Developmentally Regulated mRNAs in 3T3-Adipocytes: Analysis of Transcriptional Control

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ABSTRACT We have investigated the regulation of mRNA synthesis during 3T3-adipocyte differentiation by measuring the transcription of specific genes in isolated preadipocyte and adipocyte nuclei. Transcription was assayed by hybridization of newly synthesized RNA to cDNA clones coding for glycerophosphate dehydrogenase (GPD), the induced protein of 13K which is shown here to be related to myelin protein P-2, the induced protein of 28K, actin, and two RNAs that are not developmentally regulated. Transcription of GPD and 13K was observed in adipocyte but not preadipocyte nuclei. Actin was transcribed in both types of nuclei but at a lower level in adipocytes. For most of the RNAs examined, there was a consistent relationship between amounts of nuclear transcription and the abundance of the corresponding cytoplasmic mRNA in adipocytes. However, 13K and 28K mRNAs are 10–100 times more abundant than would be predicted by their nuclear transcription alone. Preliminary mRNA turnover experiments in which 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was used to inhibit mRNA synthesis suggest that these mRNAs are much more stable in the adipocyte cytoplasm than the other mRNAs examined. These results indicate that the transcription of specific genes is increased during adipocyte differentiation and suggest that other levels of control, particularly mRNA stability, may contribute to the relative abundance of certain developmentally-regulated mRNAs in adipocytes.

The differentiation of preadipocytes derived from mouse 3T3 cells into adipocytes involves many changes in lipogenic enzyme activities, acquisition of increased hormone sensitivities (reviewed in reference 1), and the production of signals related to tissue angiogenesis (2). These changes in cellular metabolism are accompanied by the synthesis of many new proteins that, together, represent a large fraction of the total adipocyte protein (3, 4). Furthermore, alterations in protein synthesis were shown to correspond to changes in the levels of particular translatable mRNAs (4, 5). More recently, very large alterations in concentrations of several specific mRNAs were demonstrated with cDNA probes isolated from a 3T3-adipocyte clone bank (6). These probes include sequences encoding glycerophosphate dehydrogenase, a key lipogenic enzyme, the abundant previously unidentified proteins of 13K and 28K, and actin.

In the present study, we have examined the mechanisms that regulate the extensive differentiation-dependent changes in mRNA levels by measuring the transcription of specific genes in isolated preadipocyte and adipocyte nuclei. In doing so, we have attempted to determine (*a*) whether transcription of developmentally-related genes is increased during adipocyte differentiation and (*b*) whether the levels of transcription correlate with the amounts of these RNAs in adipocytes. The results presented here indicate that transcription of GPD and 13K genes is enhanced during differentiation and suggest that additional levels of control may contribute to the accumulation of 13K and 28K mRNAs.

MATERIALS AND METHODS

Materials: Plasmids containing cDNA inserts of 400, 940, 300, and 1,200 base pairs that are complementary to 13K, 28K, GPD, and actin RNAs, respectively, were described previously (6). Two other plasmids, pC1 and pC2, were selected from a cDNA clone bank as controls for this study because the level of their complementary RNAs changed little upon differentiation. pC1 and pC2 contain inserts of 2.2 and 1.1 kilobases, respectively. DNase I and RNase A were purchased from Worthington Enzymes, Freehold, NJ. RNase T1, proteinase K and all chemicals and enzymes for molecular cloning and DNA sequencing were from Bethesda Research Laboratories (Bethesda, MD).

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Polynucleotide kinase was from New England Biolabs, Beverly, MA. Vanadium ribonucleoside complexes were prepared as described by Berger and Birkenmeier (7). 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was purchased from Sigma Chemical Co., St. Louis, MO.

Cell Culture Conditions: 3T3-F442A cells (8) were grown in the Dulbecco-Vogt modification of Eagle's medium supplemented with a mixture of 1% calf serum and 9% cat serum to maintain cells as preadipocytes (9) or with 10% fetal calf serum and 5 μg/ml of insulin to promote the formation of adipocytes. Cells were collected 6–7 d after confluence, at which time the number of cells converted to adipocytes had reached a maximum (~80–90%). Preadipocyte cultures contained 1–2% adipocytes. Cells were fed 18–24 h before harvesting.

Isolation of Nuclei: Nuclei were isolated from twenty 100-mm cultures of preadipocytes and adipocytes by lysis of cells in 20 vol of 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 0.3 M sucrose, and 0.4% NP-40 (lysis buffer). Nuclei were collected by centrifugation at 1,000 *g* for 5 min and were washed twice with lysis buffer. The nuclear pellet was suspended in 20 vol of lysis buffer, dounced three times using a B pestle, and layered over a 1-ml cushion of lysis buffer containing 0.88 M sucrose. Nuclei were collected by centrifugation at 3,000 *g* for 10 min. After washing once, nuclei were suspended in a solution of 26% glycerol, 33 mM Tris-HCl, pH 7.5, 3.3 mM $MgCl_2$, and 60 μM EDTA at DNA concentrations of 0.4 to 2.0 mg/ml. The isolation of nuclei was carried out at 4°C. DNA concentrations were measured by the diphenylamine reaction (10).

In Vitro Transcription: Transcription in nuclei and the isolation of RNA were performed by modifications of the procedures of McKnight and Palmiter (11). Nuclei from 10 100-mm cultures of adipocytes or preadipocytes were incubated at 30°C in 200 μl reaction mixtures containing 20% glycerol, 25 mM Tris-HCl, pH 7.5, 5.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 70 mM KCl, 50 μM EDTA, 2.5 mM dithiothreitol, 5 mM GTP, 5 mM CTP, 10 mM ATP, and 1 mCi of [α-^{32}P]UTP (600 Ci/mmol). After 30 min, 50 μg of tRNA was added, and the reactions were terminated by treatment with DNase I at 20 μg/ml for 5 min. Proteins in the mixtures were then digested for 30 min at 37°C with 100 μg/ml proteinase K in buffer containing 0.1 M Tris-HCl, pH 7.5, 12 mM EDTA, 0.15 M NaCl, and 1.0% SDS. The reaction mixtures were diluted to 1.0 ml with 10 mM Tris-HCl, pH 7.5, passed through a 23 gauge needle, extracted with phenol/chloroform, and precipitated at −20°C with 0.3 M sodium acetate, and 2.5 vol of ethanol. The precipitate was dissolved in 50 mM Tris-HCl, pH 7.5, treated for 40 min at 30°C with 20 μg/ml DNase I in the presence of 10 mM vanadium ribonucleoside complexes and 10 mM $MgCl_2$, digested with proteinase K as described above and then extracted with phenol, phenol/chloroform, and chloroform. RNA was collected by centrifugation after precipitation at 4°C with 5% trichloroacetic acid containing 15 mM sodium pyrophosphate. The pellets were dissolved in 300–400 μl of 0.1 M sodium acetate and precipitated at −20°C with 2.5 vol of ethanol. The pellets were dissolved, reprecipitated with ethanol, and suspended in 100 μl of H_2O.

Hybridizations of Nuclear Transcripts: 5 μg of plasmid DNAs were immobilized on nitrocellulose filters using a Schleicher and Schuell filtration manifold (Keene, NH) as recommended by the manufacturer. The filters were dried at room temperature and baked for 2 h at 80°C under vacuum. The blots were prehybridized at 37°C for 8 to 14 h in 5 ml of a solution of 50% deionized formamide, 50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM EDTA, 20 mM sodium phosphate, pH 6.5, 0.1% SDS, 0.04% (wt/vol) each of BSA, polyvinylpyrrolidone, and Ficoll 400, 100 μg/ml denatured salmon testis DNA, and 40 μg/ml polyadenylic acid. Hybridizations were carried out for 3 d at 37°C in 0.5–0.6 ml of the same solution containing ^{32}P-labeled nuclear RNA from adipocytes or preadipocytes and an equal amount of unlabeled nuclear RNA from the opposite form of the cells. The addition of unlabeled RNA ensured that the hybridization conditions in both reactions were identical, thus allowing direct comparison of the signals for a given RNA from adipocyte and preadipocyte nuclei. After hybridization, filters were digested with RNase as described by McKnight and Palmiter (11) and were exposed to preflashed Kodak X-Omat film at −70°C with a Dupont Lightening Plus intensifying screen. The amount of radioactivtiy in the spots was determined by liquid scintillation counting of the excised dots. Values for signals that were too low to accurately count were determined by scanning the dots with a Helena Laboratories quick scan densitometer (Beaumont, TX) and correlating their relative intensities with those of dots that had been counted. Hybridizations with different amounts of ^{32}P-labeled RNA demonstrated that the hybridized radioactivity was proportional to input RNA.

The extent to which RNA hybridizes to each of the cDNAs was measured using ^{32}P-labeled RNA probes complementary to the cDNAs. The probes were prepared by hydrolyzing 1 μg of poly A+ RNA to 200 nucleotide pieces in 100 mM Tris-HCl, pH 9.5, at 90°C for 5 min, and then end-labeling the RNA using [γ-^{32}P]ATP (3,000 Ci/mmol) and polynucleotide kinase (12). The RNA (specific activity of 4×10^6 cpm/μg) was hybridized to 5 μg of immobilized DNAs for 30 h at 48°C in the same hybridization solution as that employed for nuclear transcripts except that tRNA was substituted for the salmon testis DNA. After washing the filters, the RNAs were eluted by heating the excised dots in 100 μl of 1 mM EDTA, pH 7.4, at 100°C for 2 min. Known amounts of the RNA probes were combined and rehybridized to immobilized cDNAs in the presence of carrier nuclear RNA from adipocytes and preadipocytes under the exact conditions used for nuclear transcription experiments. The extent of hybridization to the cDNAs ranged from 7–17% before treatment with RNase, except for 28K which was 37%.

Isolation of Cytoplasmic RNA and RNA Blotting: Cytoplasmic RNA for dot blotting was isolated from the supernatant of the nuclear isolation (see above). Solid guanidine HCl was added to the cytoplasmic extracts to give a final concentration of 6 M and the RNA was extracted as described previously (4, 13). The cytoplasmic RNA was quantitated by absorbance at 260 nm and hybridization to ^{3}H-poly[U]. The purified RNAs were denatured by heating at 60°C with formaldehyde, diluted, and immobilized on nitrocellulose according to the procedure of White and Bancroft (14). Cytoplasmic RNA was isolated from cultures treated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole by the NP-40 lysis procedure (15), electrophoresed on 1.5% agarose gels containing formaldehyde, and transferred to nitrocellulose as described by Maniatis et al. (15). The nitrocellulose filters were baked at 80°C under vacuum for 2 h, prehybridized, hybridized to nick-translated probes ($1.2–1.7 \times 10^8$ cpm/μg DNA), and washed using the conditions suggested by Maniatis et al. (15). Autoradiography and quantitation of hybridization signals by densitometry and liquid scintillation counting were carried out as described above.

DNA Sequence Determination and Analysis: The 13K plasmid used for transcription studies, pAd-5 (6), and an overlapping clone isolated from our adipocyte cDNA library were used to determine the sequence of the 13K cDNA. Inserts were excised from the plasmids by *PstI* digestion and isolated by PAGE. The purified fragments were redigested with *EcoRI*, *SalI* or *AhaIII* and subcloned into the M13 sequencing vectors developed by Messing (16). Sequencing was done according to the method of Sanger et al. (17) on 80-cm gels. Both strands of the amino acid coding portion of the cDNAs were determined. Analysis of the DNA sequence data was done on a Digital 2060 computer with the Intelligenetics program or the Lipman-Wilbur programs (18).

RESULTS

Transcription in Isolated Nuclei

For our studies of transcription in isolated nuclei, preadipocytes and adipocytes were collected 6 to 7 d after confluence. This time point was selected because most morphological and biochemical changes that accompany adipocyte differentiation have reached a maximum by then (4). The time course of UMP incorporation into transcripts synthesized by endogenous RNA polymerases in preadipocyte and adipocyte nuclei is shown in Fig. 1. Adipocyte and preadipocyte nuclei show similar patterns of UMP incorporation; incorporation increases for 30–40 min with half of the total incorporation occurring in the first 7 min. The initial rates of transcription for preadipocyte and adipocyte nuclei are ~0.037 and 0.018 pmol/min/μg of DNA, respectively. Adipocyte nuclei appear to be less transcriptionally active than preadipocyte nuclei since the activity of adipocyte nuclei per microgram of DNA was consistently 0.5 to 0.65 times lower than that of preadipocyte nuclei. α-Amanitin at 2 μg/ml in the transcription reactions inhibits incorporation by 50% in both preadipocyte and adipocyte nuclei (data not shown) indicating that RNA polymerase II transcribes the same percentage of the total transcripts in both types of nuclei. This result is important because the hybridizations used to analyze specific transcription were performed with equal amounts of incorporated radioactivity from preadipocyte and adipocyte nuclei. In addition, the ^{32}P-labeled transcripts from both preadipocyte and adipocyte nuclei ranged in length from 80 to 1,000 bases and averaged ~300 bases (Fig. 2).

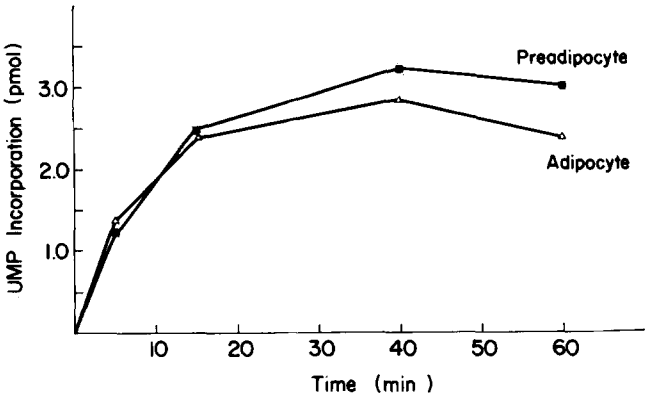

FIGURE 1 Time-course of transcription in isolated adipocyte and preadipocyte nuclei. Transcription reactions were carried out using the conditions described in Materials and Methods except that 10 μl of nuclei was used in a reaction volume of 20 μl and the UTP concentration was 5 μM (8 Ci/mmol). Reactions were terminated at the indicated times by adding 0.5 ml of 5% trichloroacetic acid. After 20 min at 4°C, precipitates were collected by filtration through Millipore HAWP 0.45-μm filters and radioactivity was measured by liquid scintillation counting. The preparations of preadipocyte and adipocyte nuclei contained 0.65 and 1.5 mg/ml of DNA, respectively.

Specific Gene Transcription and RNA Abundance

To assay the transcription of specific genes, ^{32}P-labeled transcripts from preadipocyte and adipocyte nuclei were hybridized to immobilized cDNA probes in identical reaction mixtures (see Materials and Methods). Autoradiograms of the resulting hybridization of nuclear transcripts are shown in Fig. 3. Transcription of GPD and 13K was increased in adipocyte nuclei while no signals for these transcripts were detected in preadipocyte nuclei. The corresponding cytoplasmic RNA dot blots show a large increase in RNA concentration for both of these sequences, although 13K RNA is clearly far more abundant than GPD RNA (Fig. 4). Surprisingly, 28K was not transcribed at detectable levels in either adipocyte or preadipocyte nuclei even though 28K mRNA is very abundant in adipocytes (reference 6 and Fig. 4). Unlike these induced sequences, actin transcripts were synthesized in both types of nuclei although at a lower relative rate in the adipocyte nuclei. Similarly, actin RNA levels decreased during differentiation. Transcription of sequences complementary to the control plasmids, pC1 and pC2, was observed in both types of nuclei. RNA levels for these control clones show little change during differentiation. pC2 is transcribed at a very high level in both adipocyte and preadipocyte nuclei. Transcription of this sequence was not inhibited by α-amanitin at 2 μg/ml (not shown), suggesting that it is not transcribed by RNA polymerase II.

Differentiation-dependent transcriptional control as described above was observed in three separate experiments. Occasionally lower signals were observed with all of the cDNA probes and in these cases, the less abundant nuclear transcripts (13K, GPD, and pC1) were too low to be detected in either adipose or preadipose nuclei. Transcription signals for GPD and 13K were never observed from preadipocyte nuclei.

Quantitation of the results shown in Figs. 3 and 4 is presented in Table I, columns A and B. 13K and GPD transcription are increased during differentiation at least 11- and 6-fold, respectively. These are minimum estimates limited by the sensitivity of the assay; the actual enhancement of transcription is likely to be much higher. The differentiation-dependent increases at the RNA level are 41-fold for 13K and at least 17-fold for GPD. Decreases of 1.7 and 1.8-fold were observed, respectively, for actin transcription and actin RNA levels. Transcription of RNA complementary to pC1 and pC2 changes only slightly on differentiation with pC1 decreasing 2.3-fold and pC2 increasing 1.2-fold. The amount of both sequences decreased 1.4-fold at the RNA level.

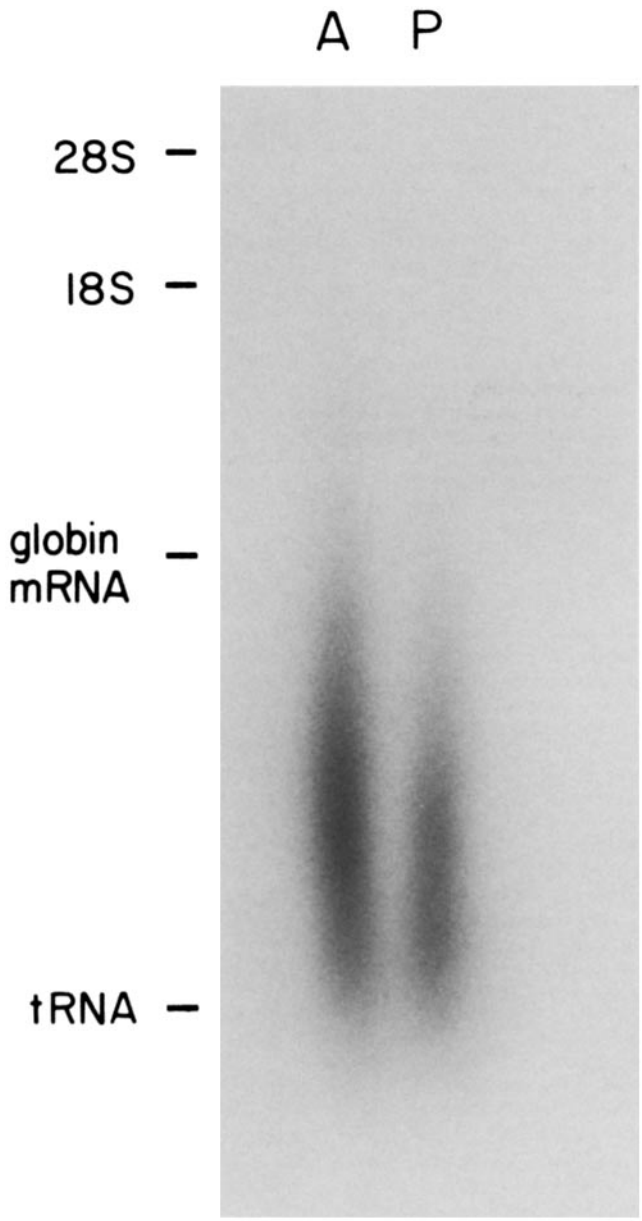

FIGURE 2 Size of the RNA synthesized in isolated adipocyte (*A*) and preadipocyte (*P*) nuclei. The RNA isolated from transcription reactions carried out for 30 min was denatured by heating with formaldehyde and formamide and resolved by electrophoresis on a 1.8% agarose gel containing formaldehyde according to the method described by Maniatis et al. (15). The gel was stained with 1 μg/ml ethidium bromide to visualize the markers and exposed to Kodak X-Omat film to detect the ^{32}P-labeled transcripts.

The correlation between transcription signals and RNA levels for each sequence is reflected in the ratio of these two measurements (Table I, column C). In the cases of the 13K, 28K, and GPD, the ratios could only be calculated for adipocytes since transcription of these sequences was not detect-

TABLE I

Specific Nuclear Transcription and Cytoplasmic RNA Levels during Adipocyte Differentiaton

	A			B			C	
	Nuclear transcription*			Cytoplasmic RNA levels‡			Nuclear transcription/cytoplasmic RNA level§	
Probe	Preadipose	Adipose	-fold change	Preadipose	Adipose	-fold change	Preadipose	Adipose
	cpm	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*		
13K	—	112	>11,↑‖	12	491	41,↑	—	0.23
28K	—	—	—	1.5	172	115,↑	—	<0.06‖
GPD	—	62	>6,↑‖	—	13	>17,↑‖	—	4.7
Actin	824	481	1.7,↓	183	104	1.8,↓	4.5	4.6
pC1	91	39	2.3,↓	11.3	8	1.4,↓	8.1	4.8
pC2	8,927	11,145	1.2,↑	6,661	4,818	1.4,↓	1.3	2.3

In vitro nuclear transcription and cytoplasmic RNA dot blots were carried out as described in Materials and Methods. In all cases only those signals >25% above the pBR 322 control were considered positive. Data in columns A and B were derived with the same probes and were not corrected for probe length. A dashed line indicates no detectable positive signal.

* These data were obtained from the experiment shown in Fig. 3. Since extent of hybridization for the different probes was similar (see Materials and Methods), data were not normalized for these values.

‡ These data were obtained from the experiment shown in Fig. 4.

§ Respective values in column A and column B.

‖ These values were estimated on the basis of the lowest detectable signal (10 cpm for nuclear transcription and 0.75 cpm for cytoplasmic RNA levels).

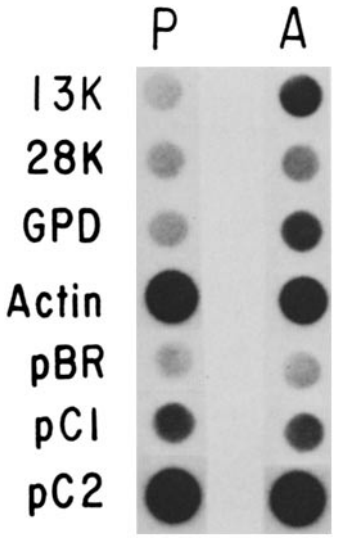

FIGURE 3 Transcription of specific genes in isolated preadipocyte and adipocyte nuclei. 5.5×10^7 cpm of purified RNA transcribed in nuclei from preadipocytes (*P*) and adipocytes (*A*) were hybridized to the immobilized DNAs. After hybridization, the filters were treated with RNase, washed, and exposed to X-ray film as described in Materials and Methods. A resulting autoradiogram is shown. The exposure of pC2 was one seventh as long as that of the other dots.

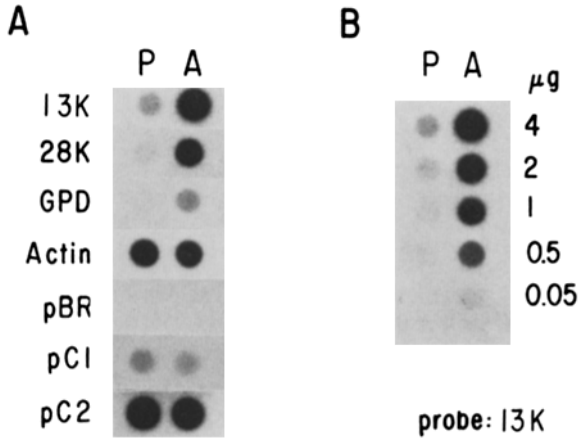

FIGURE 4 Dot blot analysis of specific RNA levels in preadipocytes (*P*) and adipocytes (*A*). (*A*) 4 μg of immobilized preadipocyte and adipocyte cytoplasmic RNAs were hybridized to cDNA probes nick-translated to specific activities of $1.2–1.7 \times 10^8$ cpm/μg. The blots were washed, exposed to X-ray film, and a resulting autoradiogram is shown. For pC2, hybridizations to 0.5 μg of RNA are shown. (*B*) Hybridization of nick-translated 13K cDNA to dilutions of preadipocyte and adipocyte RNA. The amounts of cytoplasmic RNA in the dots are indicated on the right side of the blot.

TABLE II

Turnover of Cytoplasmic RNAs in Adipocytes

RNA species	Apparent half-time of degradation
	h
GPD	2
Actin	4
13K	12
28K	>30

3T3-F442A adipocytes were maintained 7 d post-confluence in 10% fetal calf serum + 5 μg/ml insulin. 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was added to 25 μg/ml in culture medium and cytoplasmic RNA was isolated at 0, 3, 8, 12, 24, and 30 h after the addition of drug. Adipocytes maintained normal morphology and attachment throughout the drug treatment period. The amounts of particular RNA species remaining at various times after the addition of DRB were analyzed by electrophoresing RNA from an equivalent number of cells in formaldehyde/agarose gels, blotting onto nitrocellulose and hybridizing to probes as described in Materials and Methods.

able in preadipocytes. The ratios of transcription to cytoplasmic RNA level for actin and control clones pC1 and pC2 fall in the range of 1–8 in both preadipocytes and adipocytes, indicating that the abundance of these RNAs is related to their transcription rates in a relatively consistent manner. Similarly, the ratio of transcription to RNA level for GPD is 4.7 in the adipocyte. In clear contrast, the ratios for the 13K and 28K protein are 0.23 and <0.06, respectively, reflecting the low transcription signals obtained for these very abundant RNAs.

RNA Half-lives in the Presence of DRB

The data presented above suggest that one or more post-transcriptional steps contribute significantly to the accumulation of the 13K and 28K mRNAs. Since the rate of RNA degradation may greatly influence RNA abundance, we have performed a preliminary measurement of RNA half-lives in adipocytes by inhibiting mRNA synthesis with DRB (19, 20) and subsequently measuring loss of these mRNAs by Northern blotting. As shown in Table II, we estimate actin mRNA half-life to be 4 h, while GPD mRNA has a half-life of only 2 h. In contrast, 13K and 28K mRNAs are degraded with half-times of 12 and greater than 30 h, respectively. While these experiments must be interpreted with caution because

of possible secondary effects due to the use of an RNA synthesis inhibitor (21), the results suggest that 13K and 28K mRNAs are very stable in the adipocyte, compared with the other mRNAs examined.

13K Protein Is Closely Related to Myelin Protein P2

The functions of the 13K and 28K proteins are unknown at this time. Since the regulation of 13K and 28K mRNA biosynthesis may be related to the roles of the corresponding proteins in adipocytes, we sought to identify one of the proteins, 13K, by cDNA sequence analysis. Fig. 5 shows 550 bases of the nucleotide sequence obtained from two overlapping cDNA clones and the corresponding derived amino acid sequence. A computer-assisted search of amino acid sequence libraries was performed and three classes of proteins were found to match 13K: P2 myelin proteins, retinoic acid binding proteins, and fatty acid binding proteins. The alignment of the amino acid sequence of 13K with that of rabbit myelin protein P2 (22) (Fig. 6), shows sequence homology of 70% but 80% if conservative amino acid substitutions are allowed. Similarly, a strong homology (48%) is observed between a 32 amino acid fragment of rat testis retinoic acid binding protein (23) and the corresponding portion of the 13K protein (not shown). This result is expected because close amino acid sequence homology between myelin protein P2 and retinoic acid binding protein was described previously (24). Less striking homology, 25 and 47% with conservative substitutions, is observed between the 13K amino acid sequence and that of rat intestinal fatty acid binding protein (25) (Fig. 6). Significantly, myelin protein P2, retinoic acid binding protein, and fatty acid binding protein all appear to interact with lipids (26–28).

DISCUSSION

Our results clearly demonstrate that transcription of GPD and 13K genes in isolated nuclei is greatly increased upon adipocyte differentiation. This is the first demonstration that adipocyte differentiation involves enhanced transcription of specific genes. The transcription of GPD and 13K in adipo-

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                          15                  30                  45
CTT GTC TCC AGT GAA AAC TTC GAT GAT TAC ATG AAA GAA GTG GGA GTG GGC
Leu Val Ser Ser Glu Asn Phe Asp Asp Tyr MET Lys Glu Val Gly Val Gly

         60                  75                  90                 105
TTT GCC ACA AGG AAA GTG GCA GGC ATG GCC AAG CCC AAC ATG ATC ATC AGC GTA
Phe Ala Thr Arg Lys Val Ala Gly MET Ala Lys Pro Asn MET Ile Ile Ser Val

                 120                 135                 150
AAT GGG GAT TTG GTC ACC ATC CGG TCA GAG AGT ACT TTT AAA AAC ACC GAG ATT
Asn Gly Asp Leu Val Thr Ile Arg Ser Glu Ser Thr Phe Lys Asn Thr Glu Ile

    165                 180                 195                 210
TCC TTC AAA CTG GGC GTG GAA TTC GAT GAA ATC ACC GCA GAC GAC AGG AAG GTG
Ser Phe Lys Leu Gly Val Glu Phe Asp Glu Ile Thr Ala Asp Asp Arg Lys Val

            225                 240                 255
AAG AGC ATC ATA ACC CTA GAT GGC GGG GCC CTG GTG CAG GTG CAG AAG TGG GAT
Lys Ser Ile Ile Thr Leu Asp Gly Gly Ala Leu Val Gln Val Gln Lys Trp Asp

270                 285                 300                 315
GGA AAG TCG ACC ACA ATA AAG AGA AAA CGA GAT GGT GAC AAG CTG GTG GTG GAA
Gly Lys Ser Thr Thr Ile Lys Arg Lys Arg Asp Gly Asp Lys Leu Val Val Glu

        330                 345                 360                 379
TGT GTT ATG AAA GGC GTG ACT TCC ACA AGA GTT TAT GAA AGG GCA TGA GCCAAAGGAA
Cys Val MET Lys Gly Val Thr Ser Thr Arg Val Tyr Glu Arg Ala  .

       389         399         409         419         429         439         449
GAGGCCTGGA TGGAAATTTG CATCAAACAC TACAATAGTC AGTCGGATTT ATTGTTTTTT TTTAAAGATA

       459         469         479         489         499         509         519
TGATTTTCCA CTAATAAGCA AGCAATTAAT CTTTTCTGAA GATGCATTTT ATTGGATATG GTTATGTTGA

       529         539         549
TTAAATAAAA CCTTTTTAGA CTTAAAAAAA A
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FIGURE 5 Nucleic acid sequence and corresponding protein sequence of the 13K cDNA. The cDNA inserts from two unique plasmid clones, containing sequences complementary to 13K mRNA, were sequenced by the method of Sanger et al. (17) as described in Materials and Methods. The inserted sequences were found to overlap by 95 nucleotides. The composite cDNA sequence is given above (first line). The total deduced sequence of 550 base pairs can be translated into a polypeptide of 122 amino acids (second line) encoded by nucleotides 1 through 372. This segment is followed by an untranslated 3′ sequence containing 187 nucleotides. A consensus polyadenylation signal sequence, AATAAA, at position 523 is followed after 14 bases by a run of adenine residues indicating the end of the mRNA.

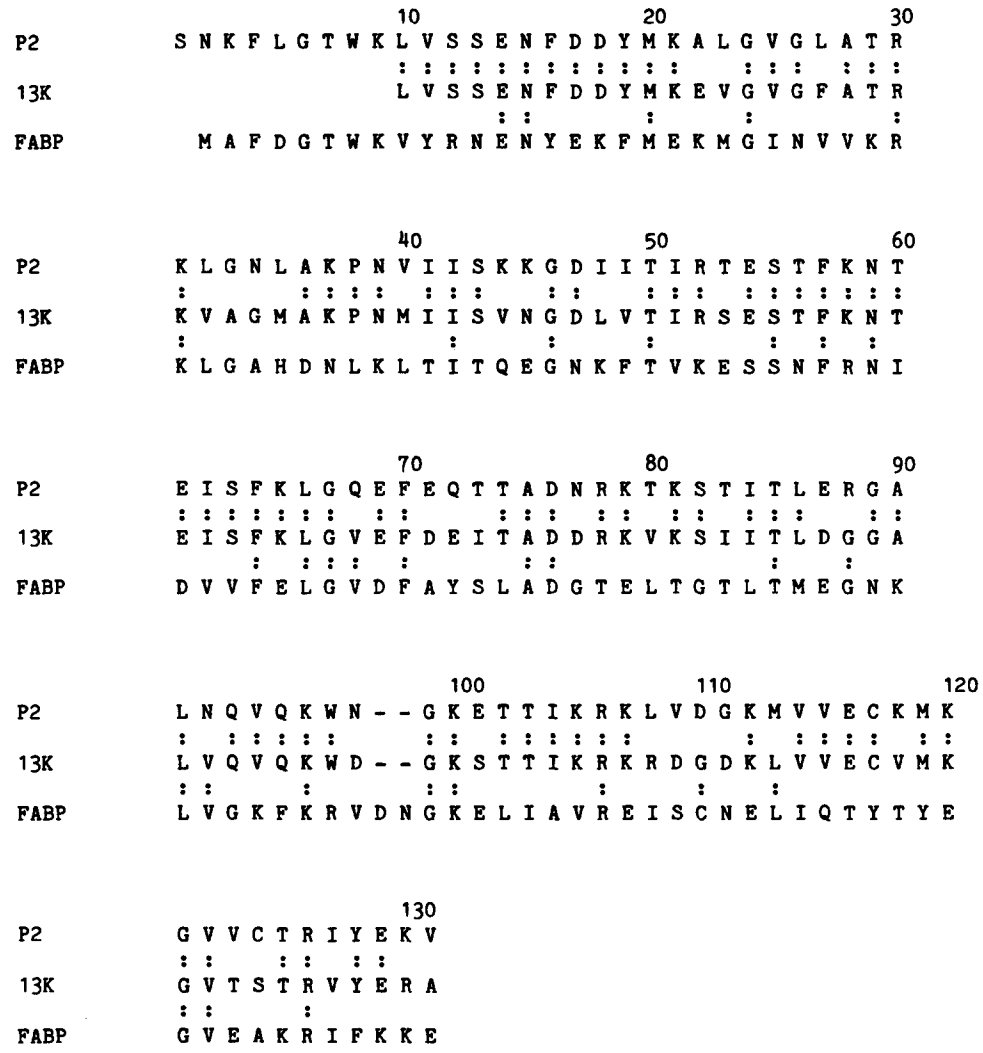

FIGURE 6 Protein sequence comparison between 13K, myelin P2, and fatty acid binding protein. The 13K amino acid sequence shown in Fig. 5 was used to search the NBRF Protein Sequence Data Bank for homologous proteins. Shown above in single letter amino acid code is the alignment of rabbit myelin P2 to the 13K sequence as well as the best alignment of 13K to rat intestinal fatty acid binding protein (FABP). Amino acid matches are indicated by colons (:) and gaps introduced into the sequence by dashes (–). Protein homology searches were done with the Intelligenetics program. Final alignments were done with the program developed by Wilbur and Lipman (18).

cytes may be due to either activation of previously untranscribed genes or to enhancement of transcription which, if it occurs in preadipocytes, is below detectable limits. Due to the limited sensitivity of the transcription assays we cannot discriminate between these possibilities. While increased transcription undoubtedly plays a large role in the build up of 13K protein and GPD RNAs in adipocytes, potential differentiation-dependent changes at other levels of regulation cannot be ruled out. Surprisingly, no transcription signal could be obtained for the abundant, strongly differentiation-dependent 28K mRNA (see below). The decrease in actin transcription agrees well with the decrease in actin mRNA observed in these experiments. Although a larger decrease in actin biosynthesis (and presumably mRNA) can be obtained by longer culture and by isolation of pure adipocytes (13), we found the present culture conditions optimal for the routine isolation of active preadipocyte and adipocyte nuclei in good yield. Nuclear transcription results similar to those presented in this study have been observed by M. Phillips, P. Djian, and H. Green (personal communication).

The correlation between relative transcription rate and the steady state abundance differ markedly for particular RNAs in adipocytes (Table I). The ratios of transcription to RNA abundance are similar for GPD, actin, pC1, and pC2. In contrast, 13K and 28K mRNAs are far more abundant than would be expected from their relative transcription rates. It is unlikely that our transcription results reflect some peculiarity of the cDNA probes since the extent of hybridization of RNA fragments to each of these sequences was similar (Materials and Methods). Instead, other steps in mRNA metabolism appear to contribute to the accumulation of 13K and 28K mRNAs. The preliminary results presented in Table II suggest that the abundance of the 13K and 28K mRNAs is due, at least in part, to their turnover times which are long relative to those of GPD and actin. Measurements of the 13K, 28K, and GPD mRNA half lives in preadipocytes are not feasible because of the extremely low abundance of the RNAs in these cells. Hence, our experiments do not indicate whether the apparent stability of the 13K and 28K RNAs is dependent on differentiation or whether it is an intrinsic property of the RNA molecules. Our current experiments are aimed at determining the half-lives of these mRNAs more precisely and defining the factors that control mRNA stability in preadipocytes and adipocytes.

Many studies have previously demonstrated developmentally related changes in specific gene transcription in other systems (reviewed in reference 29). While a good quantitative correlation is generally observed between relative transcription levels and RNA abundance (30) there are now several examples of mRNAs whose abundance cannot be accounted

for by relative transcription levels alone (31–33). In most of these cases, the mRNA species have an intrinsically long half-life or a developmentally-related increase in specific mRNA half-life (29, 31, 32). In adipocytes, what physiological consequences could arise from the different contributions made by transcription and RNA turnover to the abundance of 13K and 28K and GPD mRNAs? An mRNA which is transcribed relatively rapidly and degraded rapidly, such as GPD, could be quickly removed by stopping transcription when its presence is deleterious and later quickly restored by reactivating transcription. This control is probably useful to the adipocyte because while glycerol-3-phosphate, the product of GPD, is required in large amounts when cells are in a lipogenic mode, removal of this molecule during lipolysis may minimize re-esterification of the newly formed free fatty acids. While the physiological role for 13K has not been determined, it is likely to function as a lipid binding protein because it is related by amino acid sequence to three proteins that interact with lipids: myelin protein P2, retinoic acid binding protein and fatty acid binding protein (Figs. 5 and 6, 26–28). The sequence of an adipocyte mRNA related to myelin protein P2 has also been recently described by Bernlohr et al. (34). Since fatty acids and triglycerides are very abundant when adipocytes are in either lipogenic or lipolytic modes, rapid regulation lipid-binding proteins may not be necessary. Some support for this rationale is the decreased synthesis previously observed in GPD and fatty acid synthetase but not in 13K protein when differentiating adipocytes were challenged with drugs which raise the concentration of intracellular cyclic AMP, a key lipolytic agent (5, 35). It is likely that the ability of the adipocyte to meet its multiple metabolic roles will require several regulatory mechanisms for different mRNAs.

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