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EXPRESSION OF THE PHOSPHOENOLPYRUVATE CARBOXYKINASE GENE AND ITS INSULIN REGULATION DURING DIFFERENTIATION OF PREADIPOSE CELL LINES

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The adipose conversion of Ob1771 and 3T3-F442A preadipose cells is accompanied by the expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene. The PEPCK mRNA is absent from growing, undifferentiated Ob1771 and 3T3-F442A cells as well as from non-differentiating 3T3-C2 cells. It is present in differentiated Ob1771 and 3T3-F442A cells as well as in liver, kidney and white adipose tissue from mouse. Transcriptional run-off measurements in nuclei isolated from undifferentiated and differentiated Ob1771 and 3T3-F442A cells reveal that the PEPCK gene transcription is activated during differentiation. Studies of the time course of changes indicate that the emergence of PEPCK mRNA takes place in parallel to mRNA encoding for a 28 kDa protein (28 K mRNA) but later than that encoding for glycerol-3-phosphate dehydrogenase (GPDH mRNA). Insulin leads to an increase in the content of PEPCK and GPDH mRNAs with half-maximally effective concentrations of 0.5 and 5 nM for GPDH mRNA and PEPCK mRNA, respectively. Thus, in contrast to rat hepatoma cells, insulin exerts in adipose cells a positive regulation on the expression of the PEPCK gene.

The adipose conversion process of Ob17 cells (1) and 3T3 cells (2) in vitro appears to be a faithfull model of the adipose tissue development observed in vivo. Differentiation is accompanied in vitro by large changes in the cellular protein composition and by the emergence of adipocyte markers detected both at the protein and mRNA level (3). These changes takes place in 3T3 cells (4), TA1 cells (5) and Ob17 cells (6) through corresponding changes in mRNA content determined by translation and/or hybridization using cDNA probes. Increased transcription of the corresponding genes is primarily responsible in 3T3 and TA1 cells for the accumulation of differentiation-dependent mRNAs (5,7). We have shown recently that the adipose conversion of Ob17 cells

ABBREVIATIONS

PEPCK, phosphoenolpyruvate carboxykinase ; GPDH, glycerol-3-phosphate dehydrogenase ; LPL, lipoprotein lipase ; T_3, triiodothyronine ; cAMP, cyclic AMP ; DME medium, Dulbecco's modified Eagle's medium ; FBS, fetal bovine serum ; IBMX, isobutyl methylxanthine.

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involves the sequential expression of at least two separate sets of markers -early and late-during the post-confluent phase (8). The emergence of early markers -lipoprotein lipase (LPL), pOb24 and pGH3 mRNAs- appears to be independent of insulin, triiodothyronine (T_3) and growth hormone supplementation (9) and solely due to growth arrest (10). By contrast the emergence of late markers -GPDH and its corresponding mRNA as well as mRNAs encoding for a myelin P_2-like protein of M_r 13000 (13 K mRNA, ref.11) and an homologue of serine proteases of M_r 28000 (28 K mRNA, ref.12)- occurs after a period of limited proliferation and requires the presence of T_3 and growth hormone (13) whereas insulin acts merely as a modulator in the expression of late enzyme markers (14). Exposure of Ob1771 cells (subcloned from Ob17 cells, ref.13) to physiological concentrations of insulin leads to an accumulation of mRNA encoding for GPDH ; this accumulation was not due to an effect on transcription but due, at least in part, to a post-transcriptional event as shown by the significant increase of the mRNA half-life (6). Cytosolic phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is known to be present at high activity levels in liver, kidney and white adipose tissue (15). This enzyme is rate-limiting for hepatic and renal gluconeogenesis and is required in adipose tissue for the synthesis of glycerol-3-phosphate from pyruvate (16). Insulin acts in hepatoma cells as a specific and potent inhibitor of the PEPCK gene transcription whereas cAMP and analogues show the opposite effect (15). The insulin regulation of the PEPCK gene expression has not been reported so far in adipose tissue and in preadipose clonal lines. Moreover the relationships between adipose cell differentiation and PEPCK gene expression are still unknown. In this report it is shown that the transcriptional activation of the PEPCK gene is a differentiation-related event and that insulin plays a **positive** role in the regulation of the PEPCK mRNA content.

MATERIALS AND METHODS

Clonal lines and cell culture

The characterization of Ob1771, 3T3-F442A and 3T3-C_2 clonal lines has been previously reported (2,13). Cells were plated at 1-5x10^3 cells/cm^2 in 100-mm dishes and grown until confluence in DME medium supplemented with 10% FBS, 200 units/ml penicillin, 50 µg/ml streptomycin, 33 µM biotin and 17 µM pantothenate (defined as the standard medium). Cells were exposed to 100 µM IBMX during the last three days of the growth phase. After confluence, 2 nM T_3 and, unless otherwise stated, 17 nM insulin were chronically added to the standard medium (now defined as the differentiation medium).

RNA isolation and Northern blot hybridization

The preparation of total RNA and poly (A) -containing RNA has been previously described (13). Northern blot of 6 µg of poly (A) -containing RNA electrophoresed on

denaturing 1.2% agarose gels were obtained on nylon membranes (Hybond-N, Amersham International, Buckinghamshire, U.K.) as described by Thomas (17). The cDNA probes were labelled by nick-translation (18). After hybridization for 48 hours at 42°C (13) the membranes were washed and exposed to XAR5 Kodak films at -70°C with intensifying screens.

Elongation of nascent chains in isolated nuclei

Isolation of nuclei and nuclear transcription in vitro assays have been extensively described (13). Briefly incubation of 10^7 nuclei for 30 minutes at 30°C in the presence of 200 µCi [α-^{32}P]UTP (Amersham International, Buckinghamshire, U.K.) leads to the incorporation of about 10^5 cpm per µg of input DNA. 10^7 cpm of the [^{32}P]reaction products were hybridized for 48 hours at 65°C to nitrocellulose filters (Schleicher and Schüll GmbH, Dassel, F.R.G.) containing 10 µg of each denaturated plasmid DNA. The washed filters were exposed to XAR5 Kodak films for 48 hours at -70°C with intensifying screens.

RESULTS

As shown in Figure 1, the pC116 probe for rat cytosolic PEPCK (18) is able to hybridize with a single 2.8 Kb mRNA species in liver, kidney and white adipose tissue from mouse but not to any mRNA species in heart (Fig.1), skeletal muscle, spleen and testis (not shown). These results are in agreement with observations reported in chicken and rat (19). Figure 1 shows also that the PEPCK mRNA is expressed in confluent and differentiated Ob1771 and 3T3-F442A cells. By contrast this mRNA is absent in the corresponding preconfluent, undifferentiated cells as well as in the non-differentiating 3T3-C_2 cells, either before or after confluence. This result excludes that the emergence of PEPCK mRNA is merely due to growth arrest at confluence and/or to the addition of insulin and T_3. This result strongly suggests that the

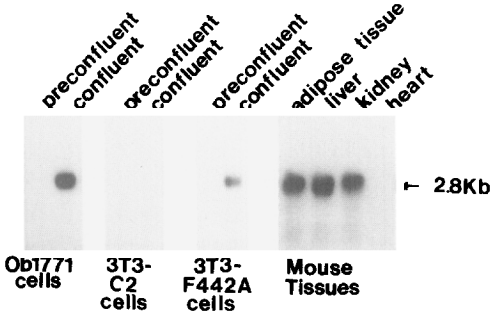

Figure 1 : Distribution of PEPCK mRNA in growing and late post-confluent Ob1771, 3T3-C_2 and 3T3-F442A cells and in various mouse tissues

Northern blots of 6 µg of polyadenylated RNA were probed with the pC116 labelled cDNA as described in the Materials and Methods section. Cells were cultured in the standard medium during the growth phase and maintained after confluence in the differentiation medium for 10 days. Tissues were obtained from 5 week-old OFI mice fed ad libitum with a Purina chow diet. The size of the mRNA band was determined from standards of ribosomal RNAs and HinfI fragments of digested pBr322 DNA.

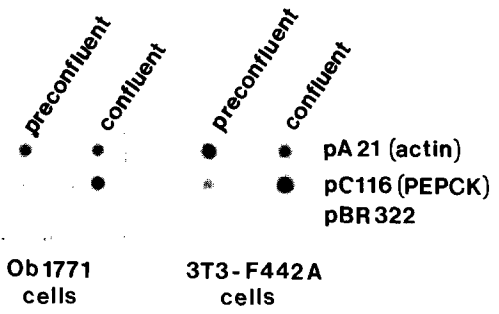

Figure 2 : Transcription of PEPCK mRNA during adipose conversion of Ob1771 and 3T3-F442A cells

Cells were maintained in the standard medium until confluence and then exposed during 11 days to the differentiation medium. Preparation of nuclei and in vitro nuclear transcription assays were as described in ref.13. Identical results were obtained in a further experiment for undifferentiated cells and at least in three further experiments for differentiated cells.

emergence of PEPCK mRNA is a differentiation-dependent event. The results of in vitro nuclear transcription reported in Figure 2 support this hypothesis. Dot-blot analysis shows no difference for the transcription of the actin gene (pA21 probe) in Ob1771 cells (13) and some decrease for that of the same gene in 3T3-F442A cells (20). In contrast a transcription signal of the PEPCK gene is absent in growing, undifferentiated Ob1771 cells and appears in confluent, differentiated cells. Similar results are obtained with 3T3-F442A cells, although a low but significant transcription is observed in undifferentiated cells. This latter observation is likely due to the small percentage of already differentiated lipid-containing 3T3-F442A cells which are present during the growth phase. The results obtained with the pBr322 DNA plasmid exclude any significant unspecific hybridization. Taken together the results of Figures 1 and 2 indicate that the expression of the PEPCK gene during differentiation is likely due to an activation of this previously untranscribed gene more than an enhancement of transcription which, if it occurs in preadipocytes, is below detectable limits. This conclusion is supported by the fact that the low transcription signal observed with nuclei of undifferentiated Ob1771 cells is identical to that observed in α-amanitin-pretreated nuclei of undifferentiated or differentiated Ob1771 cells in which the transcription of mRNAs is abolished (not shown and ref.13). Therefore the kinetics of the appearance of PEPCK mRNA has been next investigated and compared to those of mRNAs encoding for GPDH (detected with the pC8 probe, ref.21) and for the

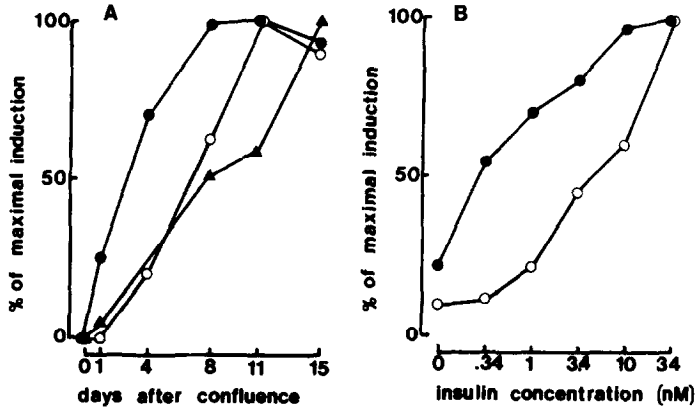

Figure 3 : **(A) Time course of changes in PEPCK (○), GPDH (●) and 28 K (▲) mRNAs during adipose conversion of Ob1771 cells**

Cells were maintained since confluence in the differentiation medium. RNAs were prepared at the indicated days and analyzed by Northern blot by using sequentially pC8 (GPDH mRNA), pC116 (PEPCK mRNA), pAd20 (28 K mRNA) and pA21 (β-actin mRNA) labelled probes. After scanning by densitometry, the results were normalized to actin signals and expressed by taking as 100% the maximal signal obtained for each probe. The curves shown are representative of two independent experiments.

(B) Dose-response relationship of insulin to PEPCK (○) and GPDH (●) mRNAs accumulation in post-confluent Ob1771 cells

Post-confluent cells were exposed for 11 days to standard medium supplemented with 2 nM T_3 and various concentrations of insulin as indicated. RNAs were analyzed as described in Figure 3A. The results are normalized to actin mRNA signals and expressed by taking as 100% the maximal signal obtained for each probe. Similar results were obtained in two further experiments.

28 K mRNA (detected with the pAd20 probe, ref.12). The curves of Figure 3A have been obtained with Ob1771 cells treated with IBMX for the last three days which precede confluence. Under these conditions, the time course of differentiation is dramatically shortened and thus it is not surprising that GPDH mRNA is already present at day 1 instead of emerging in untreated cells at later days (6). Clearly the emergence of GPDH mRNA takes place significantly earlier than that of PEPCK mRNA, and both mRNAs reach a plateau at day 11. Both the PEPCK mRNA and 28K mRNA emerge in parallel but the maximal accumulation of the latter mRNA occurs a few days later than that of the PEPCK mRNA. This result indicates that PEPCK mRNA and thus most likely the enzyme itself behaves as a very late marker of adipose conversion. This conclusion is in agreement with the very low content in PEPCK mRNA and 28 K mRNA in cells of the stromal-vascular fraction of mouse adipose tissue (containing adipose precursor cells and a few adipocytes) compared to the high content of these mRNAs in the adipocyte fraction (containing mainly, if not all, mature adipocytes) (Dani et al.,

submitted for publication). Since both GPDH and PEPCK mRNAs are actually expressed in Ob1771 cells exposed to insulin-supplemented medium, the effect of this hormone on the regulation of the expression of the corresponding genes has been investigated. The curves of Figure 3B show clearly a dose-response relationship between insulin concentration and mRNA content determined at day 11. As already reported (6), some expression of the GPDH gene occurs in the absence of insulin merely acting as a modulator (22). This is also observed for the PEPCK gene. In both cases a strong **positive** and modulating effect of insulin is seen with respect to the content of GPDH and PEPCK mRNAs, with half-maximally effective concentrations of 0.5 and 5 nM, respectively. This shift by one order of magnitude observed between a late marker (GPDH mRNA) and a very late marker (PEPCK mRNA) has been observed identically for 3T3-F442A cells and is reminiscent of a similar shift observed at the protein level between an early (LPL) and a late (GPDH) enzyme marker during differentiation of adipose precursor cells of inguinal fat tissue in primary culture (Deslex et al., submitted for publication).

DISCUSSION

Our previous work has indicated a sequential emergence which allowed us to define early markers (LPL, pOb24 and pGH_3 mRNAs) and late markers (GPDH and its corresponding mRNA, acid:CoA ligase, 13 K mRNA) (8,13). The present results are in favour of PEPCK and 28 K mRNAs as representative of very late markers of adipose conversion. The completion of terminal differentiation via the acquisition of these markers may be indicative of the formation of fully mature adipose cells, in agreement with our observations made on adipocytes isolated from mouse periepididymal fat tissue (Dani et al., submitted for publication). The finding (Fig.2) that the PEPCK gene is expressed in differentiated Ob1771 and 3T3-F442A cells exposed to 17 nM insulin indicates clearly that, in contrast to rat H411E hepatoma cells exposed to this hormone (23), the transcription of this gene is not abolished in adipose cells. This indicates that the same gene is regulated differently at the transcriptional level in both cell types. The additional finding that insulin within a physiological range of concentrations exerts a positive effect on the accumulation of the PEPCK mRNA is at variance with a previous work performed at the enzyme level (24) but is in accordance with the role

postulated in adipose tissue for PEPCK in furnishing dihydroxyacetone phosphate and subsequently glycerol-3-phosphate which is required for the insulin-stimulated triacylglycerol synthesis (25). This interpretation is strengthened by preliminary investigations showing that, in fasted mice refed with a high-carbohydrate diet, a fast and potent increase in white adipose tissue and, as expected, a strong and rapid decrease in liver are observed in the PEPCK mRNA content when compared to that of mice fed with a standard diet (Grimaldi et al., manuscript in preparation). Therefore, it would seem that the insulin regulation of the PEPCK gene expression in liver and adipose tissue is in agreement with that observed in cultured hepatoma and adipose cells but this regulation is taking place in a reciprocal manner in liver (or hepatoma cells) and white adipose tissue (or adipose cells). Clearly, the level at which the expression of the PEPCK gene is regulated by insulin in adipose cells remains to be investigated. Whether such regulation would occur at a transcriptional level, as demonstrated in rat hepatoma cells (23), the possibility of distinct regulatory sequences in the PEPCK gene of liver and adipose cells or different regulatory factors acting on the same sequence in both cell types could be envisionned.

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