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Decreases in Tubulin and Actin Gene Expression prior to Morphological Differentiation of 3T3 Adipocytes

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Summary

The differentiation of 3T3-F442A preadipocytes is characterized by numerous enzymatic events and by a programmed change in cell morphology from a fibroblastic form to a nearly spherical shape. Accompanying this morphological change are large and specific decreases in biosynthetic rates for β and γ actin, vimentin and α and β tubulin, as detected by one- and two-dimensional gel electrophoresis. In cells undergoing adipose differentiation, actin synthesis decreases by 90%, while the decrease in tubulin synthesis is more than 95%. Translation in vitro of mRNA isolated from differentiating cultures indicates that the decreases in biosynthetic rate for cytoskeletal proteins result from altered levels of active mRNA. Using cloned cDNA probes for β actin and α tubulin, we show that changes in mRNA activity correspond to a specific loss of these sequences during cellular differentiation. Quantitatively, this loss of tubulin and actin mRNA sequences accounts virtually completely for the changes in protein biosynthetic rates. Examination of the synthesis and accumulation of cytoskeletal proteins and of their temporal relation to morphological conversion indicates that the biosynthetic changes are very early events in the differentiation, and suggests strongly that they participate in the development of the adipocyte morphology. The early occurrence of decreased cytoskeletal-protein synthesis also suggests that subsequent biosynthetic events specific to adipocyte differentiation may be influenced by alterations in the cytoskeleton.

Introduction

Certain sublines of 3T3 cells undergo adipose differentiation with high frequency when they reach a resting state under appropriate conditions (Green and Kehinde, 1974, 1976). During the development of these cells in culture, they acquire increased activity and increased amounts of numerous enzymes related to lipid synthesis (Mackall et al., 1976; Kuri-Harcuch and Green, 1977; Coleman et al., 1978; Grimaldi et al., 1978; Spiegelman and Green, 1980). They also develop increased hormone sensitivity (Rubin et al., 1977, 1978; Reed et al., 1977; Karlsson et al., 1979) and produce factors related to tissue vascularization (Castellot et al., 1980). Further evidence that these cells represent true adipocyte precursors was provided by the demonstration that they give rise to fat pads composed of histologically normal white fat cells when injected into animals (Green and Kehinde, 1979).

During the differentiation of 3T3 preadipocytes, cells undergo a marked change in morphology, from a flat fibroblastic form to a nearly spherical shape. This morphological change is a programmed event and not merely a passive consequence of lipid accumulation, since shape change occurs even if lipid accumulation is blocked (Kuri-Harcuch et al., 1978). Accompanying this morphological change are a decrease in the synthesis and accumulated level of actin (Sidhu, 1979; Spiegelman and Green, 1979, 1980) and a decrease in the actin cytoskeleton observed with immunofluorescence (Spiegelman and Green, 1980) or electron microscopy (Novikoff et al., 1980). The alteration in the biosynthesis of actin results from decreased levels of its mRNA and is regulated independently of many other differentiation-dependent biosynthetic events (Spiegelman and Green, 1980, 1981).

The programmed cell-shape changes and alterations in actin biosynthesis already described suggest that the differentiation of 3T3 adipocytes may be a useful system for understanding the control of cell morphology during animal cell development, especially with respect to the cytoskeleton. With this goal in mind, we have specifically examined changes in the three major cytoskeletal proteins: actin, tubulin and vimentin. Very early in the differentiation process, all of these proteins undergo large, specific decreases controlled at the mRNA level, and the resulting changes in cytoskeletal-protein biosynthesis appear to play a role in cellular morphological change and may influence subsequent biosynthetic events.

Results

Changes in Cytoskeletal-Protein Synthesis during Adipocyte Differentiation

The 3T3-F442A preadipocytes underwent differentiation in culture medium containing 10% fetal calf serum, the optimal condition for these cells. A phase-contrast micrograph of a differentiating culture 7 days after confluence is shown in Figure 1. At this time, a maximum number of cells (80%–90%) had acquired the nearly spherical morphology characteristic of the adipocytes and contained numerous small lipid droplets. A few cells in the field shown retained the flat fibroblastic morphology characteristic of preadipocytes. With increasing culture time, the adipocytes

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accumulated more lipid, but there was no substantial increase in the number of cells that underwent morphological change.

The biosynthesis of cytoskeletal proteins by the 3T3-F442A cells as they underwent differentiation was determined by pulse labeling with ^{35}S-methionine, fractionation of proteins on two-dimensional gels (O'Farrel, 1975) and fluorography. The mobility of tubulins, actins and vimentin was determined with hamster fibroblast cytoskeleton preparations enriched in these proteins, provided by F. Solomon. The relative positions of vimentin, β and γ actin and α and β tubulin were assigned according to our previous study of these fibroblast proteins (Ben-Ze'ev et al., 1979). As shown in Figure 2, top, as cells went from the preadipose to the adipose state there was a decrease in the relative rates of synthesis of β and γ actin, α and β tubulin and vimentin. This decrease in biosynthesis of cytoskeletal proteins was highly specific in that most of the other cellular proteins were not greatly affected by the differentiation. In addition, there were many new species made in the adipocyte, as described earlier (Sidhu, 1979; Spiegelman and Green, 1980).

We quantitated these changes in cytoskeletal-protein biosynthesis by densitometry of prefogged fluorographs of one-dimensional SDS-polyacrylamide gels. As shown in Table 1, during the transition from the growing preadipose state to the adipose state, the cultures underwent an 80% decrease in actin biosynthesis (12.6% of total synthesis to 2.7%) and a decrease in β-tubulin biosynthesis of more than 90% (2.3% to 0.2%). Alpha tubulin and vimentin were not resolved sufficiently to permit quantitative analysis on one-dimensional gels. We were concerned that some or all of the changes in cytoskeleton synthesis described in these experiments may have been a consequence of maintaining cells in the postconfluent state for several days, rather than a consequence of the adipose differentiation. As a control for this, cells were kept at confluence for an equivalent period of time in medium containing cat serum, which supports very little adipose differentiation (Kuri-Harcuch and Green, 1978). Under these conditions, there was no decrease in β-tubulin synthesis, but a reproducible 25% reduction in actin synthesis (Table 1). This is consistent with the known effects of the resting state on actin synthesis (Riddle et al., 1979). Identical results were observed when 3T3-C2, a 3T3 clone not susceptible to adipose differentiation, was kept at confluence under the conditions permissive for adipocyte differentiation (data not shown). Thus all of the decrease in synthesis of tubulin and most of the decrease in synthesis of actin can reasonably be ascribed to differentiation and not merely to the confluent state.

The changes in cytoskeletal-protein biosynthesis during differentiation are likely to be larger than the estimates made above, because no more than 80%–90% of the cells actually underwent adipose differentiation. To obtain a more accurate assessment, we isolated pure adipocytes by a new procedure that

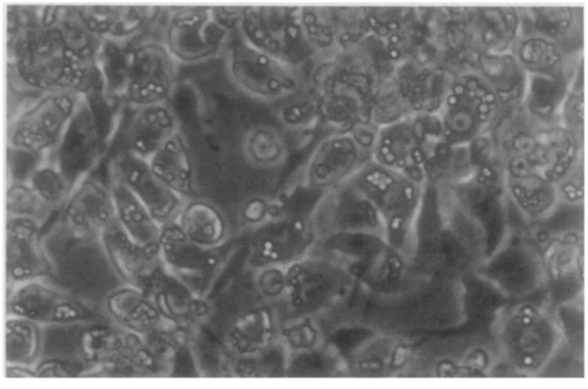

Figure 1. Phase-Contrast Micrograph of Differentiating 3T3-F442A Cells

Cells were maintained 7 days after confluence in medium containing 10% fetal calf serum. Micrographs were taken with 10× objective (0.22 numerical aperture) to get the nearly spherical adipocytes and flatter preadipocytes in the same plane of focus (total magnification, 100×).

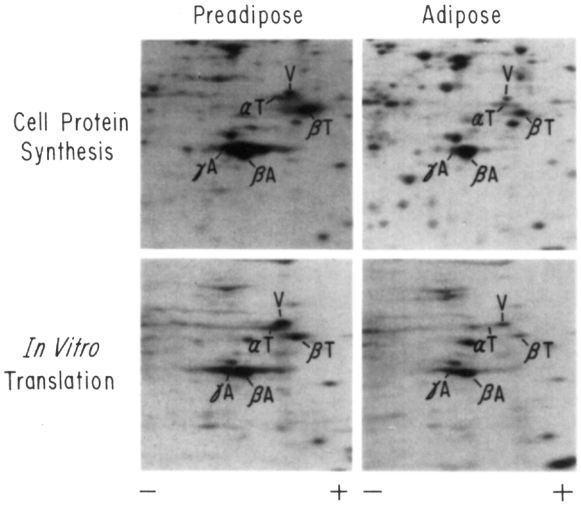

Figure 2. Biosynthesis of Cytoskeletal Proteins in Vivo and in Vitro

Preadipose cells were grown in 10% cat serum, while adipose cells were maintained 7 days after confluence in 10% fetal calf serum. Cell proteins were labeled for 90 min with 33 μCi/ml of ^{35}S-methionine in medium containing 1 mg/l of nonradioactive methionine. Extracts were prepared by sonication and were centrifuged for 5 min at 8000 × g to remove large debris. Poly(A)$^+$-selected mRNA was isolated as described in Experimental Procedures, and equal amounts (0.4 μg) from preadipose and adipose cells were translated in a 20 μl reticulocyte lysate preparation. Two-dimensional gels were run according to the method of O'Farrel (1975) and fluorographed. The isoelectric focusing gels contained ampholines in the ratio of 60% pH 3–10 to 40% pH 5–7. The mobility of the proteins was determined with hamster fibroblast standards. V: vimentin. αT: α tubulin. βT: β tubulin. βA: β actin. γA: γ actin. Note the decrease in the cytoskeletal proteins as cells went from preadipose to adipose state, while most other species remain constant or increase.

Table 1. Tubulin and Actin Synthesis during Adipocyte Differentiation

	Actin	β Tubulin
Growing preadipose cultures[a]	12.6	2.3
Confluent preadipose cultures[a]	9.4	2.3
Adipose cultures[b]	2.7	0.2
Isolated adipocytes[c]	1.2	0.1

Data are given as percentage of total cellular-protein biosynthesis. Cells were radioactively labeled with ^{35}S-L-methionine, and extracts were prepared as described in the legend to Figure 2. Protein biosynthesis rates were determined by densitometry of prefogged fluorographs of one-dimensional SDS-polyacrylamide gels.

[a] Preadipose cells (growing or 7 days after confluence) were cultured in 10% cat serum.

[b] Adipose cells were maintained 7 days after confluence in 10% fetal calf serum.

[c] Adipocytes were isolated from insulin-treated cultures as described in Experimental Procedures.

utilizes buffers containing bromobenzene to manipulate the density of fatty cells (see Experimental Procedures). When preadipocytes were compared with pure isolated adipocytes (Table 1), the decrease in actin synthesis was more than 90%, while β-tubulin synthesis was reduced by more than 95%. Because of the possibility of minor comigrating proteins, even these amounts must be considered minimum estimates. While it is possible that these changes in cytoskeletal-protein synthesis could have been affected somewhat by changes in extractability of newly synthesized subunits as a consequence of the differentiation, the close correlation between the pattern of synthesis in whole cells and that in vitro (see below) indicates that this was not a major factor.

Regulation of Cytoskeleton Synthesis

The means of regulation of cytoskeletal-protein synthesis were investigated first by isolation of poly(A)$^+$ mRNA from the preadipocytes and adipocytes and translation of equal amounts in vitro in a reticulocyte lysate preparation (Figure 2, bottom). It is apparent from this fluorograph that there was a specific decrease in vitro in the synthesis of vimentin, α and β tubulin and β and γ actin, indicating that the changes observed in rates of cellular synthesis of these proteins were a consequence of altered mRNA activity. This decrease in mRNA activity was shown to reflect a relative decrease in cytoplasmic mRNA concentration for β actin and α tubulin by Northern blotting with cloned cDNA sequences. Equivalent amounts of total poly(A)$^+$ mRNA from preadipocytes and adipocytes (see legend to Figure 3) were subjected to electrophoresis on an agarose gel containing methylmercury hydroxide. The RNA was then transferred to activated diazobenzyloxymethyl–cellulose paper and hybridized to ^{32}P-labeled plasmid DNA containing inserted cDNA sequences for rat α tubulin (Lemischka et al., 1981) or chicken β actin (Cleveland et al., 1980). The

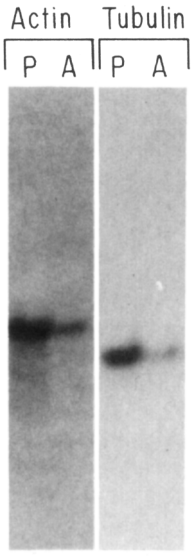

Figure 3. Determination of β- and γ-Actin and α-Tubulin mRNA Content by Northern Blots

Messenger RNAs from preadipose and adipose cells were isolated, subjected to electrophoresis on agarose gels containing methylmercury and blotted onto diazobenzyloxymethyl paper as described in Experimental Procedures. We used equal amounts of mRNA from both sources as determined by absorbance at 260 nm, hybridization to ^{3}H-poly(U), stimulation of in vitro protein synthesis and banding patterns on ethidium-bromide-stained gels. The RNA covalently linked to diazobenzyloxymethyl paper was hybridized to linearized plasmids containing β-actin or α-tubulin sequence, labeled with ^{32}P by nick translation (Maniatis et al., 1975). A single blot was used for both determinations, with the tubulin probe used first and subsequently eluted with 99% formamide at 65°C. (Lanes P) Preadipocyte mRNA. (Lanes A) Adipocyte mRNA.

β-actin clone hybridized to both β- and γ-actin mRNA sequences (Cleveland et al., 1980). As shown in Figure 3, a large decrease in autoradiographic signals corresponding to β- and γ-actin and α-tubulin mRNAs could be observed as cells progressed from the preadipose to adipose form. A single blot was used for both determinations, with the tubulin probe used first and eluted before hybridization to the actin clone. As noted earlier, the actin mRNA was somewhat larger than tubulin mRNA (2000 bases versus 1750 bases; Cleveland et al., 1980; Lemischka et al., 1981). Autoradiographs were quantitated by densitometry of prefogged films exposed with ^{32}P-labeled intensifying screens. In two separate experiments, the decrease in actin mRNA was between 75% and 85%, while the decrease in tubulin mRNA was 90%–92%. These densitometric data were confirmed by serial dilution of preadipocyte mRNA on a single blot until a signal visually equivalent to that from the adipocyte mRNA was obtained. Since similar amounts of mRNA were isolated from the preadipocytes and adipocytes (maintained in the absence of insulin; see Experimental Procedures), these changes reflect an absolute as well as a relative decrease in these messages. That tubulin and actin mRNAs were decreased specifically

rather than as a result of general mRNA degradation was indicated by the fact that the adipocyte mRNA supported in vitro translation of both high and low molecular weight proteins to a comparable extent as the preadipocyte mRNA. In addition, we have recently used cloned adipocyte cDNAs and Northern blots to demonstrate specific mRNA species that are unchanged during differentiation and several that are significantly increased (B. Spiegelman and H. Green, manuscript in preparation).

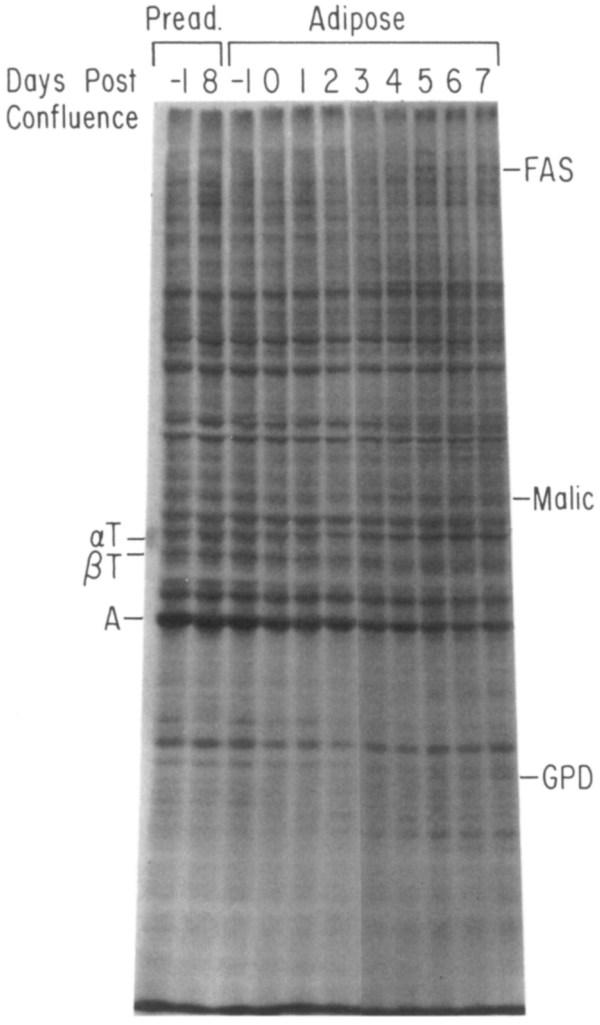

Figure 4. Protein Synthesis during Time Course of Differentiation

3T3-F442A cells (Adipose) were plated in medium containing 10% fetal calf serum and were pulse-labeled for 90 min with 33 μCi/ml of ^{35}S-methionine every day from 1 day before confluence (3 days after plating) to 7 days after confluence. Control preadipocytes (Pread.), maintained in medium containing 10% cat serum, were pulse-labeled at 1 day before confluence and 8 days after confluence. Soluble cell extracts were prepared and fractionated on a 9% SDS-polyacrylamide gel, fluorographed, dried and allowed to expose prefogged Kodak XR-2 film at −70°C (Laskey and Mills, 1975). αT: α tubulin. βT: β tubulin. A: actin. FAS: fatty acid synthetase. Malic: malic enzyme. GPD: glycerophosphate dehydrogenase. Vimentin was not resolved on this gel.

Temporal Relation of Tubulin and Actin Synthesis to Morphological Differentiation

The decreasing synthesis of cytoskeletal proteins that accompanies cellular differentiation is likely to be related to morphological change in the cells, but it is not clear whether altered protein biosynthesis influences cell shape or, conversely, whether cell shape affects biosynthetic rates of cytoskeletal proteins. Supporting the view that cell shape can directly or indirectly affect cytoskeleton synthesis is the finding that actin synthesis is induced when spherical, suspended fibroblasts flatten during replating (Benecke et al., 1978; Farmer et al., 1978). To investigate the temporal relation between adipocyte morphological change and cytoskeleton synthesis, we have followed both changes during differentiation. A fluorograph of an SDS-polyacrylamide gel on which actin and β tubulin were clearly resolved and α tubulin was incompletely resolved is shown in Figure 4. The percentage of cells showing morphological change in phase-contrast microscopy is shown in Figure 5 (see legend for method of scoring). It can be seen from these data that whereas morphological change began at 2–3 days after confluence and reached 50% of the cells at 4–5 days after confluence, decreases in α- and β-

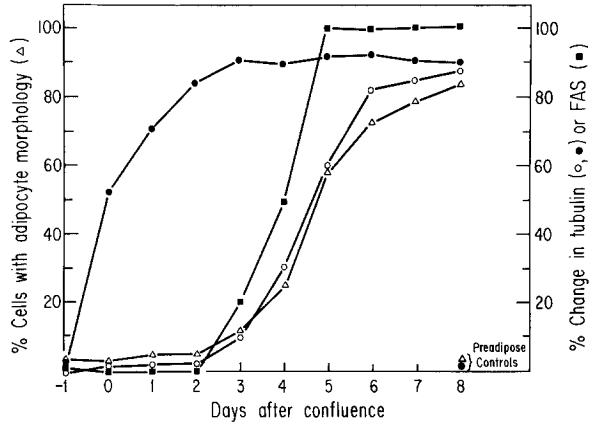

Figure 5. Time Course of Changes in Tubulin, Fatty Acid Synthetase and Cell Morphology during Adipocyte Differentiation

The rates of synthesis of β tubulin and fatty acid synthetase (FAS) were determined by densitometry of the fluorograph shown in Figure 4. The accumulated level of β tubulin was measured by densitometry of the Coomassie-blue-stained gels. The percentage of change during differentiation was calculated from the maximum rates of synthesis or of accumulation for these proteins. This was in preconfluent cells for tubulin and 8 days after confluence for FAS. The percentage of cells showing morphological change was followed by phase-contrast microscopy, with 25× objective and a grid eyepiece, on living cultures. Cells were scored as adipocytes if they had retracted their cell borders and were more spherical and refractile, while preadipocytes remained flat and had tight boundaries with neighboring cells. Cells of ambiguous morphology (always less than 5% of the total) were excluded from consideration. At least ten random fields and 2500 cells were counted for each data point, and this process was repeated. Data shown are the means of two duplicate estimates, which were always within 15% of each other. (○——○) β Tubulin protein. (●——●) β Tubulin synthesis. (■——■) Fatty acid synthetase synthesis. (△——△) Percentage of cells showing adipocyte morphology.

tubulin and actin synthesis preceded this. The decrease in tubulin synthesis began virtually as soon as the cells became confluent, and was nearly complete by 2 days after confluence. Actin synthesis also appeared to decrease significantly at confluence, but the loss appeared somewhat more gradual, approaching a minimum at 3 days after confluence. Preadipose controls, kept at confluence for 8 days, underwent a small decrease in actin and no decrease in tubulin (Figure 4, Pread.). In three separate experiments, the onset of the decreases in tubulin and actin synthesis preceded the onset of morphological change by at least 2 days. Because of the difficulty in resolving vimentin on SDS-polyacrylamide gels, the time course of its biosynthesis was not analyzed.

To facilitate direct comparisons of the time course of decreases in β-tubulin synthesis, in accumulated β-tubulin protein (as determined by Coomassie blue staining) and in percentage of cells undergoing morphological change, we plotted all three parameters together in Figure 5. The time course of change in tubulin protein approximately paralleled the appearance of cells showing adipocyte morphology. The decrease in tubulin synthesis preceded both of these by approximately 4 days. This time span, between the decrease in tubulin synthesis and loss of tubulin protein, was roughly consistent with the known half-life of this protein, 32–48 hr (Fine and Taylor, 1976; Spiegelman et al., 1977). Evident also from the time-course data in Figures 4 and 5 is that decreases in tubulin and actin biosynthesis preceded several other molecular events related to adipose differentiation, such as the increases in synthesis of fatty acid synthetase, malic enzyme and glycerophosphate dehydrogenase (Spiegelman and Green, 1980). It is apparent in Figure 5 that the increase in synthesis of fatty acid synthetase preceded morphological change by approximately 1 day but occurred about 3 days after the initial turn-down of tubulin (and actin) synthesis. To ensure that the later change in synthesis of fatty acid synthetase was not an artifact due to difficulty in detecting the turn-on of this protein on gels of total soluble cell extracts, we also measured the time course of fatty acid synthetase biosynthesis in an immunoprecipitation assay (Spiegelman and Green, 1981) and obtained similar results. Thus it appears that the altered biosynthesis of tubulin and actin during adipocyte differentiation not only precedes morphological change but also occurs before the biosynthesis of several important enzymes of the adipocyte.

Discussion

Cytoskeletal-Protein Synthesis during Differentiation

We have shown that there is a marked decrease in the synthesis of the major cytoskeletal proteins during differentiation of 3T3 adipocytes. These changes correlate with cellular differentiation rather than the confluent state per se, as preadipose cells maintained at confluence for prolonged periods did not show similar alterations. Quantitatively, the decrease in cytoskeletal-protein synthesis during differentiation was large. The synthesis of β and γ actin (combined) decreased by 90% and that of β tubulin decreased by 95% when pure preadipose cells were compared with isolated adipocytes. Although more difficult to analyze quantitatively, the changes in vimentin and α-tubulin synthesis certainly appeared to be of similar magnitude. The altered synthesis of cytoskeletal proteins was also quite specific in that most preadipocyte proteins did not undergo large decreases during differentiation. The down-regulation of tubulin and actin biosynthesis was controlled by changes in their respective mRNA levels, as shown by in vitro translation and hybridization to cloned cDNA probes. The specific decrease in these mRNAs could be a consequence of altered mRNA production or turnover rates.

Do these changes in protein biosynthetic rates and accumulated levels actually result in alterations in the assembled cytoskeletal components? The fact that a significant portion of cellular tubulin, vimentin and actin is usually found in assembled form (30%–100%; Pipeleers et al., 1977; Hiller and Weber, 1978; Hynes and Destree, 1978) suggests very strongly that a reduction in these cytoskeletal elements must occur during adipocyte differentiation. Direct evidence for quantitative and qualitative changes in the actin cytoskeleton has been provided by indirect immunofluorescence (Spiegelman and Green, 1980) and electron microscopy (Novikoff et al., 1980). Using immunofluorescence, we have also observed a sharp differentiation-dependent decrease in microtubules at or near the cell periphery (data not shown). However, high background levels in these thick cells has prevented a clear view of the entire microtubule array.

Cytoskeletal Proteins and Morphological Change

Clearly, the time-course data presented here rule out the possibility that the gross morphological changes during adipocyte differentiation caused the observed alterations in cytoskeletal-protein biosynthesis. Of course, the possibility exists that very subtle changes in morphology not observable in light microscopy may initiate the biosynthetic changes. However they are initiated, the important role these proteins play in the control of cell morphology (Brunser and Luft, 1970; Hsie and Puck, 1971; Porter et al., 1974; Mooseker and Tilney, 1975; Piatigorsky, 1975) make it very likely that alterations in the biosynthesis and accumulated levels of actins, tubulins and vimentin influence or cause the gross morphological change that accompanies adipocyte differentiation.

Control of morphology via quantitative regulation of cytoskeletal-protein biosynthesis would seem an obvious mechanism, but there has been surprisingly little

evidence that it is widely used by animal cells. This may be because these proteins are needed for other important cellular functions in addition to the control of morphology, such as mitosis and intracellular transport. Much more common mechanisms utilized for morphological change appear to be control of the extent and localization of intracellular microtubule and actin polymerization. This seems true for cyclic-AMP-induced shape changes in Chinese hamster ovary cells (Hsie and Puck, 1971; Patterson and Waldren, 1973; Porter et al., 1974), epithelial lens cell elongation (Piatigorsky, 1975), acrosome elongation in the sperm (Tilney et al., 1973) and neurite elongation in neuroblastoma (Hiller and Weber, 1978; Spiegelman et al., 1979). Quantitative modulation of tubulin synthesis is an important component of flagellar regeneration (Weeks and Collis, 1976; Weeks et al., 1977; Lefebvre et al., 1978), but our results in differentiating adipocytes may be the clearest example of large development-related changes in cytoskeletal-protein synthesis in nonmuscle animal cells, and should prove useful for further studies of the down-regulation of these proteins. Whether tubulin, actin and vimentin synthesis can be reactivated once cells differentiate (Green and Kehinde, 1974) remains to be determined.

Relation of Cytoskeletal Changes to Other Events in Differentiation

The decrease in tubulin and actin synthesis is tied very closely to the approach to confluence, when cells are under conditions permissive for the adipose differentiation. Thus these changes are among the earliest molecular events in this program. Is it possible that changes in cytoskeleton synthesis play an important role in the expression of later events in the differentiation, such as the synthesis of lipogenic enzymes? Precedent for morphological events playing a role in the control of development-related gene expression has been presented in cultured chondrocytes, where the blocking of shape changes with exogenous fibronectin leads to suppressed synthesis of specific differentiation-dependent proteins (Pennypacker et al., 1979; West et al., 1979). In the data presented here, gross morphological change did not significantly precede the synthesis of fatty acid synthetase, malic enzyme and glycerophosphate dehydrogenase. If the alterations in tubulin, actin or vimentin synthesis play a role in the control of the expression of other differentiation-linked genes, it is most probable that this occurs through cytoskeletal changes that take place before cells take on the characteristic adipocyte morphology. An alternative view is that the rates of synthesis of the cytoskeletal components merely respond more quickly to an event, related to cell confluence, that also independently activates other elements of the program of differentiation.

Very little is currently known about the detailed mechanisms that control cytoskeletal-protein synthesis. The only relevant data described to date show that tubulin biosynthesis in many cell types decreases in response to increasing levels of depolymerized tubulin subunits (Ben-Ze'ev et al., 1979; Cleveland et al., 1981). It has been proposed that this mechanism exists to keep tight control over unpolymerized tubulin levels, and hence prevent spontaneous (non-nucleated) assembly (Kirschner, 1980; Cleveland et al., 1981). It is possible that a partial depolymerization of microtubules and perhaps of other filaments initiates the turn-down of biosynthesis of these proteins in differentiating adipocytes, which eventually results in their depletion. This speculative notion is attractive because the cytoskeletal elements interact directly or indirectly with the cell surface (Mooseker and Tilney, 1975; Yahara and Edelman, 1975), and could respond quickly to the cell-to-cell contacts formed at confluence, when conditions permissive for the differentiation exist.

Experimental Procedures

Cell Culture Conditions

Stocks of 3T3-F442A cells (Green and Kehinde, 1976) were grown in the Dulbecco-Vogt modification of Eagle's medium supplemented with a mixture of 1% calf serum and 9% cat serum to minimize adipose conversion (Kuri-Harcuch and Green, 1978). For experiments, cells were trypsinized, and 60 mm Petri dishes were inoculated with them at a density of 5×10^4 cells, with the same medium supplemented with 10% fetal calf or 10% cat serum. Unless otherwise stated, no insulin was added. Confluence was attained approximately 4 days after inoculation.

Cell Extracts

The cell layer was washed twice with phosphate-buffered saline and detached with 1 mM EDTA in the same buffer. The cells were collected by centrifugation, resuspended in ice-cold 25 mM Tris–HCl containing 1 mM EDTA (pH 7.5) and sonicated for 10 sec at 30 W with the microtip of a Branson model 185 sonifier. The extract was clarified by centrifugation at 8000 × g for 5 min at 4°C.

Isolation of Pure Adipocytes with Bromobenzene

The possibility that bromobenzene could be used to isolate adipocytes was first suggested to us by D. Zilbersmit. Cells were maintained in medium containing 10% fetal calf serum and 5 μg/ml insulin for 10 days after confluence. Insulin greatly promoted lipid accumulation, but had no significant effect on relative cytoskeletal-protein synthesis. A trypsinized single-cell suspension from two 100 mm cultures was centrifuged twice at 2500 × g for 5 min to pellet the denser preadipocytes and immature adipocytes in 7 ml of phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride. The supernatant contained fatty cells that had a wide range of densities and were dispersed throughout the tube. We then prepared a solution of phosphate-buffered saline saturated with mono-bromobenzene by vigorously vortexing 30 μl bromobenzene with 100 ml phosphate-buffered saline and centrifuging out excess bromobenzene. Seven milliliters of this saturated solution was mixed gently with the cell suspension, and this was centrifuged at 2500 × g for 5 min. After this treatment, virtually all of the previously nonsedimentable cells were rendered sedimentable, and appeared to be 100% adipocytes with no visible contamination from preadipocytes. These pure adipocytes could be gently washed and recentrifuged in phosphate-buffered saline saturated with bromobenzene. Extracts were then prepared as usual. Typically, 10% of the total cell population was isolated with this procedure, but the fraction could be increased if cultures were maintained longer so a larger percentage of cells became very fatty.

Protein Electrophoresis and Fluorography

Electrophoresis in the presence of SDS was performed on polyacrylamide slab gels (Laemmli, 1970). Proteins were stained with Coomassie blue and destained with a solution containing 5% methanol and 7.5% acetic acid. Two-dimensional gel electrophoresis was performed according to the method of O'Farrel (1975). For fluorography, gels were impregnated with 2,5-diphenyloxazole according to the method of Bonner and Laskey (1974) and dried under vacuum. Kodak XR-2 film was prefogged (Laskey and Mills, 1975) and exposed to the gels at −70°C. Bands in fluorographs were quantitated with a Zeineh soft laser densitometer equipped with an automatic peak integrator.

Isolation of Poly(A)$^+$ mRNA

mRNA was isolated from thirty 100 mm preadipose cultures grown to confluence in medium containing cat serum (Kuri-Harcuch and Green, 1978), and from the same number of adipose cultures grown and maintained in fetal calf serum. Total cellular RNA was isolated as described by Spiegelman and Green (1980), except that the initial guanidine hydrochloride extract was brought to a concentration of 0.1 M in β-mercaptoethanol, and was then extracted two times with chloroform before the first ethanol precipitation. These changes were suggested by Paterson and Roberts (1981), and improved RNA yields two to three fold. Poly(A)$^+$ mRNA was isolated by oligo(dT) chromatography (Brownlee et al., 1973) and quantitated by hybridization to ^{3}H-poly(U). Yields of mRNA from preadipose and adipose cultures (maintained in the absence of insulin) were generally within 50% of each other.

Reticulocyte Translation System

Translation of mRNA was carried out with rabbit reticulocyte lysates treated with micrococcal nuclease (Pelham and Jackson, 1976). Final concentrations of K^+ and Mg^{2+} were 125 mM and 1.2 mM, respectively. mRNA (25 μg/ml) was heated at 65°C for 5 min and cooled on ice before addition. The reaction mixture contained ^{35}S-methionine at a concentration of 250 μCi/ml. Incubation was carried out for 90 min at 30°C.

Agarose Gel Electrophoresis and Filter Hybridization of RNA

Poly(A)$^+$ RNA (exact amounts determined by poly(U) hybridization) was subjected to electrophoresis on a 1% agarose gel containing 10 mM methylmercury hydroxide at 100 V for 4–5 hr. The gel was stained for 20 min with 1 μg/ml ethidium bromide, and was visualized under an ultraviolet light. The RNA was transferred and covalently bound to diazobenzyloxymethyl–cellulose paper according to the method of Alwine et al. (1977). After completion of transfer (18–24 hr) the paper was dried at room temperature, hybridized with ^{32}P-labeled cloned cDNA and washed as described by Wahl et al. (1979). The filters were then blotted dry with 3 MM paper, wrapped in Saran wrap and allowed to expose Kodak X-Omat x-ray film at −70°C with Dupont Lightning Plus intensifying screen.

Radiolabeling of cDNA

Cloned DNA was isolated as described previously (Lemischka et al., 1981) and labeled by nick translation as described by Maniatis et al. (1975).

Materials

^{35}S-methionine and α-^{32}P-dCTP were obtained from New England Nuclear. Ampholines were from LKB. Oligo(dT) (type 3) was obtained from Collaborative Research. Mono-bromobenzene (reagent grade) was from J. T. Baker.

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