thymidine into DNA within 10 min following removal of the thymidine block. In addition, as previously shown (10), the cells were able to resume proliferation immediately after thymidine removal with a doubling time identical to that of untreated control cells. The likelihood of the block at the  $G_1/S$  boundary is also supported by the study of the expression of the DHFR gene. As in 3T6 fibroblasts (26), this gene was constitutively expressed at a low level in growing Ob1771 cells and was increased following thymidine block (Fig. 5A, lanes 1 and 2). This increase occurred in a similar way after aphidicolin block or  $\text{Ca}^{2+}$ -deprivation. Removal of the thymidine block led within 24 h to the disappearance of the bulk of pOb24 and DHFR mRNAs in Ob1771 cells (Fig. 5A, lane 3). As shown in Figs. 5, A and B, both growing Ob1771 cells arrested by thymidine block and Ob1771 cells arrested at confluence show a similar induction of pOb24 and DHFR mRNA, suggesting indirectly that early confluent cells were also arrested at the  $G_1/S$  boundary. Moreover, when growth arrest by means of confluence occurred in the presence of 1, 3, or 10% fetal bovine serum (Fig. 5B), the induction of pOb24 and DHFR mRNAs was similar, indicating that the serum components involved in this induction process, if any, are required at rather low concentrations. Comparative studies of the transcription rates of pOb24 and DHFR genes are reported in Table I (columns A and B). As expected, the transcription rate was increased 4-fold for both genes at growth arrest. Run-on assays showed that the transcription rate of both genes decreased dramatically within the first couple of hours following removal of the block (Table I, column C). Inclusion of 2  $\mu\text{g}/\text{ml}$  cycloheximide did not prevent this decrease (Table I, column D). This last observation suggests that this phenomenon was independent of protein synthesis and suggests also the inactivation in S phase cells of a cell cycle-specific factor(s) which stimulates transcription of the pOb24 gene at the  $G_1/S$  boundary. Since TNF and TGF- $\beta$  were reported to modulate this expression of several adipocyte-specific mRNAs (14, 29), the effect of both agents on pOb24 mRNA levels was next examined.

**Effects of Tumor Necrosis Factor and Transforming Growth Factor- $\beta$  on the Accumulation of pOb24 and Glycerol-3-phosphate Dehydrogenase mRNAs**—To study the effects of TNF and TGF- $\beta$  on the expression of pOb24 and glycerol-3-phosphate dehydrogenase genes, 3-day post-confluent cells were exposed for 24 h to a concentration of TNF able to cause in 3T3-L1 cells a maximal inhibitory effect on the expression of

TABLE I

Transcription of pOb24 and DHFR genes during the cell cycle of Ob1771 cells

$20 \times 10^6$  cpm of purified RNAs transcribed in nuclei obtained from  $10^7$  cells were hybridized to the immobilized DNAs. After hybridization, the procedure was identical to that described in Fig. 3. The results are given as densitometry units. A, nuclei isolated from exponentially growing cells; B, nuclei isolated from cells after growth arrest in the presence of 5 mM thymidine for 24 h; C, nuclei isolated from cells after removal of the block for 2 h; D, same experiment as in C excepted that removal of the block was performed in the presence of 2  $\mu\text{g}/\text{ml}$  cycloheximide. At that concentration, cycloheximide was able to inhibit 95% of protein synthesis (not shown).

| Gene  | A    | B    | C    | D    |
|-------|------|------|------|------|
| pOb24 | 0.16 | 0.66 | 0.20 | 0.18 |
| DHFR  | 0.16 | 0.80 | 0.22 | 0.32 |

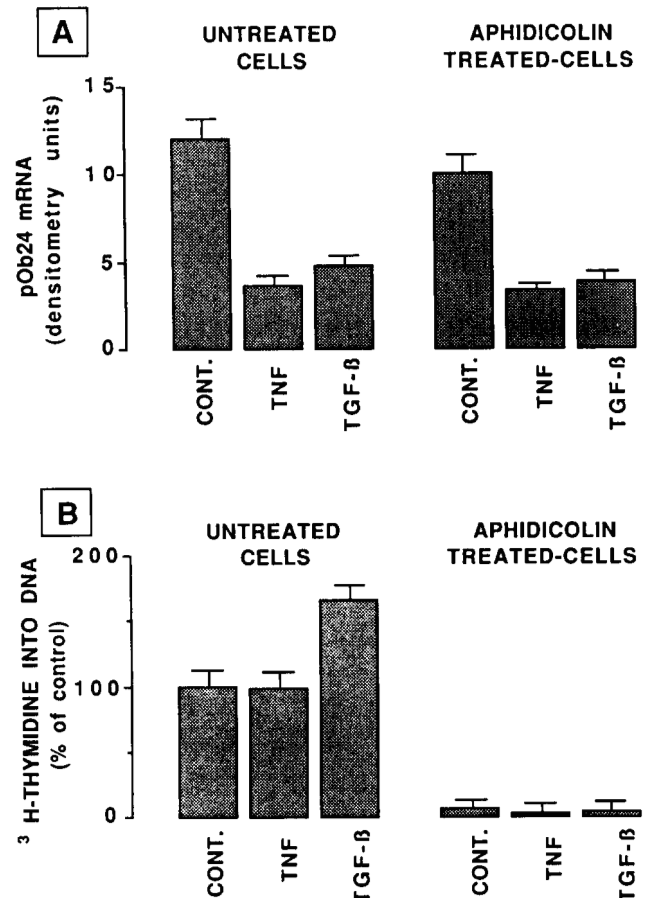


FIG. 6. Effects of TNF and TGF- $\beta$  on DNA synthesis and amount of pOb24 mRNA. A, 3-day-post-confluent Ob1771 cells were maintained in the absence (untreated) or the presence (treated cells) of 2  $\mu\text{g}/\text{ml}$  aphidicolin for 24 h to block DNA synthesis. Both untreated and treated cells were exposed with appropriate controls (cont.) during the same 24-h period to 1.5 nM TNF or 80 pM TGF- $\beta$ . Poly(A) $^+$  RNA from each culture was prepared. 6  $\mu\text{g}/\text{lane}$  was electrophoresed and analyzed on the same blot for two independent experiments. The results (mean  $\pm$  range) are expressed in densitometry units for pOb24 mRNA. B, DNA synthesis was determined by [ $^3\text{H}$ ]thymidine incorporation during 24 h of treatment in the absence or presence of aphidicolin. The results from three independent experiments are expressed (mean  $\pm$  range) by taking as 100% the values obtained for untreated control cells (150,000  $\pm$  20,000 dpm/dish).

LPL and its mRNA (12) and to a concentration of TGF- $\beta$  able to cause a maximal inhibitory effect on the expression of glycerol-3-phosphate dehydrogenase activity (14). As shown in Fig. 6, the accumulation of pOb24 mRNA was decreased to approximately one-quarter in the presence of TNF and to one-third in the presence of TGF- $\beta$  (panel A). The effect of TNF was not due to overproliferation since [ $^3\text{H}$ ]thymidine incorporation into DNA was not increased over control $^3$  (panel B). A similar conclusion could not be drawn for TGF- $\beta$  since a 2-fold increase in [ $^3\text{H}$ ]thymidine incorporation was observed (panel B). Therefore, further experiments were performed in early confluent cells treated with aphidicolin to block DNA synthesis (24). Under these conditions, it should be pointed out that no further accumulation of pOb24 mRNA

$^3$  Autoradiography experiments indicated that approximately 50–60% of control and TNF-treated cells were labeled. The fact that pOb24 mRNA remained expressed in DNA-synthesizing cells which undergo a limited growth resumption (approximately one cell doubling) is reminiscent of our previous results on the expression of LPL (10).

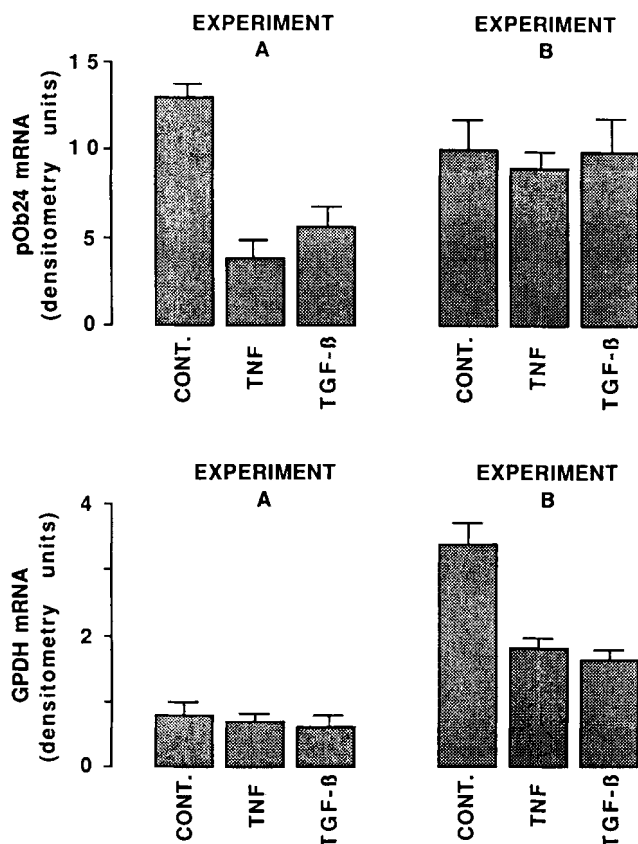


FIG. 7. Effects of TNF and TGF- $\beta$  on the expression of pOb24 and glycerol-3-phosphate dehydrogenase genes in early differentiating and late differentiating Ob1771 cells. 3-day (experiment A) and 6-day (experiment B) post-confluent Ob1771 cells were treated with appropriate controls (cont.) for 24 h with 1.5 nM TNF or 80 pM TGF- $\beta$ . Poly(A)<sup>+</sup> RNA was prepared under each condition and 6  $\mu$ g/lane was electrophoresed. The same blot was successively hybridized with the pOb24 and glycerol-3-phosphate dehydrogenase cDNA probes. After autoradiography (12 h for glycerol-3-phosphate dehydrogenase mRNA signal and 24 h for pOb24 mRNA signal) and analysis by densitometry of the same blot, the results from three independent experiments were obtained and are expressed in densitometry units. The reported values are means  $\pm$  ranges.

occurred, suggesting that these cells were previously blocked at the G<sub>1</sub>/S boundary and that no further recruitment of cells blocked at that stage was taking place (see below). A significant inhibitory effect of TNF and TGF- $\beta$  on pOb24 mRNA accumulation was still observed (panel A) whereas, in aphidicolin-treated cells, no [<sup>3</sup>H]thymidine incorporation into DNA could be noted both in control cells and those treated with TNF and TGF- $\beta$  (panel B). Taken together, these experiments indicate that both TNF and TGF- $\beta$  were able to cause a loss of pOb24 mRNA as an early marker of differentiation-specific genes, in a way which appeared to be independent of DNA synthesis.

It was important to know whether the inhibitory effects of TNF and TGF- $\beta$  on pOb24 mRNA accumulation were effective throughout the adipose conversion process. As shown in Fig. 7 the decrease in pOb24 mRNA content was observed in a population of Ob1771 cells (3 days after confluence), treated with TNF and TGF- $\beta$  during 24 h, in which the glycerol-3-phosphate dehydrogenase mRNA content was low (experiment A). No corresponding decrease in the pOb24 mRNA content was observed in Ob1771 cells (6 days after confluence), in which the glycerol-3-phosphate dehydrogenase mRNA content was 4.5-fold higher (experiment B) and rep-

resentative of a higher degree of cell maturation (30). The lack of effect of TNF and TGF- $\beta$  on pOb24 mRNA accumulation in 6-day post-confluent cells was not due to a lack of cell sensitivity as a 2-fold decrease in glycerol-3-phosphate dehydrogenase mRNA content could be observed in cells treated for 24 h. It is of interest that, after 24 h exposure, the LPL mRNA content was decreased  $4.1 \pm 0.8$ -fold in TNF- or TGF- $\beta$ -treated cells both in 3- and 6-day post-confluent cells (not shown), as compared with 2.5-fold in 3T3-L1 cells treated with TNF for 18 h (12). These results indicate that the expression of pOb24 gene but not that of LPL and glycerol-3-phosphate dehydrogenase genes appeared to become insensitive to the action of TNF and TGF- $\beta$  as a function of cell maturation.

#### DISCUSSION

The understanding of the mechanisms underlying adipose cell commitment requires the study of very early events which occur during adipose cell differentiation. The molecular cloning of a cDNA probe has allowed us to show that pOb24 mRNA of 6 kb in length is specifically expressed during the preadipose state. *In vitro*, the transcription of pOb24 gene was inactive in exponentially growing Ob1771 cells and was activated at the growth arrest taking place at confluence. The pOb24 mRNA content was maximal in early differentiating, *i.e.* glycerol-3-phosphate dehydrogenase-negative cells, and was maintained at a relatively high level in differentiated, *i.e.* glycerol-3-phosphate dehydrogenase-positive, triglyceride-filled cells. *In vivo*, pOb24 mRNA was confined in mouse to adipose tissue. This mRNA was heavily concentrated in cells of the stromal-vascular fraction ( $\approx 90\%$ ) known to contain adipose precursor cells (31) whereas the glycerol-3-phosphate dehydrogenase mRNA was heavily concentrated in adipocytes ( $\approx 80\%$ ). Among hypotheses to explain this difference between *in vitro* and *in vivo* observations, it is possible that the pOb24 mRNA content varied according to the maturation degree of adipose cells. This maturation process might not be complete in adipose cells after differentiation in culture as compared with adipocytes after differentiation *in vivo*.

*In vitro*, the presence of GH, T<sub>3</sub>, and insulin was not required to activate the pOb24 gene which is expressed early in confluent cells but was required to activate another set of differentiation-related genes, *i.e.* glycerol-3-phosphate dehydrogenase and adipocyte P<sub>2</sub> genes which are expressed later (5, 8 and this paper). These results indicate that the obligatory role of GH and T<sub>3</sub> and the modulating role of insulin during adipose cell differentiation are related to late markers only. Taken together, the data of Fig. 5 and Table I indicate clearly that growth arrest at the G<sub>1</sub>/S boundary was sufficient to express the pOb24 gene. In many instances, it is now well established that growth arrest during the G<sub>1</sub> phase of the cell cycle is required for the commitment into a given cell type (32). Using 3T3 T mouse preadipocytes, Scott and co-workers (33, 34) have made the proposal that, in the G<sub>1</sub> phase, there is a growth arrest at a distinct state designated G<sub>D</sub> which was required before glycerol-3-phosphate dehydrogenase was expressed; at that state, LPL is already expressed at 50% of its maximal value (33). It is difficult to compare their and our own data as the G<sub>D</sub> state was only defined by comparison with growth arrest induced by deprivation of growth factor or serum or by deprivation of nutrients or by using selected batches of human plasma (34). Both our present and previous results (10) indicate that Ob17 cells arrested at the G<sub>1</sub>/S boundary express pOb24 and LPL mRNAs (and LPL activity) but not glycerol-3-phosphate dehydrogenase mRNA (and glycerol-3-phosphate dehydrogenase activity), and become

subsequently able to synthesize DNA, divide and later to express glycerol-3-phosphate dehydrogenase.

Transcription studies of pOb24 and DHFR genes reported in Table I suggest the inactivation in the S phase of a cell cycle-specific factor(s) which stimulates their transcription at the G<sub>1</sub>/S boundary. Identification of a cell cycle-specific transcription factor(s) for the pOb24 gene, as already performed in 3T6 cells for the DHRF gene (26), would be of interest. In any event, it must be stressed that separate regulatory factors are probably involved for pOb24 and DHFR genes since, in 3T3-C2 cells, no expression of the pOb24 gene could be observed whereas that of the DHFR gene was similar, if not identical, to that in Ob1771 cells.

The molecular actions of TNF and TGF- $\beta$  on the expression of the differentiation-related genes of adipose cells are unknown. Their effect does not appear to be coupled to growth resumption (14, 29, and our results). Several levels of regulation could be involved such as modifications of the extracellular matrix, as demonstrated for other TGF- $\beta$  target cells (35, 36). Alternatively, both TNF and TGF- $\beta$  could act by causing the cells to return to a point in the G<sub>1</sub> phase where they are unable to express the differentiated phenotype, in a way similar to the inhibitory effect of fibroblast growth factor on myoblast differentiation (37).

The molecular weight and the functional properties of the protein corresponding to the pOb24 mRNA will have to be investigated by sequencing the full length cDNA probe. Sequence studies of both strands of the 1.2-kb cDNA insert have revealed no homology with nucleotide sequences reported in mouse, rat, and human libraries (Genbank and EMBL libraries) and have shown that the insert corresponds to the 3'-untranslated region. At the present time it is premature to claim that pOb24 triggers the terminal differentiation of adipose cells but, in any event, the availability of the 1.2-kb cDNA insert should render studies on the cellularity of adipose tissue in mouse during development and adult life feasible, since *in situ* hybridization techniques should allow the determination of the pOb24 mRNA-positive cells before any triacylglycerol accumulation.

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