Regulation of Collagen Gene Expression in 3T3-L1 Cells. Effects of Adipocyte Differentiation and Tumor Necrosis Factor α[†]

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ABSTRACT: An early feature in the development of adipocytes from fibroblast-like precursor cells is the biogenesis of an extracellular basement membrane (Napolitano, 1963; Kuri-Harcuch et al., 1984). Interactions between components of the basement membrane (e.g., collagens) and the surfaces of differentiating adipocytes are thought to regulate subsequent phases of the developmental program. Since fibroblasts principally secrete type I and III collagens whereas type IV collagen is abundant in basement membrane, it appears that a switch in collagen gene expression is a key element in adipocyte differentiation. Little is known about the mechanisms underlying differentiation-dependent changes in collagen expression or the effects of the potent lipolytic cytokine TNF-α on collagen mRNA accumulation in preadipocytes and adipocytes. In this study, 3T3-L1 preadipocytes were found to express mRNAs encoding type I, III, and IV procollagens. When 3T3-L1 cells were stimulated to differentiate into adipocytes, the relative concentrations of type I and type III procollagen mRNAs declined by 80–90%. Parallel decreases in the rates of transcription of the procollagen I and procollagen III genes appear to account for the diminished levels of these mRNAs. In contrast, the relative rate of transcription of the procollagen IV gene increased 2.6-fold during adipocyte development. As a consequence, the abundance of type IV procollagen transcripts was elevated in adipocytes. Tumor necrosis factor α (TNF-α) is a cytokine that stimulates lipolysis, an apparent "dedifferentiation" of adipocytes, and inhibits transcription of certain adipocyte-specific genes. The effects of TNF-α on collagen mRNA levels were dependent upon the state of differentiation of 3T3-L1 cells. In preadipocytes, 5 nM TNF-α elicited 60–80% decreases in the transcription rates of the three procollagen genes, thereby promoting a coordinated decline of 75% in the amounts of type I, III, and IV procollagen mRNAs. However, TNF-α provoked the accumulation of all three procollagen mRNAs in 3T3-L1 adipocytes via posttranscriptional mechanism(s). Thus, the responsiveness of procollagen genes to TNF-α is drastically altered in degree, direction, and underlying mechanism as preadipocytes differentiate into adipocytes. Transforming growth factor β (TGF-β) also inhibits adipocyte differentiation and alters extracellular matrix protein synthesis. While TGF-β mRNA was expressed in preadipocytes, its abundance declined markedly (3.3-fold) during adipogenesis. In contrast, TNF-α effected a moderate (2-fold) increase in TGF-β mRNA content in both preadipocytes and adipocytes by a posttranscriptional process.

The process of cell differentiation involves ordered and coordinated changes in the expression of limited sets of proteins and their cognate mRNAs. 3T3-L1 preadipocytes, which differentiate into adipocytes, provide a suitable system for investigating the regulation of expression of specific mRNAs that are markedly increased or decreased during differentiation (Cook et al., 1985; Spiegelman et al., 1983). Previous studies on 3T3-L1 cells (Cook et al., 1985; Chapman et al., 1984; Bernlohr et al., 1985) have focused on the regulation of genes that are associated with the development of the adipocyte phenotype. However, little attention has been given to genes that are expressed at substantial levels in the fibroblast-like 3T3-L1 preadipocytes. The collagens are abundant secreted proteins produced by fibroblasts and, along with other extracellular matrix proteins, have been implicated as regulators of cell growth, differentiation, and development (Yoshizato et al., 1985; Yamada, 1982; Culp et al., 1979; Kraemer & Tobey, 1972; Spiegelman & Ginty, 1983). One objective of the present study was to investigate collagen gene regulation during the differentiation of 3T3-L1 preadipocytes into adipocytes.

Tumor necrosis factor α (TNF-α)[1] is a 17-kDa polypeptide with multiple biological actions. Macrophages are the principal source of TNF-α, and the major target tissues include the liver, skin, kidney, lungs, gastrointestinal tract, and adipose tissue (Beutler et al., 1985). TNF-α is thought to play a role in the wasting associated with chronic disease (Beutler & Cerami, 1986), and it appears to be a potent negative regulator of adipocyte differentiation. Torti et al. (1985) demonstrated that TNF inhibits the differentiation of another mesenchymal cell line (TA1) into adipocytes. Furthermore, exposure of the differentiated TA1 adipocytes to TNF-α elicited a marked decrease in several adipocyte-specific mRNAs and a gradual loss of microscopically visualized triacylglycerol droplets (Torti

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[1] Abbreviations: TNF α, tumor necrosis factor α; TGF β, transforming growth factor β; Col I, pro-α_2 type I collagen; Col III, type III procollagen; Col IV, type IV procollagen; T-Arg, arginine transfer RNA.

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et al., 1985). After prolonged exposure to TNF-α, the cells appear to be phenotypically similar to fibroblasts or TA1 preadipocytes. A second goal of our studies was to examine the influence of TNF-α on collagen mRNA content in both preadipocytes and adipocytes and to determine the molecular level at which TNF-α-mediated effects are exerted.

Materials and Methods

Cell Culture, Differentiation, and TNF-α Treatment. 3T3-L1 preadipocytes were grown in 150-mm culture dishes in Dulbecco's modified Eagle's medium (DME) (Gibco) supplemented with 2 mM glutamine (Rubin et al., 1978) and 10% fetal calf serum (Gibco). Cultures were fed 18 mL of medium every 2–3 days during exponential growth and according to the schedule described below during differentiation (Smith et al., 1988). The cells were maintained in an atmosphere of 10% CO_2 and 90% air at 37 °C.

Preadipocytes that were grown to confluence in 150-mm culture dishes were subjected to the differentiation protocol (Rubin et al., 1978). Confluent cells were fed with 18 mL of fresh standard medium containing 0.5 mM 1-methyl-3-isobutylxanthine and 0.5 μM dexamethasone. After 48 h, the medium containing the steroid and 1-methyl-3-isobutylxanthine was aspirated. The cells were then given 8 mL of standard medium and were allowed to differentiate for an additional 3 days.

Confluent 3T3-L1 preadipocytes and 3T3-L1 adipocytes were exposed to 5 nM TNF-α (Genentech, South San Francisco, CA), in DME and 10% fetal calf serum for 24 h. After 24 h of treatment, the cells were harvested for molecular studies.

RNA Extraction and Northern Blot Hybridization Analysis. Control and treated cells were washed twice in cold Hank's balanced salt solution, removed from the plates with a rubber policeman, and pelleted in cold Hank's balanced salt solution in a clinical centrifuge. Three to eight 150-mm dishes were pooled per condition. Total RNA was extracted from 3T3-L1 cells by a modification of the Chirgwin procedure (Chirgwin et al., 1979) as previously described (Czaja et al., 1987). Cells were homogenized in 3.5 mL of a 4 M guanidine thiocyanate solution and cleared of cellular debris by low-speed centrifugation. The RNA was then pelleted through a cesium chloride gradient by centrifugation at 35000 rpm in an SW60 rotor for 17 h at 14 °C. The resultant RNA pellet was redissolved, precipitated with ethanol, quantitated by its absorbance at 260 nm, and used for molecular hybridization studies. Messenger RNA levels were determined by Northern blot hybridization analysis as previously described (Czaja et al., 1987). Samples (20 μg) of total RNA were denatured in 0.5 M glyoxal, 50% dimethyl sulfoxide, and 10 mM sodium phosphate buffer, pH 7.0, electrophoresed in a 1% agarose gel, transferred to a GeneScreen filter (New England Nuclear, Boston, MA), and baked for 2 h at 80 °C. The filters were prehybridized and then hybridized under stringent conditions (Zern et al., 1985) with cDNA inserts that were labeled to a specific activity of (2–10) $\times$ 10^8 cpm/μg of DNA with [α-^{32}P]dCTP by random priming (Amersham Corp., Arlington Heights, IL). The following cDNA clones were used: rat pro-α_2(I) collagen (Genovese et al., 1984), mouse type III procollagen (Liau et al., 1985), mouse type α_2(IV) procollagen (Wang & Gudas, 1983), chicken β-actin (Cleveland et al., 1980), human transforming growth factor β (Derynck et al., 1985), and clones 1 and 28, which are cDNAs complementary to mRNAs whose expression increases during adipocyte differentiation (Chapman et al., 1984). cDNA clones 1 and 28 were generously supplied by Dr. Gordon Ringold, Syntex Corp. (Palo Alto, CA). Following hybridization, the filters were washed and exposed to X-ray film, and the developed film was scanned in a densitometer (Zern et al., 1985). The presence of equal amounts of RNA among samples was substantiated by ethidium bromide staining of the GeneScreen filters (Maniatis et al., 1982) following exposure to X-ray film.

Transcription Rate Analysis. Nuclear run-on assays were performed as previously described (Weiner et al., 1987), using nuclei isolated from four to six 150-mm dishes of control or treated cells. Nuclei were labeled for 15 min at 30 °C with high specific activity [α-^{32}P]UTP (Jefferson et al., 1984; Clayton & Darnell, 1983), and the labeled RNA transcripts were isolated by lysis of the nuclei in a hypotonic solution (Jefferson et al., 1984), followed by DNA digestion with RNase-free DNase (100 units/mL), proteinase K digestion, phenol extraction, and ethanol precipitation with an intervening precipitation with 10% trichloroacetic acid to remove the unincorporated radiolabeled UTP. RNA transcripts were then hybridized with at least a 10-fold excess of the same cDNA probes used for Northern blots (Clayton & Darnell, 1983). The plasmid pBR322 was used as a negative control, and a mouse cDNA probe of arginine transfer RNA (T-Arg) (provided by Dr. J. Darnell, Jr., Rockefeller University, NY) was used as an indirect marker to normalize transcription among various samples (Clayton & Darnell, 1983).

Results

3T3-L1 preadipocytes have a fibroblast-like morphology. Following exposure to 0.5 mM methylisobutylxanthine and 0.5 μM dexamethasone in standard culture medium for 48 h, the cells displayed a more polygonal shape with triglyceride droplets surrounding the nucleus as previously described (Rubin et al., 1978). These droplets subsequently coalesced and occupied a major portion of the cell cytoplasm as the adipocytes became more spherical in shape (Rubin et al., 1978). Exposure of either undifferentiated 3T3-L1 cells or adipocytes to 5 nM TNF-α for 24 h did not alter the characteristic morphology or viability of the cells.

A low level of clone 1 mRNA was present in preadipocytes whereas clone 28 mRNA was not detected (Figure 1). Upon differentiation of the 3T3-L1 cells into fat cells, the amounts of "adipocyte specific" mRNAs corresponding to clone 28 and 1 cDNAs increased markedly whereas β-actin mRNA content decreased (Figure 1), as expected on the basis of previous studies on TA1 and 3T3-L1 cells (Chapman et al., 1984; Bernlohr et al., 1985). Quantitation of these differences by densitometric scanning of three sets of experiments showed that the differentiation-dependent increase in clone 1 mRNA content was 19-fold, while β-actin levels were only 21% of the control value. Nuclear run-on assays demonstrated that these changes in mRNA levels were associated with corresponding changes in transcription rates (Figure 2, Table I).

A high level of type I procollagen mRNA and lower levels of type III and IV procollagen mRNAs were observed in 3T3-L1 preadipocytes (Figure 1). Upon differentiation of 3T3-L1 cells into adipocytes, there were 80–90% decreases in the mRNA levels for type I and III procollagens (Figure 1). In contrast, type IV procollagen mRNA content increased by 70% in adipocytes. These results correlate the loss of the fibroblast-like phenotype of 3T3-L1 cells during differentiation with a decline in type I and III collagen mRNAs and indicate that collagen gene expression is controlled pretranslationally. Nuclear run-on analysis revealed that the changes in mRNA levels for the three collagens were associated with similar alterations in the rates of transcription (Figure 2, Table I). There were 75–95% decreases in types I and III procollagen

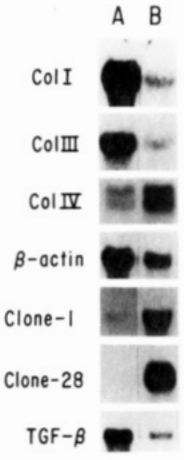

FIGURE 1: Effects of adipocyte differentiation on the relative levels of selected mRNAs. Northern blots of total RNA (20 μg) from 3T3-L1 preadipocytes (A) and adipocytes (B) were hybridized with the indicated ^{32}P-labeled cDNA probes as described under Materials and Methods. Portions of the autoradiograms containing the hybridized mRNAs are shown. Abbreviations for the cDNA probes indicated in the figure are as follows: pro-α_2 type I collagen, ColI; type III procollagen, ColIII; type IV procollagen, ColIV; transforming growth factor β, TGF-β.

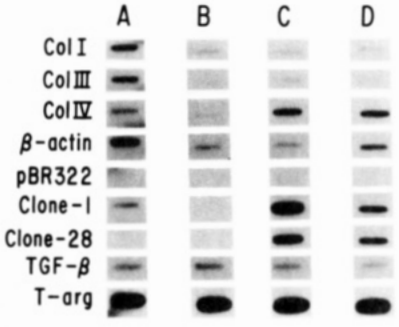

FIGURE 2: Effects of differentiation and TNF-α on gene transcription. A representative autoradiogram of a nuclear run-on assay demonstrating the relative levels of transcription for several genes in (A) untreated 3T3-L1 preadipocytes, (B) 3T3-L1 preadipocytes treated with 5 nM TNF-α, (C) untreated 3T3-L1 adipocytes, and (D) 3T3-L1 adipocytes treated with 5 nM TNF-α. Nuclei were isolated and used for nuclear run-on analysis as described under Materials and Methods. The filters contained the cDNA probes indicated in Figure 1 and pBR322 and mouse arginine transfer RNA (T-arg).

Table I: Effects of Adipocyte Differentiation on Gene Transcription[a]

gene assayed	transcription in 3T3-L1 adipocytes as % of transcription in 3T3-L1 preadipocytes
Col I	7 ± 1
Col III	25 ± 9
Col IV	260 ± 70
β-actin	41 ± 22
clone 1	1560 ± 65
clone 28	*b*
TGF-β	89 ± 5

[a] Results of densitometric scanning of autoradiograms from nuclear run-on experiments using 3T3-L1 preadipocytes and adipocytes. Data expressed as the mean ± the standard error for three experiments. [b] Not determined because of the absence of a signal in preadipocytes.

gene transcription and a 2.6-fold increase in type IV collagen gene transcription associated with adipocyte differentiation (Table I).

When 3T3-L1 adipocytes were treated with 5 nM TNF-α for 24 h, there was a marked depletion in the mRNA levels of transcripts encoded by clones 1 and 28 (Figure 3), a result

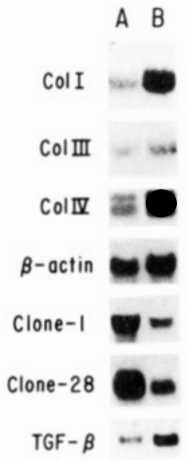

FIGURE 3: Effects of TNF-α on the relative levels of selected mRNAs in adipocytes. Northern blots with total RNA (20 μg) extracted from untreated 3T3-L1 adipocytes (control cells) (A) and 3T3-L1 adipocytes treated with 5 nM TNF-α (B) were hybridized with the indicated ^{32}P-labeled cDNA probes as described under Materials and Methods. Portions of autoradiograms containing the hybridized mRNAs are shown. Abbreviations for the cDNA probes are as indicated in Figure 1.

Table II: Effects of TNF-α on Preadipocyte and Adipocyte Gene Transcription[a]

gene assayed	transcription in treated 3T3-L1 preadipocytes as % of control[b,c]	transcription in treated 3T3-L1 adipocytes as % of control[b,d]
Col I	20 ± 3	115 ± 9
Col III	29 ± 12	81 ± 2
Col IV	40 ± 10	95 ± 7
β-actin	44 ± 21	220 ± 59
clone 1	*e*	35 ± 3
clone 28	*e*	51 ± 7
TGF-β	96 ± 13	98 ± 10

[a] Results of densitometric scanning of nuclear run-on experiments for 3T3-L1 preadipocytes and adipocytes treated with TNF-α. [b] Data expressed as the mean ± the standard error for three experiments. [c] Control transcription was measured in untreated preadipocytes. [d] Control transcription was measured in untreated adipocytes. [e] Not determined because of the low or absent signals obtained from preadipocytes.

consistent with a previous report (Torti et al., 1985). Quantification of three sets of experiments revealed a 75% decrease in the mRNA content for clone 1 and 28 proteins. Depressed gene transcription rates were also associated with changes in mRNA levels for clones 28 and 1 (Figure 2, Table II).

Exposure of undifferentiated 3T3-L1 cells to 5 nM TNF-α for 24 h resulted in decreases in the mRNA content for types I, III, and IV procollagen (Figure 4). A decrease in the mRNA content of β-actin was also observed. Scanning densitometry of three such experiments revealed a 70–75% decrease in type I, III, and IV procollagen mRNA levels, and a 57% decrease in β-actin mRNA content compared with untreated control cells. TNF-α treatment also lowered the transcription rates for these four genes (Figure 2, Table II). On the other hand, exposure of 3T3-L1 adipocytes to 5 nM TNF-α for 24 h resulted in 2.9-, 1.4-, and 2-fold increases in the mRNA content for type I, III, and IV procollagens, as well as a 1.6-fold increase in the β-actin mRNA content (Figure 3). The increases in types I and III procollagen and β-actin mRNAs are consistent with the TNF-induced dedifferentiation of 3T3-L1 adipocytes. However, the increase in type IV collagen mRNA content is not characteristic of a dedifferentiation effect. Nuclear run-on analysis revealed that the

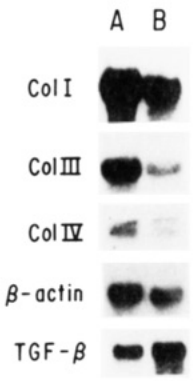

FIGURE 4: Effects of TNF-α on the relative levels of selected mRNAs in preadipocytes. Northern blots with total RNA extracted from untreated 3T3-L1 preadipocytes (control cells) (A) and 3T3-L1 preadipocytes treated with 5 nM TNF-α (B) were hybridized with the indicated ^{32}P-labeled cDNA probes as described under Materials and Methods. Portions of an autoradiogram containing the hybridized mRNAs are shown. Abbreviations for the cDNA probes are indicated in Figure 1.

transcription rates for the three collagen genes were unaffected by the cytokine (Figure 2, Table II). Thus, the effect of TNF-α on the expression of these extracellular matrix protein mRNAs in adipocytes occurs at a posttranscriptional level. However, a 2-fold increase in the β-actin gene transcription in TNF-α-treated adipocytes (Figure 2, Table II) was observed.

Finally, the effect of TNF-α on transforming growth factor β (TGF-β) expression in 3T3-L1 cells was examined. TGF-β is a cytokine which inhibits 3T3-L1 adipocyte differentiation (Ignotz & Massagué, 1985). The effect of TNF-α on TGF-β gene expression was explored in 3T3-L1 preadipocytes and adipocytes because (1) both cytokines have similar effects in adipocyte differentiation (Torti et al., 1985; Ignotz & Massagué, 1985), (2) it has been shown that the effect of one cytokine may be mediated by the synthesis, secretion, and paracrine action of a second cytokine (Patton et al., 1986), and (3) TGF-β alters extracellular matrix protein synthesis (Sporn et al., 1987). We found that while 3T3-L1 adipocytes contained TGF-β mRNA, the content was 70% lower than in the undifferentiated cells (Figure 1), and posttranscriptional mechanisms appear to mediate this change in TGF-β mRNA content (Figure 2, Table I). Upon treatment of 3T3-L1 preadipocytes or adipocytes with 5 nM TNF for 24 h, there was a 2-fold increase in the TGF-β mRNA content (Figures 3 and 4). Nuclear run-on analysis revealed that a posttranscriptional mechanism also appeared to be responsible for this effect (Figure 2, Table II).

Discussion

Changes in collagen mRNA expression during adipocyte differentiation have not been previously described. In this study, we have examined the expression of three collagen genes in 3T3-L1 preadipocytes and adipocytes. Preadipocytes express principally type I procollagen mRNA and lower amounts of type III and IV procollagen mRNAs. Upon differentiation into adipocytes, the expression of fibrillar collagen mRNAs (type I and III) declines as mRNAs for basement membrane collagen (type IV) and adipocyte-specific genes accumulate. These changes are consistent with loss of the fibroblast phenotype of 3T3-L1 preadipocytes upon differentiation into adipocytes, whose specialized cellular function is lipid metabolism and storage (Green & Meuth, 1974). Moreover, the increased transcription of the type IV collagen gene and the accumulation of type IV procollagen mRNA complement morphological studies that link adipocyte differentiation with the production of a basement membrane (Napolitano, 1963; Kuri-Harcuch et al., 1984). The differentiation-dependent increase in adipocyte-specific mRNAs designated clones 1 and 28 are due to increased gene transcription. These results are consistent with the data of Chapman et al. (1984) and serve as internal controls. In addition, the loss of collagen mRNAs that are characteristic of fibroblasts also appears to be transcriptionally regulated; i.e., the decreases in type I and III procollagen mRNAs are associated with parallel decreases in transcription of the type I and III procollagen genes. In earlier studies, Green and co-workers (Green & Kehinde, 1974; Green & Meuth, 1974) demonstrated that collagens were synthesized at substantial levels in 3T3-L1 preadipocytes. More recently, Djian et al. (1985) reported that the levels of type I collagen mRNA and gene transcription were not altered during the differentiation of 3T3-F442A adipocytes. The basis for the difference between our results and those of Djian et al. (1985) is not yet known. One possibility is that the 3T3-F442A cell line expresses type I collagen in an unregulated (constitutive) manner, thereby reflecting differences in the origins of the 3T3-L1 and 3T3-F442A lineages. Future experiments will be required to address this point. Djian and co-workers (Djian et al., 1985) did not study the expression of type III and IV collagen genes during adipogenesis or the effects of TNF-α on the control of collagen gene transcription and mRNA levels. The differentiation of 3T3-L1 preadipocytes was also accompanied by a decrease in the mRNA for the cytoskeletal protein, β-actin (Figure 1). This decrease in β-actin levels was also due to a lower level of transcription.

Tumor necrosis factor α (TNF-α) inhibits 3T3-L1 preadipocyte differentiation into adipocytes and causes decreases in the levels of clone 1 and 28 mRNAs and their gene transcription rates in TA1 adipocytes (Torti et al., 1985). TNF-α exerts differential effects on type I, III, and IV procollagen mRNA levels in 3T3-L1 preadipocytes and adipocytes. In preadipocytes, TNF-α elicited decreases in the levels of all procollagen mRNAs, and this effect was exerted at the transcriptional level. Thus, susceptibility to TNF-α-mediated transcriptional regulation in 3T3-L1 cells is not dependent on cell differentiation. TNF-α-mediated suppression of 3T3-L1 preadipocyte collagen mRNA accumulation did not alter cell morphology within 24 h. This observation is consistent with a previous study which showed that TNF-α treatment of preadipocytes does not affect cell growth or viability (Torti et al., 1985) and indicates that the effect of TNF-α on preadipocytes was not due to cytotoxicity. Solis-Herruzo et al. (1988) also showed that TNF-α inhibited pro-α_2 type I collagen gene transcription and reduced the content of type I procollagen mRNA levels and collagen production in human fibroblasts. In 3T3-L1 preadipocytes, it also appears that the transcription of type III and IV procollagen genes is coordinately inhibited by TNF-α, thereby promoting large decreases in their corresponding mRNAs.

The effects of TNF-α on 3T3-L1 adipocyte collagen mRNA levels were markedly different from its effects on preadipocyte collagen mRNAs. Although treatment of 3T3-L1 adipocytes with TNF-α caused a decrease in the transcription and steady-state mRNA levels of two adipocyte-specific proteins (Figures 2 and 3, Table II), the relative concentrations of type I, III, and IV procollagen mRNAs increased following cytokine treatment. The accumulation of collagen mRNA was controlled at a posttranscriptional level. Thus, the mechanisms by which TNF-α modulates collagen gene expression and mRNA content in 3T3-L1 cells vary depending upon the state

of differentiation of these cells. It has been suggested that TNF-α causes a "dedifferentiation" of adipocytes on the basis of the decrease in transcription and content of adipocyte-"specific" mRNAs (Torti et al., 1985). However, TNF-α treatment of 3T3-L1 adipocytes only partially reproduces the preadipocyte phenotype in terms of collagen mRNA levels. Moreover, regulation is exerted at different levels in preadipocytes and adipocytes. There is, as yet, no physiological explanation for the finding that collagen gene expression is stimulated in 3T3-L1 adipocytes and inhibited in 3T3-L1 preadipocytes. The increases in type I, III, and IV procollagen mRNA levels caused by TNF-α treatment, and the decrease in the adipocyte-specific mRNAs, indicate that TNF-α can simultaneously cause positive and negative alterations in gene expression in 3T3-L1 adipocytes.

Another alteration in gene expression that could possibly affect the expression of adipocyte genes is the increase in β-actin mRNA levels caused by TNF-α. A decrease in actin synthesis was found prior to lipogenic enzyme accumulation (Spiegelman & Farmer, 1982; Sidhu, 1979; Spiegelman & Green, 1980). The apparent requirement for morphological and cytoskeletal reorganization for the normal expression of the mRNAs for lipogenic enzymes and proteins in differentiating adipocytes (Spiegelman & Green, 1980; Novikoff et al., 1980; Spiegelman & Farmer, 1982) suggests a similar process could also be partly mediating the effect of TNF-α observed in this study. Further studies will be necessary to determine if the TNF-α-induced increase in β-actin mRNA levels results in increases in β-actin protein levels and filament assembly.

Finally, TGF-β has been shown to play a role in the regulation of cell growth and differentiation in many systems (Sporn et al., 1987). In addition, it appears that TGF-β induces type I procollagen gene expression (Ignotz & Massagué, 1986). Although both 3T3-L1 preadipocytes and adipocytes contained TGF-β mRNA, TGF-β mRNA content decreased with the differentiation of 3T3-L1 cells into adipocytes. Since exogenous TGF-β potently inhibits adipocyte differentiation, the decrease in TGF-β mRNA levels with differentiation may reflect a component of adipogenesis in 3T3-L1 cells. The role TGF-β plays in vivo in adipogenesis will require further studies to determine whether differentiating adipocytes in vivo modulate TGF-β expression in a manner that parallels the modulation of expression in 3T3-L1 cells. The decrease in TGF-β gene expression may also explain the decrease in fibrillar collagen synthesis in these cells. TNF-α also increased TGF-β mRNA content in preadipocytes and adipocytes by a posttranscriptional process. This TNF-α-induced increase in TGF-β mRNA content is consistent with the idea that part of the effect of TNF-α on these cells could be due to the elevation in TGF-β mRNA levels. A concomitant elevation in TGF-β protein levels could then subsequently affect the state of differentiation and collagen gene expression. It is possible that the stimulatory efect of TNF-α on the production of this second cytokine is part of a cascade that regulates the expression of a multiplicity of genes associated with the acute and chronic metabolic derangements observed in certain pathophysiologic states in vivo (Patton et al., 1986; Beutler & Cerami, 1986). Further experiments will be required to determine the validity of this hypothesis and the mechanisms involved in linking the TNF-α-dependent cell surface signal to the regulation of specific gene expression.

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Comparative Cross-Linking Study on the 50S Ribosomal Subunit from *Escherichia coli*

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ABSTRACT: We have carried out an extensive protein–protein cross-linking study on the 50S ribosomal subunit of *Escherichia coli* using four different cross-linking reagents of varying length and specificity. For the unambiguous identification of the members of the cross-linked protein complexes, immunoblotting techniques using antisera specific for each individual ribosomal protein have been used, and for each cross-link, the cross-linking yield has been determined. With the smallest cross-linking reagent diepoxybutane (4 Å), four cross-links have been identified, namely, L3–L19, L10–L11, L13–L21, and L14–L19. With the sulfhydryl-specific cross-linking reagent *o*-phenylenedimaleimide (5.2 Å) and *p*-phenylenedimaleimide (12 Å), the cross-links L2–L9, L3–L13, L3–L19, L9–L28, L13–L20, L14–L19, L16–L27, L17–L32, and L20–L21 were formed; in addition, the cross-link L23–L29 was exclusively found with the shorter *o*-phenylenedimaleimide. The cross-links obtained with dithiobis(succinimidyl propionate) (12 Å) were L1–L33, L2–L9, L2–L9–L28, L3–L19, L9–L28, L13–L21, L14–L19, L16–L27, L17–L32, L19–L25, L20–L21, and L23–L34. The good agreement of the cross-links obtained with the different cross-linking reagents used in this study demonstrates the reliability of our cross-linking approach. Incorporation of our cross-linking results into the three-dimensional model of the 50S ribosomal subunit derived from immunoelectron microscopy yields the locations for 29 of the 33 proteins within the larger ribosomal subunit.

The catalysis of peptide bonds to form proteins from aminoacyl-tRNAs under direction of mRNAs occurs on ribosomes. In order to understand this mechanism, which is fundamental to all organisms, a detailed knowledge of the structure of the ribosome is essential.

Whereas the overall topography of the 30S subunit has been revealed (Stöffler-Meilicke & Stöffler, 1987; Capel et al., 1988; Brimacombe et al., 1988), the knowledge of the topography of the 50S subunit is by far not as complete (Stöffler & Stöffler-Meilicke, 1986; Nowotny et al., 1986). Therefore, we initiated an extensive protein–protein cross-linking study with the aim of providing the missing topographical data, necessary for constructing a considerably more complete three-dimensional model of the protein topography of the 50S subunit. In this study, we have used four different cross-linking reagents of varying length and specificity, namely, diepoxybutane (DEB),[1] *o*-phenylenedimaleimide (oPDM), *p*-phenylenedimaleimide (pPDM), and dithiobis(succinimidyl propionate) (DSP). A total of 12 cross-links have been identified with DSP, 10 with oPDM, 9 with pPDM, and 4 with DEB as cross-linking reagent. As we have pointed out previously, two-dimensional diagonal gel electrophoresis (Kenny et al., 1979; Traut et al., 1980, 1986) can yield ambiguous results when used for the identification of cross-linked complexes (Walleczek et al., 1989). For an unambiguous identification, we have thus used specific immunoreaction in the analysis of the individual members of the cross-linked protein complexes (Stöffler et al., 1988). In addition, the cross-linking yield of each cross-linked protein complex has been determined in order to minimize the possibility of identifying cross-linked complexes that are derived from a small subpopulation of protein-depleted or functionally inactive ribosomal particles (Walleczek et al., 1989).

Together with the cross-linking reagents used by us in previous cross-linking studies (Walleczek et al., 1989; Redl et al., 1989), a total of six different cross-linking reagents have

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[1] Abbreviations: DEB, diepoxybutane; oPDM, *o*-phenylenedimaleimide; pPDM, *p*-phenylenedimaleimide; DSP, dithiobis(succinimidyl propionate); DMS, dimethyl suberimidate; SDS–PAG, sodium dodecyl sulfate–polyacrylamide gel; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IEM, immunoelectron microscopy; TP_{50}, total protein from 50S ribosomal subunits; TP_{50_X}, total protein from cross-linked 50S ribosomal subunits.

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