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

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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 G1/S boundary was critical for the entry into the preadipose state. Tumor necrosis factor and transforming growth factor-β 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-β 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 P₃-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 adipocyte 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) regulates the lipoprotein lipid mRNA content (12, 13) and that exposure to transforming growth factor-β (TGF-β) prevents the expression of glycerol-3-phosphate dehydrogenase activity and the acquisition of the adipocyte phenotype (14), the effects of TNF and TGF-β on pOb24 gene expression have been also investigated.

EXPERIMENTAL PROCEDURES

Animals—Four-week-old male OF-1 mice were obtained from Laffa-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 × 10⁵ cells/cm² in 100-

kibobase; DHFR, dihydrofolate reductase.

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mm diameter dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 200 units/ml penicillin, 50 μg/ml streptomycin, 33 μM biotin, and 17 μM 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-C3H10T1/2 cells) were added to the standard medium (defined in 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 μM thymidine to Ob1771 cells (100-mm culture dishes) inoculated at 106 cells/cm2 and allowed to grow 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-β 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-β for 24 h, in the absence or presence of 2 μg/ml aphidicolin. Poly(A)+ 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 [3H] thymidine (6 μM; 1 Ci/dish) in the absence or in the presence of 2 μg/ml aphidicolin, with or without 1.5 nM TNF or 80 pM TGF-β. After exposure, cell extracts were processed for counting the radioactivity incorporated into DNA (10).

Expression of pOb24 mRNA during Adipose Cell Differentiation and in Different Mouse Tissues—A cDNA library was screened by sequential hybridization using 32P-labeled cDNAs from poly(A)+ 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 the 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 β-actin mRNA. Since no significant change in the β-actin mRNA content was observed, this signal was subsequently used to correct for possible differences in the amounts of poly(A)+ RNA loaded and in transfer efficiency. The pOb24 mRNA was present in adipose tissue of 4-week-old mice 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 β-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.

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 β-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...

Results

Expression of pOb24 mRNA in Adipose Cells

Specific pOb24 mRNA in Adipose Cells

Cell-The acute treatment of adipocytes with 50 mM Tris-HCl buffer, pH 8.3, containing 5 mM MgCl2, 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-1-14C, 2-3H]Thymidine, [6-3H]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 thiocyanate was a product of Fluka (Bucks, Switzerland). Porcine TGF-β (batch GF 1471) was a product of R&D Systems, Inc. (Minneapolis, MN). Recombinant mouse TNF (4 × 106 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 32P-labeled cDNAs from poly(A)+ 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 the 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 β-actin mRNA. Since no significant change in the β-actin mRNA content was observed, this signal was subsequently used to correct for possible differences in the amounts of poly(A)+ RNA loaded and in transfer efficiency. The pOb24 mRNA was present in adipose tissue of 4-week-old mice 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 β-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 β-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.

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5 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 adipins were present at higher proportions in the adipocyte fraction than in the stromal-vascular fraction. These observations were not unique to the peripididymal fat tissue, as almost identical results were found for the inguinal fat tissue of the same mice (not shown).

The relative transcription rates of β-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 α-amanitin at 2 μg/ml for 10 min before addition of labeled UTP and ribonucleotide triphosphates did not transcribe the genes corre-
stromal-vascular cells and adipocytes. pocytes
given in x-ray film in parallel with 1 pg/ml of a-amanitin to determine the basal value.
hybridized to the immobilized DNAs. Adipocyte nuclei were treated
hydrogenase, and @-actin genes in isolated nuclei from
RNase T1 (20 units/ml), washed, and exposed for 4 days to Kodak
0-Actin RNAs transcribed in nuclei from stromal-vascular cells
phosphate dehydrogenase gene expression by growth hor-
 conceivable that 10% fetal bovine serum or 10% bovine serum. At
expression of the dihydrofolate reductase (DHFR) gene was performed. As shown in Fig. 5A, after
the presence of 10% bovine serum. At confluence, these culture media were further supplemented
mRNAs. The results of Fig. 3 indicate that the transcription rate of the
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-
sponding to @-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 a-amanitin-treated nuclei, strongly suggesting that the transcription rate of the pOb24 gene was low or absent in nuclei of adipocytes.

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 T3-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 horm-
ones, 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 T3 whereas insulin was acting as a mere modulator.

Expression of pOb24 mRNA in G1, 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-
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To block the cells near or at the G1/S stage of the cell cycle, a specific inhibitor of DNA polymerase-α (24), and after Ca²⁺-deprivation. These various treatments are known to block the cells near or at the G1/S stage of the cell cycle (25, 26). This interpretation of the block at the G1/S boundary is supported by control experiments showing an incorporation of [3H]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 G1/S boundary is also supported by the study of the expression of the DHFR gene. As in 3T3 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 Ca²⁺-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 G1/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 µg/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 G1/S boundary. Since TNF and TGF-β 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-β on the Accumulation of pOb24 and Glycerol-3-phosphate Dehydrogenase mRNAs—To study the effects of TNF and TGF-β 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 TNFable to cause in 3T3-L1 cells a maximal inhibitory effect on the expression of LPL and its mRNA (12) and to a concentration of TGF-β 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-β (panel A). The effect of TNF was not due to overproliferation since [3H]thymidine incorporation into DNA was not increased over control (panel B). A similar conclusion could not be drawn for TGF-β since a 2-fold increase in [3H]thymidine incorporation during 24 h of treatment in the absence or presence of aphidicolin. The results from three independent experiments are expressed (mean ± range) by taking as 100% the values obtained for untreated control cells (150,000 ± 20,000 dpm/dish).

TABLE I

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

 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).
specific pOb24 mRNA in adipose cells

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, 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 (90%) known to contain adipose precursor cells (31) whereas the glycerol-3-phosphate dehydrogenase mRNA was heavily concentrated in adipocytes (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 complete in adipose cells after differentiation in culture as compared with adipocytes after differentiation in vivo.

In vitro, the presence of GH, T₃, 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 P2 genes which are expressed later (5, 8 and this paper). These results indicate that the obligatory role of GH and T₃ 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 G₁/S boundary was sufficient to express the pOb24 gene. In many instances, it is now well established that growth arrest during the G₁ 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 G₁ phase, there is a growth arrest at a distinct state designated G₂ which was required before glycerol-3-phosphate dehydrogenase was expressed; at that state, LPL is already expressed at 

When taken together, these experiments indicate that growth arrest at the G₁/S boundary was sufficient to express the pOb24 gene. In many instances, it is now well established that growth arrest during the G₁ 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 G₁ phase, there is a growth arrest at a distinct state designated G₂ 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 G₂ 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 G₁/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 representative of a higher degree of cell maturation (30). The lack of effect of TNF and TGF-β 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 by 4.1 ± 0.8-fold in TNF- or TGF-β-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-β as a function of cell maturation.

![Fig. 7. Effects of TNF and TGF-β on the expression of pOb24 and glycerol-3-phosphate dehydrogenase genes in early differentiating and late differentiated 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-β. Poly(A)+ RNA was prepared under each condition and 6 μg/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 ± ranges.](attachment://image.png)

It was important to know whether the inhibitory effects of TNF and TGF-β on pOb24 mRNA accumulation was still observed (panel A) whereas, in adipocin-treated cells, no [³H]thymidine incorporation into DNA could be noted both in control cells and those treated with TNF and TGF-β (panel B). Taken together, these experiments indicate that both TNF and TGF-β 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-β 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-β 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
subsequently able to synthesize DNA, divide and later to express glycerol-3-phosphate dehydrogenase.

Transcription studies of pOb24 and DHFR genes reported in Table 1 suggest the inactivation in the S phase of a cell cycle-specific factor(s) which stimulates their transcription at the G1/S boundary. Identification of a cell cycle-specific transcription factor(s) which stimulates their transcription at identical, to that in Ob1771 cells.

In any event, it must be stressed that separate regulatory factors in 3T6 cells for the DHRF gene (26), would be of interest. 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-β 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-β target cells (35, 36). Alternatively, both TNF and TGF-β could act by causing the cells to return to a point in the G1 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 cells feasible, since in situ hybridization techniques should allow the determination of the pOb24 mRNA-positive cells before any triacylglycerol accumulation.

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REFERENCES

1. Kozak, L. P., and Birkenmeier, E. H. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3020-3024
2. Bernlohr, D. A., Angus, C. W., Lane, D. L., Bolanowski, M. A., and Kelly, T. J., Jr. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5468-5472
3. Spiegelman, B. M., Frank, M., and Green, H. (1983) J. Biol. Chem. 258, 10083-10089
4. Chapman, A. B., Knight, D. M., Dieckmann, B. S., and Ringold, G. M. (1984) J. Biol. Chem. 259, 15548-15555
5. Doglio, A., Dani, C., Grimaldi, P., and Ailhaud, G. (1986) Biochem. J. 238, 123-129
6. Grimaldi, P., Dijian, P., Négre, R., and Ailhaud, G. (1982) Embo J. 1, 687-692
7. Dani, C., Doglio, A., Grimaldi, P., and Ailhaud, G. (1986) Biochem. Biophys. Res. Commun. 138, 468-475
8. Dani, C., Grimaldi, P., and Ailhaud, G. (1986) in Mechanisms of Insulin Action (Belfrage, P., Donner, J., and Strålfors, P., eds), pp. 383-393, Elsevier, Scientific Publishing Co., Amsterdam
9. Chapman, A. B., Knight, D. M., and Ringold, G. M. (1985) J. Cell Biol. 101, 1297-1235
10. Amri, E. Z., Dani, C., Doglio, A., Grimaldi, P., and Ailhaud, G. (1986) Biochem. Biophys. Res. Commun. 137, 903-910
11. Krawisz, B. R., and Scott, R. E. (1982) J. Cell Biol. 94, 394-399
12. Cornelius, P., Enerback, S., Bjursell, G., Olivecrona, T., and Pekala, P. H. (1988) Biochem. J. 249, 765-769
13. Zechner, R., Newman, T. C., Sherry, B., Cerami, A., and Breslow, J. L. (1988) Mol. Cell. Biol. 8, 2394-2401
14. Ignatz, R., and Massagué, J. (1986) Proc. Natl. Acad. Sci. U. S. A. 82, 8530-8534
15. Green, H., and Kehinde, O. (1976) Cell 7, 105-113
16. Gubler, U., and Hoffman, B. J. (1983) Gene (Amst.) 25, 263-269
17. Land, H., Grez, M., Hauser, H., Lindenmaier, W., and Schütz, G. (1981) Nucleic Acids Res. 9, 2251-2266
18. Marniati, T., Fritsch, E. F., and Sambrook, J. (1982a) in Molecular Cloning: A Laboratory Manual, pp. 313, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Marniati, T., Fritsch, E. F., and Sambrook, J. (1982b) in Molecular Cloning: A Laboratory Manual, pp. 90, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Amri, E., Dani, C., Doglio, A., Etienne, J., Grimaldi, P., and Ailhaud, G. (1986) Biochem. J. 236, 115-122
21. Spencer, I. M., Hutchinson, A., and Robinson, D. S. (1978) Biochim. Biophys. Acta 530, 375-384
22. Bernlohr, D. A., Bolanowski, M. A., Kelly, T. J., Jr., and Lane, M. D. (1985) J. Biol. Chem. 260, 5563-5567
23. Cook, K. S., Clayton, R. H., and Spiegelman, B. M. (1985) J. Cell Biol. 100, 514-520
24. Ikegami, S., Taguchi, T., Ohashi, M., Oguro, M., Nagano, H., and Mano, Y. (1978) Nature 275, 458-460
25. Ashihara, T., and Baserga, R. (1979) Methods Enzymol. LVIII, 248-262
26. Farnham, P. J., and Schimke, R. T. (1986) Mol. Cell. Biol. 6, 2392-2401
27. Farnham, P. J., and Schimke, R. T. (1985) J. Biol. Chem. 260, 7675-7680
28. Setzer, D. R., McGrogan, M., and Schimke, R. T. (1982) J. Biol. Chem. 257, 5143-5147
29. Torti, F. M., Dieckmann, B., Beutler, B., Cerami, A., and Ringold, G. M. (1980) Science 209, 867-869
30. Ratner, P. L., Fisher, M., Burkart, D., Cook, R., and Kozak, L. P. (1981) J. Biol. Chem. 256, 3571-3579
31. Rodbell, M. (1964) J. Biol. Chem. 239, 375-380
32. Nadal-Ginard, B. (1978) Cell 15, 855-864
33. Scott, R. E., Florine, D. L., Wille, J. J., Jr., and Yun, K. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 845-849
34. Scott, R. E., Hoeri, B. J., Wille, J. J., Jr., Florine, D. L., Krawisz, B. R., and Yun, K. (1982) J. Cell Biol. 94, 400-405
35. Ignatz, R. A., and Massague, J. (1986) J. Biol. Chem. 261, 4337-4345
36. Montesano, R., and Orci, L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4894-4897
37. Lathrop, B., Thomas, K., and Glaser, L. (1985) J. Cell. Biol. 101, 2194-2198