

## Transcriptional Regulation of Acetyl Coenzyme A Carboxylase Gene Expression by Tumor Necrosis Factor in 30A-5 Preadipocytes†

MICHAEL E. PAPE AND KI-HAN KIM\*

*Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907*

Received 23 August 1988/Accepted 3 December 1988

Acetyl coenzyme A (acetyl-CoA) carboxylase activity, amount, and mRNA levels increase during the differentiation of 30A-5 preadipocytes to adipocytes. Tumor necrosis factor (TNF) completely prevents this differentiation, with concomitant inhibition of acetyl-CoA carboxylase mRNA accumulation. To investigate the mechanisms by which TNF prevents acetyl-CoA carboxylase mRNA accumulation, we determined the effect of TNF on the transcription rate of the carboxylase gene and the half-life of carboxylase mRNA. Nuclear runoff transcription assays revealed no differences in the number of RNA polymerase molecules actively engaged in transcription of the acetyl-CoA carboxylase gene in preadipocytes, adipocytes, TNF-treated preadipocytes, or at any time during the course of differentiation. However, changes in adipin, glycerophosphate dehydrogenase, and actin mRNAs, whose levels are also differentiation dependent, can be accounted for in part by changes in the number of polymerase complexes on their respective genes. To determine whether TNF caused a decrease in the stability of carboxylase RNA transcripts, we measured the rate of decay of prelabeled acetyl-CoA carboxylase mRNA. Control and TNF-treated cells showed no difference between the apparent half-lives of acetyl-CoA carboxylase mRNAs (9 h). However, the rate of acetyl-CoA carboxylase mRNA synthesis *in vivo* was decreased three- to fourfold in the presence of TNF. These data demonstrate that TNF prevents accumulation of acetyl-CoA carboxylase mRNA during preadipocyte differentiation by decreasing the rate of acetyl-CoA carboxylase gene transcription. However, transcriptional control is not due to a change in the number of RNA polymerase complexes actively engaged in carboxylase transcript elongation which could be measured by a nuclear runoff assay. Instead, transcriptional control may be related to the rate at which RNA polymerase traverses the acetyl-CoA carboxylase gene.

Tumor necrosis factor (TNF) or cachectin is part of a polypeptide network made up of several cytokines and growth factors that have wide and varied effects on the growth, differentiation, and functions of immune system and normal cells (4, 26). With the availability of cloned TNF and TNF-specific antibodies, it has been possible to assess the specific role that TNF plays in mediating the broad range of effects elicited by these polypeptides and also the synergism and antagonism TNF displays with respect to factors of the network. Recent work has implicated TNF in mediating such diverse immunological and inflammatory processes as macrophage-induced angiogenesis (13), endotoxic and septic shock due to bacterial infection (5, 35), hemorrhagic necrosis of certain transplanted tumors (25), prevention of encephalomyocarditis virus replication in human fibroblasts (11), monocyte-mediated cytotoxicity (29), and cytolysis of certain murine and human cell lines (33). Most of these effects are due to TNF action on reticuloendothelial cells and lymphocytes; however, TNF also alters the growth, differentiation, and functions of normal cells, such as fibroblasts, myocytes, and adipocytes. For instance, TNF is mitogenic for human fibroblasts (33, 38) and prevents the differentiation of myoblasts (19) and preadipocytes (27, 34) in culture. Furthermore, TNF dramatically alters the cellular functions of fully differentiated adipocytes (28, 34).

The wide-ranging effects of TNF on these various cell types may ultimately be due to alterations in the expression of key gene products. There are several examples of TNF affecting the levels of mRNAs that encode key proteins.

TNF stimulates the production of granulocyte-monocyte, granulocyte, and monocyte colony-stimulating factor mRNAs in endothelial cells and other cell types by increasing the rates of transcription of their respective genes (21, 31). TNF also regulates oncogene expression, since the monokine has been shown to cause a rapid and transient increase in *c-fos* and *c-myc* mRNA levels in human fibroblasts (14) while reducing *c-myc* mRNA levels in HeLa cells through a transcriptional control mechanism (40). TNF inhibits the expression of alpha-cardiac actin, alpha-skeletal actin, and myosin heavy-chain mRNAs during myogenesis (19) but increases beta 2 interferon mRNA in myoblasts, myotubes (19), and human fibroblasts (11). Finally, TNF has been shown to decrease the amounts of acetyl coenzyme A (acetyl-CoA) carboxylase (27), glycerophosphate dehydrogenase (GPD) (10, 34), lipoprotein lipase (8, 41), and adipin (20) mRNAs and also mRNAs for other developmentally regulated genes of unknown identity (34) both in fully differentiated adipocytes and during preadipocyte differentiation. In some of these cases, the decrease in mRNA amount for these lipogenic genes is due to a decreased rate of gene transcription as demonstrated by nuclear transcription assays (34, 41). A goal of the present study was to investigate in detail the mechanisms by which TNF decreases acetyl-CoA carboxylase mRNA levels during preadipocyte differentiation.

Acetyl-CoA carboxylase catalyzes the rate-limiting step in the *de novo* biosynthesis of long-chain fatty acids (12, 39). Various nutritional, hormonal, and developmental states which alter the rate of fatty acid biosynthesis have a marked effect on carboxylase activity and amount (16, 17, 22-24). In some instances, these changes in carboxylase have been shown to be due to alterations in carboxylase mRNA content

\* Corresponding author.

† Journal paper 11,505 from the Purdue University Agricultural Experiment Station.

and the amount of translatable carboxylase mRNA (2, 15, 27a). A system which is amenable to investigating the effects of hormones on the regulation of carboxylase mRNA is the 30A-5 preadipocyte cell line (27). Upon treatment with dexamethasone and insulin, 30A-5 preadipocytes differentiate into adipocytes, as demonstrated by precocious lipid accumulation. Acetyl-CoA carboxylase activity, amount, and mRNA levels increase in parallel with the number of lipid-exhibiting cells (27). TNF completely prevents the conversion process, with a corresponding inhibition of carboxylase gene expression (27).

In this paper, we report that TNF prevents accumulation of acetyl-CoA carboxylase mRNA during preadipocyte differentiation by decreasing the rate of acetyl-CoA carboxylase gene transcription, as measured by *in vivo* labeling of RNA. Furthermore, the decreased rate of synthesis is not due to fewer RNA polymerase molecules on the acetyl-CoA carboxylase gene, as determined by nuclear runoff transcription assays. Thus, we provide an example in which the nuclear runoff transcription assay does not reflect a true *in vivo* transcription rate.

### MATERIALS AND METHODS

**Materials.** The following commercial products were obtained: Eagle basal medium (MA Bioproducts, Walkersville, Md.); fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.); dexamethasone and insulin (Collaborative Research, Inc., Waltham, Mass.); RNase-free DNase I, RNase-free bovine serum albumin, and ribonucleotides (Pharmacia, Inc.; Piscataway, N.J.); RNasin (Promega Biotech); guanidine isothiocyanate (Fluka); [5,6-<sup>3</sup>H]uridine (43 Ci/mmol) and [ $\alpha$ -<sup>32</sup>P]UTP (3,000 Ci/mmol) (ICN Pharmaceuticals, Inc., Irvine, Calif.); [ $\alpha$ -<sup>32</sup>P] dCTP (3,000 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.). Recombinant human TNF- $\alpha$  ( $2.7 \times 10^7$  U/mg) was generously provided by Tatsuro Nishihara of the Suntory Institute, Osaka, Japan.

**Cell culture.** The 30A-5 preadipocyte cells were grown in Eagle basal medium containing 10% fetal bovine serum and induced to differentiate by adding insulin and dexamethasone at confluency, as previously described (27).

**Nuclear runoff transcription assays.** Nuclei were isolated from the cells of six 100-mm-diameter plates for each treatment. Cell monolayers were rinsed twice in ice-cold rinsing buffer (10 mM Tris hydrochloride [pH 7.5], 10 mM NaCl, 3 mM MgCl<sub>2</sub>), and then cells were lysed in rinsing buffer containing 0.5% Nonidet P-40, 2.75 mM dithiothreitol, and 20 U of RNasin per ml. The cells on plates were scraped, and the lysates were gently shaken for 5 s, followed by centrifugation at  $700 \times g$  for 7 min at 4°C to pellet the nuclei. All procedures were performed on ice and in the cold. The supernatant was used to make cytoplasmic RNA as described below. The nuclear pellet was washed twice in rinsing buffer containing 2.75 mM dithiothreitol and 20 U of RNasin per ml and centrifuged as before to decrease the amount of detergent present in the nuclear preparation. The final nuclear pellet was suspended in the transcription reaction buffer, which contained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5); 90 mM NH<sub>4</sub>Cl; 5 mM MgCl<sub>2</sub>; 0.5 mM MnCl<sub>2</sub>; 2 mM dithiothreitol; 0.1 mM EDTA; 0.4 mM each ATP, CTP, and GTP; 10% glycerol; 10  $\mu$ g of RNase-free bovine serum albumin per ml; and 200 U of RNasin per ml. Nuclei were counted with a hemacytometer, and  $2 \times 10^6$  to  $4 \times 10^6$  nuclei were divided into aliquots and stored at -70°C.

The *in vitro* transcription reaction was performed with an equal number of nuclei per reaction ( $2 \times 10^6$  to  $4 \times 10^6$ ) in

transcription reaction buffer containing 325  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP (0.3  $\mu$ M) in a final volume of 300  $\mu$ l. The transcription reaction was performed at 28°C and proceeded for 40 min. Newly transcribed RNA was isolated by two successive DNase I-proteinase K digestions with an ethanol precipitation step in between (18). After two more ethanol precipitations, the labeled RNA was counted. About  $3 \times 10^6$  to  $5 \times 10^6$  cpm was recovered from each reaction. The reaction products ranged from 6 kilobases to 0.5 kilobase, with an average size of 2 kilobases in nuclei isolated from all cells with the different treatments.

To assay the amounts of specific RNAs synthesized, the transcribed RNA was hybridized to plasmids containing a cDNA fragment of the specific gene. Plasmids were immobilized on Zetabind membrane (AMF-Cuno, Houston, Tex.) as follows. Plasmids were completely linearized with the appropriate restriction enzyme, phenol-chloroform extracted, and ethanol precipitated. The DNA was denatured by boiling in 0.1 N NaOH for 5 min, followed by addition of ice-cold  $20\times$  SSPE (1 $\times$  is SSPE is 0.15 M NaCl, 1 mM EDTA, and 10 mM NaH<sub>2</sub>PO<sub>4</sub> [pH 7.4]) to a final concentration of 12 $\times$ . Plasmids (3 to 5  $\mu$ g per dot) were dotted onto Zetabind membrane with dot blot apparatus (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and the filter was baked.

Plasmid filters were prewashed in 0.1 $\times$  SSPE containing 0.5% sodium dodecyl sulfate at 65°C for 1 h, followed by overnight prehybridization at 50°C in hybridization buffer which contained 50% formamide, 6 $\times$  SSPE, 1% sodium dodecyl sulfate; 0.1% Tween 20, 500  $\mu$ g of *Torula* RNA per ml, and 1 $\times$  Denhardt solution (0.2 mg each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone per ml). Equal numbers of counts of the newly transcribed RNA ( $3 \times 10^6$  to  $5 \times 10^6$  cpm) were used for hybridization in 0.5 ml of fresh hybridization buffer for 96 h at 50°C. After hybridization, the filters were washed four times for 5 min each time in 2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate at room temperature, followed by two 15-min washes in 0.1 $\times$  SSC-0.1% sodium dodecyl sulfate at 50°C. Filters were exposed to Kodak XAR-5 film at -70°C. Quantitation of all autoradiographs was performed with an LKB laser densitometer and LKB GSXL software.

In defining the hybridization conditions, we determined a minimum  $R_{gt}$  number as follows. We added equal amounts of counts to identically prepared filters containing acetyl-CoA carboxylase- or actin-specific plasmids and determined the time at which maximum hybridization was obtained. By preliminary experiments, we had determined that the actin gene was transcribed at the highest rate and the acetyl-CoA carboxylase gene was transcribed at the lowest rate, under any treatment of the 30A-5 cells, of all of the genes studied. The complete hybridization signal for actin occurred by 24 h of hybridization, while that for acetyl-CoA carboxylase occurred by 48 h, consistent with the various degrees of transcription for each gene. We chose to use 96 h for the length of hybridization to ensure that the hybridizations were complete.

**Labeling of RNA *in vivo* with [<sup>3</sup>H]uridine and measurement of the half-life of acetyl-CoA carboxylase mRNA.** Confluent monolayers of 30A-5 preadipocytes in 100-mm plates (day 0) were subjected to the standard differentiation scheme (27) with or without 200 U of TNF per ml. At day 6, [5,6-<sup>3</sup>H]uridine was added to each plate. At day 8, the medium containing the [<sup>3</sup>H]uridine was removed and the cell monolayers were washed twice with 10 ml of phosphate-buffered

saline each. Fresh medium containing 2.5 mM cytidine, 5 mM uridine, and 5  $\mu$ g of insulin per ml was then added (chase period). TNF-treated cells were exposed to the monokine at all times; from day 0 until the cells were harvested for RNA isolation. Total RNA was isolated 5, 10, 15, and 20 h after initiation of the chase, by a modification (36) of the method of Chirgwin et al. (6). Incorporation of [<sup>3</sup>H]uridine into total RNA was determined by measurement of trichloroacetic acid-precipitable counts in the final RNA preparation. About  $2 \times 10^8$  dpm was used for the quantitation of the specific mRNA species through hybridization as described above. Three and five plates were used for each time point for control and TNF data, respectively.

**Isolation and analysis of RNA.** Total RNA was isolated by directly lysing cell monolayers in a 4.5 M guanidine solution (36) and centrifuging the lysate through CsCl (36). Cytoplasmic RNA was isolated from the first supernatant fraction in the preparation of nuclei by addition of guanidine isothiocyanate to a final concentration of 4.5 M and centrifugation through CsCl. For dot blot analysis, RNA was dotted onto Zetabind membrane (3) and hybridized to nick-translated plasmids containing gene-specific cDNAs. The hybridization and washing conditions were identical to those used for the nuclear runoff transcription assays and [<sup>3</sup>H]RNA experiments.

**Plasmids.** To measure labeled acetyl-CoA carboxylase mRNA in total RNA, we used three plasmids containing cDNA that spanned 6.6 kilobases of the 10-kilobase carboxylase mRNA: pCX321, pDHN2, and pKHN18 (14a). These acetyl-CoA carboxylase cDNA fragments were subcloned into pGEM3 (Promega Biotec) at the unique *Eco*RI site. Three micrograms of each plasmid constituted the acetyl-CoA carboxylase plasmid dot. The adipsin (32) and glycerophosphate dehydrogenase cDNA clones (pGPD-1 in reference 10) were in pBR322 and were generously provided by Bruce Spiegelman of the Dana-Farber Cancer Institute, Boston, Mass. The actin plasmid contained a nearly full-length chicken  $\beta$ -actin cDNA insert.

To probe RNA dot blots, linearized plasmids were nick translated and hybridized as described above. pKHR18 (2, 14a) was used as the acetyl-CoA carboxylase probe.

## RESULTS

**Changes in acetyl-CoA carboxylase mRNA content during differentiation with or without TNF.** Acetyl-CoA carboxylase mRNA levels increased 3- to 10-fold during preadipocyte differentiation, while having TNF present throughout differentiation inhibited carboxylase mRNA accumulation by 70 to 80% (Fig. 1). The increase in carboxylase mRNA during the conversion of preadipocytes to adipocytes varied, depending on the cell passage number, which has an effect on the extent of differentiation. The levels of adipsin and GPD mRNAs under the same experimental conditions responded to the differentiation and TNF treatments in a fashion analogous to that of acetyl-CoA carboxylase mRNA; differentiation increased their amounts, and TNF inhibited their accumulation (Fig. 1). On the other hand, actin mRNA levels decreased during differentiation and TNF appeared to have little effect on the actin mRNA amount.

**Measurement of the relative number of RNA polymerase complexes associated with the acetyl-CoA carboxylase gene during differentiation with or without TNF.** To determine whether changes in the number of RNA polymerase molecules actively engaged in transcribing the carboxylase gene could account for the changes in carboxylase mRNA levels,

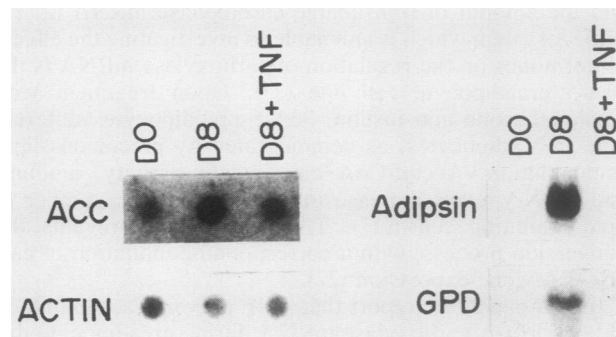


FIG. 1. Changes in acetyl-CoA carboxylase (ACC), adipsin, GPD, and actin mRNA levels during differentiation with and without TNF. The differentiation scheme described in Materials and Methods was initiated after 30A-5 cells had reached confluency (day 0; D0). Total RNA was isolated from preadipocytes (D0), fully differentiated adipocytes (day 8; D8), and 30A-5 cells grown in the presence of 200 U of TNF per ml from day 0 to day 8 (D8 + TNF) and analyzed by either Northern or RNA dot blot analysis with nick-translated cDNAs of the indicated genes.

we performed nuclear runoff transcription assays. This assay, which is widely used to estimate the rate of RNA synthesis, provides a "snapshot" of the number of polymerase complexes on a particular gene at the time of isolation of the nuclei. Since reinitiation of RNA synthesis is a very inefficient process in the *in vitro* transcription reaction, only RNA polymerase molecules associated with the gene complete transcript synthesis during the *in vitro* reaction.

The following nuclear preparations were used: nuclei from preadipocytes (day 0), adipocytes (day 8), and 30A-5 cells treated with the standard differentiation scheme but exposed to 200 U of TNF per ml from day 0 to day 8. To determine the optimum time required for the complete runoff of RNA polymerase molecules that were in the process of elongation, we performed the transcription reaction by using [ $\alpha$ -<sup>32</sup>P]UTP to label RNA transcripts that were being synthesized and determined the time course of UMP incorporation into newly transcribed RNAs. The initial rates at which UMP was incorporated were virtually identical for nuclei isolated from cells given the three different treatments; half-maximal incorporation occurred at about 5 min (Fig. 2). In addition, incorporation into newly transcribed RNA reached a plateau by 40 min for nuclei from all treatments (Fig. 2). Since less than 1% of the initial label was incorporated into RNA after the 40-min reaction (data not shown), the amount of UTP does not appear to be rate limiting. These data suggest that under our *in vitro* transcription reaction conditions, all RNA polymerase molecules associated with the DNA at the time of nuclear isolation complete their transcription of particular genes. Therefore, a reaction time of 40 min was used for all subsequent nuclear transcription reactions.

After the *in vitro* transcription reaction, newly transcribed RNA was isolated and hybridized to filters containing plasmids for acetyl-CoA carboxylase, adipsin, GPD, and actin. The amounts of acetyl-CoA carboxylase transcripts that were completed during the runoff reaction remained virtually the same for all of the cell treatments (Fig. 3). However, the number of adipsin and GPD transcripts synthesized in this assay increased during preadipocyte-to-adipocyte conversion, whereas TNF-treated preadipocytes showed reduced levels (Fig. 3). The number of actin transcripts decreased slightly during differentiation, while TNF-treated preadipocytes had levels comparable to those of day 0 preadipocytes

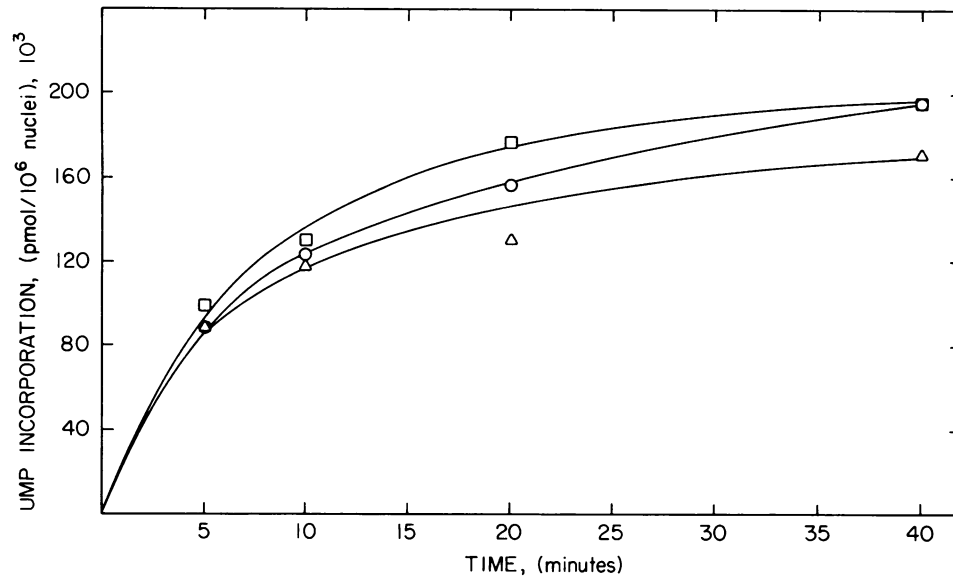


FIG. 2. Time course of UMP incorporation into newly transcribed RNAs during the nuclear transcription reaction. Equal numbers of nuclei isolated from day 0 (○) and day 8 (△) cells and cells treated with TNF from days 0 to 8 (□) were used in a 300- $\mu$ l transcription reaction. At various times after addition of [ $\alpha$ -<sup>32</sup>P]UTP, a 5- $\mu$ l sample was removed and the amount of trichloroacetic acid-precipitable counts were determined. The data represent the average of two experiments.

(Fig. 3). Control plasmids pGEM3 and pBR322 were used to assess the background; it was found that the signal level remained low in each hybridization (Fig. 3). By four separate experiments, we found that the number of transcripts synthesized in the preadipocyte nuclei compared with the

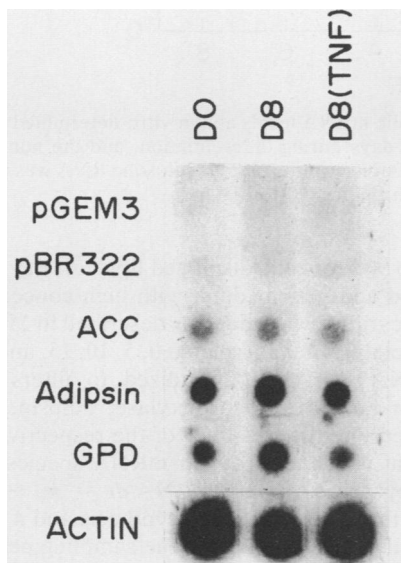


FIG. 3. Transcription of specific genes in isolated nuclei from 30A-5 cells under different hormonal treatments. Nuclei were isolated from day 0 cells (D0), day 8 cells (D8), and cells treated for 8 days with TNF [D8(TNF)]. RNA polymerase complexes actively engaged in transcription at the time of nuclear isolation were allowed to complete transcription under standard nuclear runoff transcription conditions, as described in Materials and Methods. Nuclear RNA was isolated, and equal counts per minute of newly transcribed RNA from each reaction were hybridized to immobilized plasmids containing gene-specific cDNAs as indicated. ACC, Acetyl-CoA carboxylase.

adipocyte nuclei increased  $1.2 \pm 0.2$ -fold for acetyl-CoA carboxylase,  $1.9 \pm 0.2$ -fold for adipsin, and  $3.1 \pm 0.7$ -fold for GPD and decreased by about 30% for actin. For nuclear transcription assays during differentiation of NIH 3T3-F442A preadipocytes, Cook et al. (7) reported no detectable change for adipsin, a greater-than-sixfold increase in GPD, and a 30% decrease in actin RNA transcripts. The differences from our results may be attributed to assay sensitivity or cell lines.

A comparison of transcript amounts from two experiments with adipocyte nuclei and TNF-treated preadipocyte nuclei revealed a 20% decrease in acetyl-CoA carboxylase, a 40% decrease in adipsin, a 60% decrease in GPD, and a 1.3-fold increase in actin. These data indicate that during conversion of preadipocytes to adipocytes, the number of polymerase molecules on the carboxylase gene changed insignificantly, if at all. Additionally, in the presence of TNF, a treatment that completely inhibits the conversion, the number of polymerase molecules on the acetyl-CoA carboxylase gene did not change significantly. However, the number of RNA polymerase molecules associated with the adipsin, GPD, and actin genes changed under the same experimental conditions, reflecting the levels of mRNA synthesized under these conditions.

Although these data suggested that there were no changes in the number of polymerase complexes actively engaged in transcribing the carboxylase gene under the three different conditions, we could not exclude the possibility that a transient increase in the number of polymerase molecules associated with the carboxylase gene occurs during differentiation. If TNF prevented such a transient increase, then the same data would have been obtained. Therefore, we performed the nuclear runoff transcription assay on nuclei isolated from cells at every stage of differentiation and also measured cytoplasmic RNA levels (Fig. 4). The number of polymerase molecules associated with the acetyl-CoA carboxylase gene remained the same during preadipocyte-to-adipocyte conversion, while this number increased for

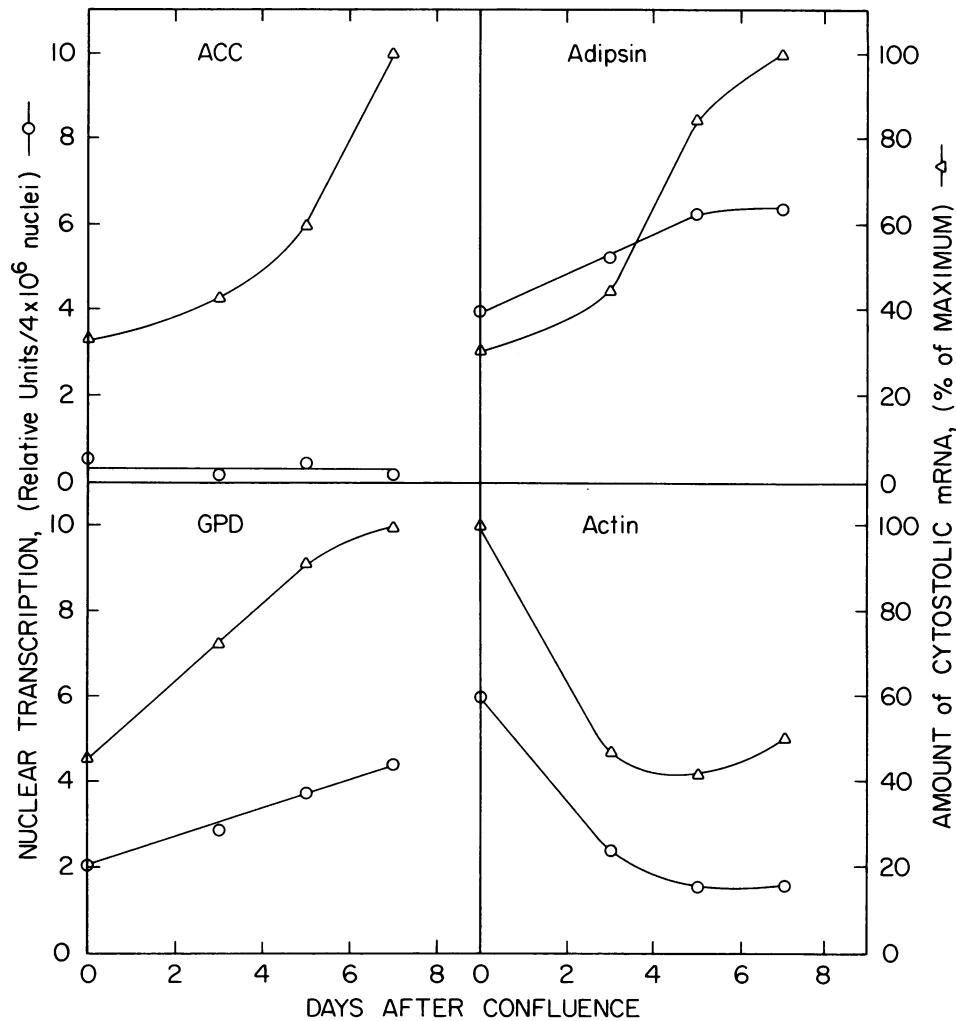


FIG. 4. Changes in acetyl-CoA carboxylase (ACC), adipsin, GPD, and actin cytosolic mRNA levels and in vitro-determined transcription rates during adipocyte conversion. Nuclei were isolated from 30A-5 cells at various days during differentiation, and the number of RNA polymerase complexes associated with each gene was determined by the nuclear runoff transcription assay. Cytosolic RNA was isolated from the same nuclear preparations and analyzed by RNA dot blot analysis as described in the legend to Fig. 1.

adipsin and GPD but decreased for actin. In the latter three cases, the changes in the amounts of cytoplasmic RNAs can be attributed in part to a change in the numbers of polymerase complexes associated with their respective genes; this presumably reflects a change in the rate of transcription of each gene. However, the increase in acetyl-CoA carboxylase mRNA during differentiation and also the decrease in carboxylase mRNA in the presence of TNF cannot be explained by the same mechanism. Therefore, we examined whether the stability of acetyl-CoA carboxylase mRNA is affected so as to control carboxylase mRNA levels.

**Effect of TNF on the half-life of acetyl-CoA carboxylase mRNA.** To measure the rate of decay of acetyl-CoA carboxylase mRNA in 30A-5 cells, we labeled the mRNA by using [<sup>3</sup>H]uridine. We avoided the use of inhibitors of transcription because of the various effects that these agents can have on other cellular processes. The 30A-5 cells were subjected to the standard differentiation scheme without TNF (control) or with TNF (TNF treated) and then exposed to [<sup>3</sup>H]uridine to label cellular RNA. The labeling period was 48 h, from days 6 to 8. [<sup>3</sup>H]uridine treatment did not noticeably affect lipid accumulation from days 6 to 8 in the control cells, since the

typical 85 to 90% of cells displayed lipid. At day 8, the cells were washed and new medium with high concentrations of uridine and cytidine was added as described in Materials and Methods. Total RNA was isolated 0, 5, 10, 15, and 20 h later. The [<sup>3</sup>H]RNA was then hybridized to filters containing plasmids for acetyl-CoA carboxylase, adipsin, GPD, and actin to determine the half-lives of the respective mRNAs.

The rate at which a particular mRNA species changes is described by the formula  $dmRNA/dt = k_s - k_d mRNA$ , where  $k_s$  is the rate constant of synthesis and  $k_d$  is the rate constant of degradation. During the sampling period,  $k_s$  can be ignored (essentially,  $k_s = 0$ ) because during this period the newly synthesized mRNA contains virtually no labeled nucleotides and the fate of the prelabeled mRNA can be examined. Thus, after rearrangement and integration, the above equation becomes a first-order rate equation for degradation:  $\ln(mRNA_t/mRNA_0) = -k_d t$ , where  $mRNA_t$  is the amount of RNA at any time after initiation of the chase and  $mRNA_0$  is the amount of mRNA at  $t = 0$ . Therefore, a plot of these data as  $\ln(mRNA_t/mRNA_0)$  versus time generates a slope that is equal to  $-k_d$ . Since the half-life ( $t_{1/2}$ ) is

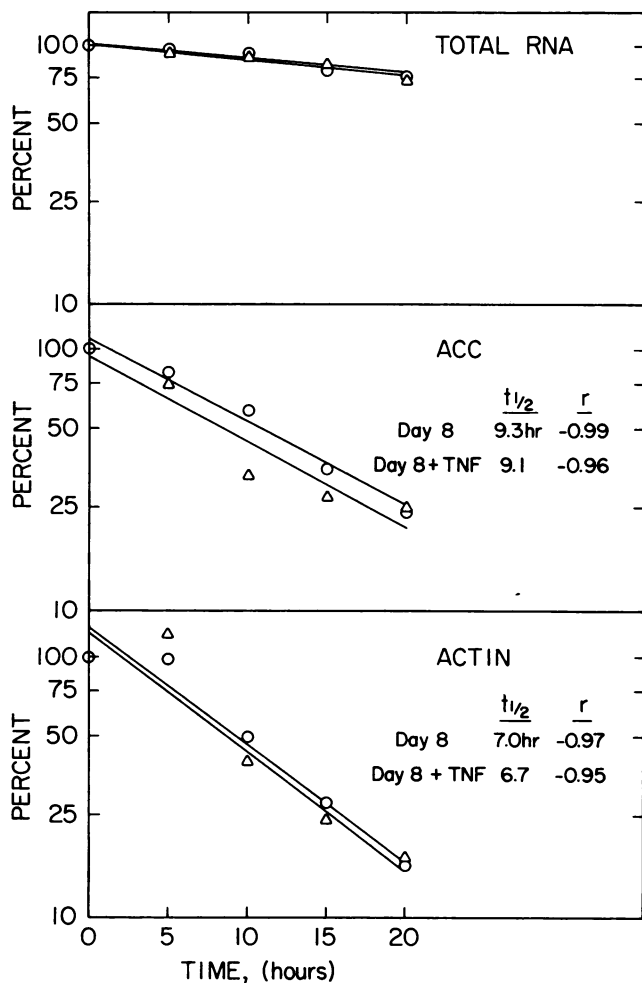


FIG. 5. Effect of TNF on the half-life of acetyl-CoA carboxylase (ACC) mRNA. Cellular RNA in 30A-5 cells at day 6 with or without TNF was labeled with [ $^3$ H]uridine for 48 h. The chase period was initiated by adding an excess of cytidine and uridine. Total RNA was isolated 0, 5, 10, 15, and 20 h later, and gene-specific [ $^3$ H]RNAs were detected under hybridization and washing conditions as described in Materials and Methods. Symbols:  $\circ$ , untreated day 8 cells;  $\Delta$ , cells treated for 8 days with TNF.

defined as the time it takes for a specific mRNA species to decay to half of its initial amount,  $\ln(0.5)/-k_d = t_{1/2}$ .

Control and TNF-treated cells showed no differences between the apparent half-lives of total RNAs (Fig. 5), which were about 50 h. The half-life of acetyl-CoA carboxylase mRNA was also not affected by TNF (Fig. 5). An apparent half-life of about 9 h was measured for both control and TNF-treated cells. Likewise, the rate of decay of actin mRNA was not changed significantly by TNF treatment, since an apparent half-life of about 7 h was measured (Fig. 5). In control cells, we were also able to determine the apparent half-lives of adipsin and GPD mRNAs, which were 23 and 5.3 h, respectively (data not shown). Unfortunately, we were unable to examine the half-lives of adipsin and GPD mRNAs in TNF-treated cells because of diminishingly low labeling of these mRNA species. Cook et al. (7) have reported apparent half-lives of 2, 4, and >30 h for GPD, actin, and adipsin, respectively, in fully differentiated NIH 3T3-F442A cells. The differences between their half-life values and ours may be attributed to the methods used to

TABLE 1. [ $^3$ H]RNA amount after pulse-labeling with [ $^3$ H]uridine for 48 h<sup>a</sup>

cDNA probe	Specific cpm/ $\mu$ g of RNA			
	Expt 1		Expt 2	
	D8	D8 + TNF	D8	D8 + TNF
Acetyl-CoA carboxylase	9.0	2.7	3.7	1.0
Adipsin	9.4	<0.6	3.4	<0.3
GPD	4.2	<0.6	1.5	<0.3
Actin	83.5	79.4	23.0	15.8

<sup>a</sup> Cell monolayers under the two treatments were exposed to [ $^3$ H]uridine for 48 h. Total RNA was isolated, and the amounts of specific RNAs for the respective genes were determined as described in Materials and Methods. Experiment 1 was performed with 1 mCi of [ $^3$ H]uridine per plate. The specific activities of total RNAs (disintegrations per minute per microgram of RNA) in experiment 1 were as follows: untreated day 8 cells (D8),  $2.2 \times 10^6$  cells treated for 8 days with TNF (D8 + TNF),  $2.6 \times 10^6$ . Experiment 2 was performed with 0.5 mCi of [ $^3$ H]uridine per plate. The corresponding specific activities of total RNAs in experiment 2 were  $1.5 \times 10^6$  and  $1.8 \times 10^6$ , respectively.

determine the half-lives. They used a transcription inhibitor, while we used an in vivo labeling approach. Regardless of the reason for the differences between the apparent half-lives, the order of increasing half-life (adipsin > actin > GPD) is the same.

We also performed another pulse-chase experiment in which we subjected cells to the standard differentiation scheme while labeling RNA from days 3 to 5. At day 5, we initiated the chase with or without TNF. This experimental design also demonstrated that the half-lives of acetyl-CoA carboxylase mRNA and actin mRNA were not affected by TNF and that the apparent half-lives were about the same as those on day 8 (8 and 7 h, respectively) (data not shown). We were unable to label adipsin and GPD mRNAs under these experimental conditions. The results of these two experiments demonstrate that TNF inhibition of accumulation of acetyl-CoA carboxylase mRNA during differentiation is not due to an increase in the rate of decay of carboxylase mRNA.

Since we were unable to detect any changes in the number of RNA polymerase molecules associated with the acetyl-CoA carboxylase gene (which presumably was a measure of the transcription rate) or a change in the stability of acetyl-CoA carboxylase mRNA under different physiological conditions, the accumulation of acetyl-CoA carboxylase mRNA during differentiation and TNF inhibition of such accumulation must be explained by some other mechanism. The most likely explanation for the paradox was that the nuclear runoff transcription assay did not measure the true in vivo transcription rate. Since the rates of degradation of carboxylase mRNA were the same in control and TNF-treated cells, a comparison of the amount of carboxylase mRNA at  $t = 0$  from the pulse-chase experiment would reflect only a difference in the rate of synthesis of the mRNA. Indeed, after the 48-h labeling period there was a three- to fourfold decrease in the rate of transcription of the carboxylase gene (Table 1), which can account for the effect of TNF on acetyl-CoA carboxylase mRNA levels during differentiation. This experiment was repeated, and a similar result was obtained (Table 1). The amount of actin RNA labeled under these conditions did not change significantly; this reflects the rate of actin RNA synthesis, because the half-life was not significantly affected by TNF. However, the amount of adipsin and GPD mRNA labeled under these conditions did change markedly. There were differences of greater than 10- and 5-fold in the

TABLE 2. [<sup>3</sup>H]RNA amounts after pulse-labeling with [<sup>3</sup>H]uridine for 9 h<sup>a</sup>

cDNA probe	Specific cpm/μg of RNA		
	Control	D8	D8 + TNF
Acetyl-CoA carboxylase	2.1	13.2	4.3
Adipsin	0.5	2.4	0.7
GPD	0.2	2.2	0.4
Actin	61.7	113.2	149.3

<sup>a</sup> Control cells were grown to 8 days postconfluence in the presence of 10% serum with no added insulin or dexamethasone. The experiment was performed with 1 mCi of [<sup>3</sup>H]uridine per plate. The specific activities of total RNAs (disintegrations per minute per microgram of RNA) were as follows: control cells,  $0.2 \times 10^6$ ; day 8 cells (D8),  $1.0 \times 10^6$ ; cells treated for 8 days with TNF (D8 + TNF),  $0.7 \times 10^6$ .

adipsin and GPD mRNA amounts, respectively, between control and TNF-treated cells. Since the labeling period was so long, the changes in adipsin and GPD mRNA levels may reflect alterations in the rates of synthesis and degradation of the mRNAs and thus should agree with studies measuring mass RNA accumulation using Northern (RNA) or RNA dot blot analysis. Indeed, a comparison of Fig. 1 with the *in vivo* labeling data bears this out.

We also labeled cells with [<sup>3</sup>H]uridine for 9 h (the half-life of carboxylase mRNA) and obtained results similar to those of the 48-h labeling period for acetyl-CoA carboxylase (Table 2). There was about a threefold difference in the amounts of carboxylase mRNA and relatively little change in the actin mRNA amounts between cells untreated for 8 days and cells treated for 8 days with TNF. Changes in adipsin and GPD mRNA amounts were also detected under the 9-h labeling period. The decrease in adipsin mRNA was only about 70% upon TNF exposure after the 9-h labeling period but decreased by greater than 90% after the 48-h labeling period. The difference may be attributed to the relatively long half-life of adipsin mRNA in relation to the length of the labeling period. Additionally, in the absence of hormonal stimulation of differentiation (dexamethasone and insulin), carboxylase gene transcription remained low as did actin gene transcription (Table 2). Thus, dexamethasone and insulin can stimulate carboxylase gene transcription, provided that the half-life is not different in control and hormone-treated cells. The *in vivo* labeling data demonstrate that TNF did decrease the rate of transcription of the carboxylase gene. However, the mechanism is not a decrease in the number of RNA polymerase molecules actively engaged in transcribing the gene, which the nuclear runoff transcription assay is designed to determine.

## DISCUSSION

We have previously shown that during the conversion of 30A-5 preadipocytes to adipocytes, the activity, amount, and mRNA levels of acetyl-CoA carboxylase increase about 10-fold (27). If the standard differentiation scheme is monitored with TNF present, the conversion to the adipocyte phenotype and the accumulation of acetyl-CoA carboxylase mRNA is inhibited by 70 to 80%. We have shown that this inhibitory effect is reversible upon withdrawal of TNF from the medium of cells exposed to the monokine. In this report, we have extended these studies to investigate the mechanisms by which TNF controls acetyl-CoA carboxylase mRNA metabolism in this system.

We demonstrated that TNF decreased the rate of transcription of the carboxylase gene three- to fourfold, as

measured by *in vivo* labeling of RNA, but TNF had no effect on the 9-h half-life of the mRNA. It is also likely that a transcriptional mode of regulation caused the accumulation of mRNA during preadipocyte differentiation, because the half-life of the carboxylase mRNA did not increase significantly as preadipocytes differentiated into adipocytes. The half-lives of the carboxylase mRNAs were about the same on days 5 and 8 at 8 to 9 h. Adipsin and GPD, which showed large increases in mRNA levels during preadipocyte differentiation, also exhibited transcriptional control. The nuclear runoff transcription assay revealed two- and threefold increases in the adipsin and GPD transcription rates, respectively. We also demonstrated that the ability of TNF to prevent accumulation of adipsin and GPD mRNAs during differentiation of preadipocytes was due in part to downregulation of adipsin and GPD gene transcription. However, the decreases in the mRNA amounts for GPD and adipsin upon TNF treatment, greater than 5- and 10-fold, respectively, cannot be accounted for by 2- and 3-fold decreases in the transcription rates of the genes. Therefore, TNF may also affect posttranscriptional regulation of the mRNAs, namely, RNA stability. Unfortunately, we were unable to assess whether TNF altered the rate of degradation of adipsin and GPD mRNAs, because we could not detect them in TNF-treated cells after [<sup>3</sup>H]uridine labeling.

We initially overlooked a transcriptional mode of regulation for the carboxylase gene by TNF because the nuclear runoff transcription assays revealed no differences in the amounts of carboxylase transcripts synthesized in isolated nuclei, although the assay did reveal differences for adipsin, GPD, and actin. Why, then, did the nuclear runoff transcription assay not reveal a difference in the acetyl-CoA carboxylase transcription rate in TNF-treated cells, whereas the *in vivo* determination of the rate of carboxylase gene transcription did? A brief discussion of the nuclear transcription assay will aid in giving a possible interpretation of our data on the effect of TNF on acetyl-CoA carboxylase gene expression in the 30A-5 preadipocyte system.

The nuclear transcription assay and various derivations have been used to roughly measure the *in vivo* rate of transcription of a particular gene. When the true *in vivo* rate has been compared with the *in vitro* data from isolated nuclei, the nuclear transcription assay has been found to be comparable to the *in vivo* rate of transcription (1, 9, 18). However, our data clearly demonstrate that the nuclear transcription assay does not reflect the *in vivo* rate of carboxylase mRNA synthesis. We attribute this discrepancy to the fact that the nuclear runoff transcription assay measures only the number of RNA polymerase molecules associated with the gene, not the rate at which the polymerase complexes transcribe the gene (i.e., the rate of transcript elongation). A measurement of the *in vivo* transcription rate inherently includes differences in the number of polymerase complexes on the gene and also the rate at which these complexes transcribe the gene. Since we have demonstrated that the number of polymerase complexes associated with the carboxylase gene does not change upon TNF treatment, a difference in the rate of transcript elongation is the most likely reason why the nuclear transcription data do not correlate with the *in vivo* data. Thus, it seems reasonable to conclude that the mechanisms by which TNF decreases acetyl-CoA carboxylase gene transcription is by reducing the rate at which the RNA polymerase molecules associated with the carboxylase gene traverse the gene. This type of transcriptional regulation is not without precedent.

We are aware of one published example of transcriptional

control by regulation of the rate of elongation of a particular gene transcript in eucaryotes (30). This period of elongation from the 5' end to the 3' end of the gene has been referred to as the transit time. Phosphoenolpyruvate carboxykinase, the rate-limiting enzyme in hepatic gluconeogenesis, displays this type of transcriptional control in H4IIE hepatocytes in response to insulin and cyclic AMP. Insulin, which decreases the number of RNA polymerase molecules associated with the gene, also decreases the rate of phosphoenolpyruvate kinase transcript elongation by about 2.5-fold in comparison with cyclic-AMP-treated cells. Since phosphoenolpyruvate kinase and acetyl-CoA carboxylase are both rate-limiting enzymes which control critical pathways in central metabolism, it seems reasonable that similar transcriptional control mechanisms work on both. A changing transit time may be a general form of transcriptional control that has largely been overlooked, especially for genes regulated by TNF. One reason for the lack of data in the literature on this type of transcriptional control may be the difficulty in assaying the rate of transcript elongation. The approaches by Sasaki and Granner (30) and Ucker and Yamamoto (37) are generally applicable to any gene, and we are currently using derivations of these approaches to determine directly whether TNF alters the rate of acetyl-CoA carboxylase transcript elongation.

#### ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA-46882 from the National Institutes of Health. M.E.P. is a trainee of National Aeronautics and Space Administration training grant NGT-50196.

#### LITERATURE CITED

- Alterman, R. M., S. Ganguly, D. H. Schulze, W. F. Marzluff, C. L. Schildkraut, and A. I. Skoultchi. 1984. Cell cycle regulation of mouse H3 histone mRNA metabolism. *Mol. Cell. Biol.* **4**:123-132.
- Bai, D. H., M. E. Pape, F. Lopez-Casillas, X. C. Luo, J. E. Dixon, and K.-H. Kim. 1986. Molecular cloning of cDNA for acetyl-CoA carboxylase. *J. Biol. Chem.* **261**:12395-12399.
- Berent, J. S. L., M. Mahmondi, R. M. Torczynski, P. J. W. Bragg, and A. P. Bollon. 1985. Comparison of oligonucleotide and long DNA fragments as probes in DNA and RNA dot. Southern, Northern, colony, and plaque hybridizations. *Biotechniques* **3**:208-220.
- Beutler, B., and A. C. Cerami. 1986. Cachectin and tumor necrosis factor as two sides of the same biological coin. *Nature (London)* **320**:584-588.
- Beutler, B., I. W. Milsark, and A. C. Cerami. 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* **229**:869-871.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
- Cook, K. S., C. R. Hunt, and B. M. Spiegelman. 1985. Developmentally regulated mRNAs in 3T3-adipocytes: analysis of transcriptional control. *J. Cell Biol.* **100**:514-520.
- Cornelius, P., S. Enerback, G. Bjursell, T. Olivecrona, and P. H. Pekala. 1988. Regulation of lipoprotein lipase mRNA content in 3T3-L1 cells by tumor necrosis factor. *Biochem. J.* **249**:765-769.
- Cross, F. R., and J. E. Darnell, Jr. 1983. Cycloheximide stimulates early adenovirus transcription if early gene expression is allowed before treatment. *J. Virol.* **45**:683-692.
- Dobson, D. E., D. L. Groves, and B. M. Spiegelman. 1987. Nucleotide sequence and hormonal regulation of mouse glycerophosphate dehydrogenase mRNA during adipocyte and muscle cell differentiation. *J. Biol. Chem.* **262**:1804-1809.
- Kohase, M., D. Henriksen-DeStefano, L. T. May, J. Vilcek, and P. B. Sehgal. 1986. Induction of beta2-interferon by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. *Cell* **45**:659-666.
- Lane, M. D., J. Moss, and S. E. Plalkis. 1974. Acetyl-CoA carboxylase. *Curr. Top. Cell. Regul.* **8**:139-195.
- Leibovich, S. J., P. J. Polverini, H. M. Shepard, D. M. Wiseman, V. Shively, and N. Nuseir. 1987. Macrophage-induced angiogenesis is mediated by tumor necrosis factor-alpha. *Nature (London)* **329**:630-632.
- Lin, J.-X., and J. Vilcek. 1987. Tumor necrosis factor and interleukin-1 cause a rapid and transient stimulation of c-fos and c-myc mRNA levels in human fibroblasts. *J. Biol. Chem.* **262**:11908-11911.
- Lopez-Casillas, F., D. H. Bai, X. Luo, I. S. Kong, M. A. Hermodson, and K.-H. Kim. 1988. Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. *Proc. Natl. Acad. Sci. USA* **85**:5784-5788.
- Lopez-Casillas, F., M. E. Pape, D. H. Bai, D. N. Kuhn, J. E. Dixon, and K.-H. Kim. 1987. Preparation of functional acetyl-CoA carboxylase mRNA from rat mammary gland. *Arch. Biochem. Biophys.* **257**:63-68.
- Mackall, J. C., and M. D. Lane. 1977. Changes in mammary-gland acetyl-CoA carboxylase associated with lactogenic differentiation. *Biochem. J.* **162**:635-642.
- Majerus, P. W., and E. Kilburn. 1969. Acetyl-coenzyme A carboxylase: the roles of synthesis and degradation in regulation of enzyme levels in rat liver. *J. Biol. Chem.* **244**:6254-6262.
- McKnight, G. S., and R. D. Palmiter. 1979. Transcriptional regulation of the ovalbumin and conalbumin genes by steroid hormones in chick oviduct. *J. Biol. Chem.* **254**:9050-9058.
- Miller, S. C., H. Ito, H. M. Blau, and F. M. Torti. 1988. Tumor necrosis factor inhibits human myogenesis in vitro. *Mol. Cell. Biol.* **8**:2295-2301.
- Min, H. Y., and B. M. Spiegelman. 1986. Adipsin, the adipocyte serine protease: gene structure and control of expression by tumor necrosis factor. *Nucleic Acids Res.* **14**:8879-8892.
- Munker, R., J. Gasson, M. Ogawa, and H. P. Koeffler. 1986. Recombinant human TNF induces production of granulocyte-macrophage colony-stimulating factor. *Nature (London)* **323**:79-82.
- Nakanishi, S., and S. Numa. 1970. Purification of rat liver acetyl-coenzyme A carboxylase and immunochemical studies on its synthesis and degradation. *Eur. J. Biochem.* **16**:161-173.
- Numa, S., and T. Tanabe. 1984. Acetyl-CoA carboxylase and its regulation, p. 1-27. *In* S. Numa (ed.), *Fatty acid metabolism and its regulation*. Elsevier Science Publishing, Inc., New York.
- Numa, S., and S. Yamashita. 1974. Regulation of lipogenesis in animal tissues. *Curr. Top. Cell. Regul.* **8**:197-246.
- Old, L. J. 1985. Tumor necrosis factor (TNF). *Science* **230**:630-632.
- Old, L. J. 1987. Polypeptide mediator network. *Nature (London)* **326**:330-331.
- Pape, M. E., and K.-H. Kim. 1988. Effect of tumor necrosis factor on acetyl-CoA carboxylase gene expression and preadipocyte differentiation. *Mol. Endocrinol.* **2**:395-403.
- Pape, M. E., F. Lopez-Casillas, and K.-H. Kim. 1988. Physiological regulation of acetyl-CoA carboxylase gene expression: effects of diet, diabetes, and lactation on acetyl-CoA carboxylase mRNA. *Arch. Biochem. Biophys.* **267**:104-109.
- Patton, J. S., H. M. Shepard, H. Welking, G. Lewis, B. B. Aggarwal, T. E. Eessalu, L. A. Gavin, and C. Grunfeld. 1986. Interferons and tumor necrosis factor have similar catabolic effects on 3T3-L1 cells. *Proc. Natl. Acad. Sci. USA* **83**:8313-8317.
- Phillip, R., and L. B. Epstein. 1986. Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon, and interleukin-1. *Nature (London)* **323**:86-89.
- Sasaki, K., and D. K. Granner. 1988. Regulation of phosphoenolpyruvate carboxykinase gene transcription by insulin and cAMP: reciprocal actions on initiation and elongation. *Proc. Natl. Acad. Sci. USA* **85**:2954-2958.
- Seelentag, W. K., J.-J. Mermod, R. Montesano, and P. Vassalli.



1987. Additive effects of interleukin 1 and tumor necrosis factor- $\alpha$  on the accumulation of the three granulocyte and macrophage colony-stimulating factor mRNAs in human endothelial cells. *EMBO J.* **6**:2261–2265.
32. Spiegelman, B. M., M. Frank, and H. Green. 1983. Molecular cloning of mRNA from 3T3 adipocytes. *J. Biol. Chem.* **258**: 10083–10089.
33. Sugarman, B. J., B. B. Aggarwal, I. S. Figari, M. A. Palladino, Jr., and H. M. Shepard. 1985. Recombinant human tumor necrosis factor- $\alpha$ : effects on proliferation of normal and transformed cells in vitro. *Science* **230**:943–945.
34. Torti, F. M., B. Dieckmann, B. Beutler, A. Cerami, and G. M. Ringold. 1985. A macrophage factor inhibits adipocyte gene expression: an in vitro model of cachexia. *Science* **229**:867–869.
35. Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature (London)* **330**:662–664.
36. Turpen, T. H., and O. M. Griffith. 1986. Rapid isolation of RNA by a guanidinium thiocyanate/cesium chloride gradient method. *Biotechniques* **4**:11–15.
37. Ucker, D. S., and K. R. Yamamoto. 1984. Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids. *J. Biol. Chem.* **259**:7416–7420.
38. Vilcek, J., V. J. Palombella, D. Henriksen-DeStefano, C. Swenson, R. Feinman, M. Hirai, and M. Tsujimoto. 1986. Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. *J. Exp. Med.* **163**:632–643.
39. Volpe, J. J., and P. R. Vagelos. 1973. Saturated fatty acid biosynthesis and its regulation. *Annu. Rev. Biochem.* **42**:21–60.
40. Yarden, A., and A. Kimchi. 1986. Tumor necrosis factor reduces c-myc expression and cooperates with interferon- $\gamma$  in HeLa cells. *Science* **234**:1419–1421.
41. Zechner, R., T. C. Newman, B. Sherry, A. Cerami, and J. L. Breslow. 1988. Recombinant human cachectin/tumor necrosis factor but not interleukin- $1\alpha$  downregulates lipoprotein lipase gene expression at the transcriptional level in mouse 3T3-L1 adipocytes. *Mol. Cell. Biol.* **8**:2394–2401.