Evidence for an Increase in Transcription of Specific mRNAs during Differentiation of 3T3-L1 Preadipocytes*

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David A. Bernlohr*, Mark A. Bolanowski§, Thomas J. Kelly, Jr.§, and M. Daniel Lane‡

From the Departments of *Biological Chemistry and §Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Differentiation of 3T3-L1 preadipocytes in culture is accompanied by alterations in the abundance of several mRNAs and by the appearance of many new adipocyte-specific mRNAs. To investigate the processes responsible for these alterations, the kinetics of accumulation of several specific mRNAs were compared with their respective rates of nuclear runoff transcription. The mRNAs for fructose-1,6-bisphosphate aldolase and an unidentified 4800-base mRNA increase in abundance only moderately (2–4-fold) during differentiation. Runoff transcription by nuclei isolated from 3T3-L1 cells during the course of differentiation revealed very little or no change in the rates of transcription of these mRNAs. Similar results were obtained for the β,α-actin and β-tubulin mRNAs where no difference in nuclear runoff transcription rates were observed even though a 2-fold decrease in the steady-state levels of these mRNAs accompanies differentiation. In contrast, the steady-state levels of mRNAs for 3T3-L1 P2 protein, an adipocyte homologue of myelin P2 protein, and an unidentified 5000-base mRNA increased dramatically (>20-fold) during adipose conversion. These large increases in abundance were correlated with marked rises (>10-fold) in nuclear runoff transcription rates for these mRNAs during differentiation of 3T3-L1 preadipocytes. No change in runoff transcription activity for these mRNAs was detected by nuclei from control nondifferentiating 3T3-C2 cells. These results strongly suggest that an increased rate of specific transcription is primarily responsible for the accumulation of these mRNAs during preadipocyte differentiation.

Both lipogenic and lipolytic hormones, consistent with the role of adipocytes in vivo in the storage and mobilization of triglyceride (3–5). Concomitant with differentiation, the activities of numerous enzymes of the glycolytic, lipogenic, and lipolytic pathways increase coordinately (6–9). The increases in many of these enzyme activities have been shown to result from increased levels of specific translatable mRNAs (10–12).

To investigate the mechanisms by which the levels of specific mRNAs are altered during the differentiation of 3T3-L1 cells, investigators in several laboratories (12–14) have isolated cDNA clones that correspond to differentially expressed mRNAs. We have isolated (14) a group of cDNA clones that correspond to differentially expressed mRNAs, one of which (pAL 422) has been identified as an adipocyte homologue of myelin P2 protein. In the present paper, we describe the relative changes in the steady-state levels and nuclear runoff transcription rates of several mRNAs during the course of differentiation of 3T3-L1 preadipocytes. The messages corresponding to clones pAL 122 (an unidentified cDNA clone) and 422 (the myelin P2 homologue), mRNAs which increase in abundance greater than 20-fold during differentiation, appear to arise via an increased rate of transcription. Two other mRNAs (pAL 421 and pRM 223), which increase in abundance only moderately, exhibit little or no change in apparent transcription rate. The mRNAs for the cytoskeletal proteins actin and tubulin were shown to decrease in abundance without changes in transcriptional activity, suggesting that the small change in mRNA abundance may result from an alteration in the stability of the message during differentiation.

EXPERIMENTAL PROCEDURES

Cell Culturing—Differentiating 3T3-L1 cells and control 3T3-C2 cells (a line which, when subjected to the differentiation protocol described below, does not undergo adipose conversion) were cultured as described previously (14). In the differentiation protocol, confluent cell monolayers (7 days post-plating, defined here as day 0) were incubated 48 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, methylisobutylxanthine (115 μg/ml), dexamethasone (380 ng/ml), and insulin (10 μg/ml). After 48 h (day 2), the cells were washed free of methylisobutylxanthine and dexamethasone and then were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and insulin (10 μg/ml) until completion of experiment, usually day 9. Cell number was determined with a Coulter Counter (Coulter Electronics Inc.), and adipocyte morphology was monitored by the appearance of cytoplasmic lipid droplets which closely mirrors triglyceride accumulation (6).

Nucleic Acid Isolation and in Vitro Translation—RNA was isolated as described by Bernlohr et al. (14). Quantitation was based upon $E_{260}^m = 250$ at 260 nm. In vitro translations were as described by Angus et al. (11). Total translation was quantitated by precipitation of [35S]methionine-labeled proteins with trichloroacetic acid and densitometric scanning of the autoradiographed gels using a Hoefer G-2.
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300 densitometer. DNA concentrations were estimated from absorbance at 260 and 230 nm (15).

Quantitation of RNA Levels—Hybridization of radiolabeled cDNA to total RNA, fractionated by agarose gel electrophoresis and transferred onto GeneScreen (New England Nuclear), was performed as previously described (14). Quantitation of RNA levels was achieved using a modification of the protocol for the total 3T3-L1 or 3T3-C2 RNA was denatured with glyoxal and diluted into 20× SSC (1× SSC is 150 mM NaCl, 0.015 M Na3 citrate). Total RNA in each sample was determined by measurement of the incorporation of [3P]UTP (3000 Ci/mmol, Amersham Corp.) to a specific radioactivity of 1×108 cpm/μg by the procedure of Feinberg and Vogelstein (17).

Hybridization probes were prepared from cDNA inserts isolated from low-melting agarose gels following restriction of plasmid DNAs. The recovered inserts were labeled with [3P]dATP (3000 Ci/mmol, Amersham Corp.) by the procedure of Feinberg and Vogelstein (17).

Protein and Enzyme Determination—Total protein was determined using the modified Lowry procedure of Markwell et al. (18). Pyruvate carboxylase activity was measured as described by Mackall and Lane (6).

Isolation of Nuclei and Runoff Transcription Assays—Nuclei were isolated, following removal of culture medium, by submerging the cell monolayers two times into ice-cold phosphate-buffered saline containing 2 mM EDTA and 0.5 mg/ml digitonin (20). This procedure rapidly chilled the cells and permeabilized the plasma membrane, thereby depleting intracellular nucleotides. The monolayers were scraped at 4°C into 2.0 ml of a buffer containing 20 mM Tris-HCl, pH 7.4, at 4°C, 10 mM NaCl, 3 mM MgCl2, 0.1% Nonidet P-40. The extract was homogenized by 20 strokes of a tight fitting pestle followed by centrifugation for 5 min at 1500 rpm. The pelleted nuclei were washed once with the extraction buffer lacking Nonidet P-40, re centrifuged as above, and frozen in 100-μl aliquots in a buffer containing 40% glycerol, 50 mM Tris-HCl, pH 8.3, at 22°C, 5 mM MgCl2, and 0.1 mM EDTA and stored at −70°C until used.

Nuclear transcription activity (referred to as runoff transcription) was determined by measurement of the incorporation of [3P]UTP (3000 Ci/mmol, Amersham Corp.) into RNA transcripts elongated in vitro using the protocol detailed by McKnight and Palmiter (19) with modifications of Groudine et al. (20). Hybridizations with isolated nuclei were conducted in 2× SSC according to the procedures of LeMeur et al. (16). Data are reported as moles of cDNA hybridized per microgram of RNA by direct measurement of radioactivity hybridized at several RNA concentrations. Hybridization data used were shown to be in the linear range of a plot of log RNA versus radioactivity hybridized.

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RESULTS

Characterization of 3T3-L1 Differentiation—Differentiation of 3T3-L1 preadipocytes in culture is associated with the acquisition of numerous morphological and biochemical characteristics of adipocytes (1, 2). To provide specific markers to which the time course of adipose conversion can be related, several events in the differentiation program were assessed. Immediately following addition of dexamethasone, methylisobutyxanthine, and insulin, 3T3-L1 but not 3T3-C2 cells divided, increasing cell number by 2-3 fold. Cell proliferation was complete within 48 h, at which time dexamethasone and methylisobutyxanthine were removed. Only after hormonal stimulation do the 3T3-L1 cells begin to express the adipocyte phenotype (Fig. 1A). A requirement for cell division has been postulated (21) as a "critical mitosis" necessary for the reorganization of chromatin structure as a prerequisite for differentiation-dependent gene expression.

As shown in Fig. 1B, total transcriptional activity, as measured by nuclear runoff transcription, begins to increase immediately after hormonal stimulation, reaching maximal levels by day 5 and declining after day 7. Hybridization of [3P]-labeled runoff RNA to 18 S and 28 S ribosomal RNA cDNA clones revealed that there is apparently no change in the rate of synthesis of ribosomal RNA during differentiation. Thus, changes in total transcriptional activity probably reflect changes in mRNA synthesis. Indeed, the results in Fig. 1B demonstrate that changes in total translatable RNA parallel the changes in transcriptional activity. In addition, the profile of translatable mRNA during differentiation is similar to the profile of total protein synthesis as measured by Student et al. (22). An increase in pyruvate carboxylase activity and lipid accumulation closely follow the rise in mRNA levels; the pyruvate carboxylase activity in differentiating 3T3-L1 cells is indicated on all subsequent figures as a reference marker for the progress of differentiation. The changes in transcriptional, translatable mRNA, and lipid accumulation occur solely...
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in differentiating 3T3-L1 cells and not in control 3T3-C2 cells, indicating that these events are relevant to adipose conversion and not the differentiation protocol per se.

Transcriptional Control of Specific mRNA Expression—The results described above profile the total runoff transcriptional activity and mRNA abundance during 3T3-L1 preadipocyte differentiation and represent the summation of all changes, both increases and decreases, in RNA expression. To determine the abundance of specific mRNAs and transcriptional activity of their respective genes, several cDNA clones were employed as specific hybridization probes. RNA was isolated from both 3T3-L1 and control 3T3-C2 cells at various times following the induction of differentiation. The abundance of specific mRNAs were determined in each sample by the quantitative dot hybridization procedure (described under “Experimental Procedures”).

Fig. 2C describes the accumulation of mRNA in 3T3-L1 and control 3T3-C2 cells for pAL 122, a clone which corresponds to a differentially expressed 5000-base mRNA (14). Very little pAL 122 mRNA was detected in undifferentiated 3T3-L1 cells and in control 3T3-C2 cells. However, by day 5, the abundance of pAL 122 mRNA in differentiating 3T3-L1 cells increased at least 30-fold and then decreased markedly over the next 4 days. No significant change in the level of pAL 122 mRNA occurred in 3T3-C2 cells subjected to the differentiation protocol.

To determine whether the increase in steady-state level of pAL 122 mRNA was attributable to an increase in transcriptional activity, nuclear runoff experiments were performed using nuclei isolated from 3T3-L1 cells at several stages of the differentiation process as well as nuclei from nondifferentiating 3T3-C2 cells in parallel cultures. Fig. 2D demonstrates that, during pAL 122 mRNA accumulation in differentiated 3T3-L1 cells, an increase in transcriptional runoff activity occurred. By day 7, the transcriptional runoff activity was 12-fold greater than in control 3T3-C2 cells. Interestingly, the nuclear runoff activity decreased dramatically by day 9. This decrease was not due to loss of cell viability, for the differentiated adipocytes remain metabolically active for many days beyond day 9. The kinetics of change in transcriptional activity differed somewhat from the kinetics of accumulation of hybridizable mRNA. The maximal levels of pAL 122 mRNA accumulated 24–48 h prior to the maximal level of pAL 122 runoff transcription activity. One possible explanation for this apparent incongruence between transcription rates and mRNA levels is that nuclear runoff transcription may not always be an accurate representation of in vivo transcription. However, in those cases where it has been measured (19, 20, 23), nuclear runoff transcription rates compared favorably to the in vivo rate of radiolabeled uridine incorporation. Alternatively, the stability of the pAL 122

Fig. 2. Kinetics of steady-state mRNA level and corresponding runoff transcription activity in differentiating 3T3-L1 and control 3T3-C2 cells for pAL 15 (A and B), pAL 122 (C and D), pAL 422, the myelin P2 homologue (E and F), and pAL 421 (G and H). In A–H, closed symbols represent data from 3T3-L1 cells, while open symbols represent data from control 3T3-C2 cells treated similarly. The activity of pyruvate carboxylase in 3T3-L1 cells (×) is indicated in all panels as a reference activity for differentiation.
mRNA may change from day 3 to 9, resulting in the observed difference between mRNA level and nuclear runoff transcription rates. Nonetheless, it is evident that the expression of pAL 122 mRNA during differentiation results primarily from an increased rate of transcription.

To ensure that the change in transcriptional runoff activity measured for pAL 122 mRNA does not merely reflect a general increase in transcription by 3T3-L1 cells, similar measurements were made with pAL 15, a cDNA clone for which the corresponding mRNA does not change in abundance during differentiation (14). No change in mRNA level or transcription runoff activity was detected in differentiating 3T3-L1 or in control 3T3-C2 cells subjected to the differentiation protocol (Fig. 2, A and B). These results support the conclusion that increased transcription of the pAL 122 gene is differentiation-specific and not a general effect of the differentiation protocol. Also, the pAL 15 mRNA level and transcriptional runoff activity from the pAL 15 gene did not decrease from day 5 to 9, indicating that the changes measured for pAL 122 accurately reflect the transcriptional activity and mRNA level.

Clone pAL 422 was shown previously (14) to correspond to a 3T3-L1 adipocyte homologue of myelin P2 protein. As shown in Fig. 2E, pAL 422 mRNA was expressed at an extremely low level in 3T3-L1 preadipocytes and in 3T3-C2 controls. Following the induction of differentiation, pAL 422 mRNA, like pAL 122 mRNA, increased in abundance about 20-fold by day 4 and then declined by day 9. Immunoblot analysis of total cellular extracts with anti-bovine myelin P2 antisera indicates that adipocyte P2 protein first appears on day 3 and then increases in abundance similar to pyruvate carboxylase.1 No anti-P2 cross-reactive material was detected in either undifferentiated 3T3-L1 cells or in control 3T3-C2 cells (results not shown).

Measurements of the transcriptional runoff activity of the gene corresponding to pAL 422 cDNA were conducted with the same RNA preparations used for the assay of pAL 122 gene transcripts. The runoff transcription activity from the pAL 422 gene on day 7 was at least 10-fold greater in nuclei from undifferentiated 3T3-L1 adipocytes than in nuclei from undifferentiated 3T3-L1 cells or in control 3T3-C2 cells subjected to the differentiation protocol (Fig. 2F). The relative rate of pAL 422 runoff transcription was very similar to that for the pAL 122 gene. These rates were, however, 10-fold lower than that for the constitutively expressed pAL 15 gene. These results and those with pAL 122 strongly suggest that transcriptional regulation accounts for the increased abundance of these mRNAs.

The mRNAs corresponding to clones pAL 421 (a cDNA clone encoding a differentially expressed unidentified mRNA from 3T3-L1 cells) and pRM 223 (a rabbit muscle fructose-1,6-diphosphate aldolase cDNA clone) represent messages that are expressed in undifferentiated 3T3-L1 and control 3T3-C2 cells whose abundance increases moderately during adipose conversion of 3T3-L1 cells. Fig. 2G demonstrates that the abundance of the mRNA corresponding to pAL 421 increases 4-fold during differentiation. The mRNA for aldolase also increased 2-fold in abundance during adipose conversion (results not shown). The small increase in aldolase mRNA level is consistent with the data of Mackall and Lane (6) who observed a 4-fold increase in aldolase activity during differentiation. In other experiments (results not shown), pRM 223 cDNA was shown to hybridize to a single 16 S RNA species from both undifferentiated and differentiated 3T3-L1 cells. This 16 S RNA is the same size as that described by Tolan et al. (24) for the rabbit muscle aldolase message.

Nuclear runoff transcription experiments revealed little or no increase in transcription runoff activity from the aldolase template (results not shown), while a 2-fold increase from the pAL 421 template was detected (Fig. 2H). The small increase in pAL 421 transcription and the failure to detect an increase in pRM 223 transcription may be due to our inability to measure small changes in transcriptional activity; alternatively, it is possible that a process other than transcription, i.e. an increase in mRNA stability, may contribute to the changes in steady-state mRNA level.

The dramatic morphological changes which occur during preadipocyte differentiation are accompanied by a decrease in the levels of the cytoskeletal proteins actin and tubulin (25). Utilizing pA1 (a cDNA clone encoding β,γ-actin sequences) and pT1 (a cDNA clone encoding β-tubulin sequences, Cleveland et al. (26)), we observed (Fig. 3, A and C) that these mRNAs decrease in abundance immediately after hormonal induction, concurrent with cell division (days 1 and 2), but prior to expression of the adipocyte phenotype. However, there was not a corresponding decrease in the rates of runoff transcription from the actin and tubulin genes (Fig. 3, B and D). These results suggest that the rate of degradation of these mRNAs may increase during differentiation.

**DISCUSSION**

The present paper examines the basis for changes in the levels of specific mRNAs during differentiation of 3T3-L1 preadipocytes into adipocytes. To address this issue, representatives of various classes of mRNA were studied which differ in the magnitude or direction of their differentiation-induced changes in abundance. These include: 1) mRNAs that are expressed nearly exclusively by the differentiated 3T3-L1 adipocyte (mRNAs encoded by pAL 122 and pAL 422), 2) mRNAs that are expressed in 3T3-L1 preadipocyte and 3T3-C2 control cells and which increase in abundance moderately upon differentiation (represented by the mRNAs encoded by pAL 421 and pRM 223), and 3) mRNAs that are expressed in preadipocyte and control cells but which decrease in abundance during differentiation of 3T3-L1 cells (represented by the mRNAs for β,γ-actin and β-tubulin encoded by pA1 and pT1, respectively).

For those mRNAs present exclusively in differentiated cells, our results indicate that increased transcription from the pAL 122 and pAL 422 genes, as measured by the nuclear runoff transcription assays, is primarily, if not solely, the basis for the observed increase in steady-state mRNA level. The increase in runoff transcription activity from the pAL 122 and pAL 422 genes occurred following hormonal induction of differentiation, was maximal by days 5–7 (Fig. 2, D and F), and, unexpectedly, decreased by day 9, paralleling a decrease in steady-state mRNA level. The nature of the biphasic increase in transcription during days 2–5 is undetermined; however, it was reproducibly detected. The basis for the decrease in runoff transcription rate from days 7–9 is also not entirely clear. The decrease may represent a common feature of all actively transcribed genes during this period for total transcriptional activity decreases coincidently (Fig. 1B). Alternatively, the decrease in runoff transcription may represent a specific regulated event, the purpose of which is to decrease the levels of certain mRNAs, e.g. pAL 122 and pAL 422 mRNA.

The possibility was also considered that a selective gene amplification may account for the increase in transcription from the pAL 122 and pAL 422 genes. Hybridization analysis

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1 D. Bernlohr and M. D. Lane, unpublished results.
of restricted genomic DNA from differentiating 3T3-L1 and similarly treated 3T3-C2 cells with \( ^{32} \)P-labeled pAL 122 and pAL 422 cDNAs indicates that the genes corresponding to these probes are probably present as single copies in the genome and that the observed increases in transcriptional activity are not the result of gene amplification or gross DNA rearrangements (results not shown). These findings are consistent with our view that an increased rate of transcription from these genes is the principle mechanism by which the differentiation-induced increase in steady-state mRNA level occurs.

The results concerning the class of mRNAs that increase only moderately during differentiation (pAL 421 and pRM 223) indicate that little, if any, increase in nuclear runoff transcription is correlated with the increase in steady-state mRNA level. However, the increases in mRNA level are relatively small, and the correspondingly small increase in nuclear runoff transcription activity may be below our limit of detection in the assay. Alternatively, the lack of any measurable increase in runoff transcription may indicate that the increase in steady-state mRNA level of pAL 422 and pRM 223 arises via a selective decrease in the rate of mRNA degradation. Similar results were obtained when the messages corresponding to the cytoskeletal proteins, tubulin and actin, were examined. Spiegelman and Farmer (25) reported that a 5-10-fold decrease in steady-state level for actin and tubulin genes remains a prerequisite for further experiments to investigate the biochemical mechanisms regulating specific gene transcription during differentiation.

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