Hormonal regulation of stability of glutamine synthetase mRNA in cultured 3T3-L1 adipocytes

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In 3T3-L1 adipocytes, glutamine synthetase (GS; EC 6.3.1.2) is subject to regulation by dexamethasone, insulin and dibutyl cyclic AMP (Bt₂cAMP). Dexamethasone increases GS-mRNA content and GS-gene transcription, whereas insulin and Bt₂cAMP prevent these increases. The effects of these modulators on the control of GS-mRNA stability were investigated. We report here that GS mRNA has a half-life of about 110 min. Bt₂cAMP increases GS-mRNA degradation by >2-fold (half-life 50 min), whereas insulin or dexamethasone have little effect on GS-mRNA stability. Down-regulation of GS-gene expression by Bt₂cAMP will involve a co-ordinate response at the level of gene transcription and mRNA stability. However, the molecular mechanisms by which insulin and dexamethasone regulate GS-gene expression in cultured adipocytes remains to be elucidated.

INTRODUCTION

Glutamine synthetase (GS) activity, cellular content, mRNA abundance and gene transcription rate increase more than 100-fold during adipocyte differentiation of confluent 3T3-L1 cells (Miller *et al.*, 1978, 1983; Miller & Carrino, 1980; Bhandari *et al.*, 1986; Bhandari & Miller, 1987). In fully differentiated 3T3-L1 adipocytes, GS-gene transcription is regulated by dexamethasone, insulin and cyclic AMP (Bhandari & Miller, 1987). However, the dexamethasone-mediated 2-fold increase in GS-gene transcription is not sufficient to account for the observed 5–10-fold increase in GS-mRNA abundance. One possible explanation for this could be that dexamethasone increases the stability of GS mRNA in the cytoplasm.

Factors affecting mRNA life-expectancy are now recognized to be, in part, responsible for regulating gene expression. In most prokaryotic mRNAs gene regulation is exerted mainly at the transcriptional level, whereas in eukaryotes all mRNAs are subject to some form of transcriptional and/or post-transcriptional control. In the mammalian cell, transcription and translation are separated topographically; thus the regulation of mRNA turnover can occur at multiple sites. The decay of a mRNA molecule represents a fine balance between the transcriptional activity and the intracellular degradative processes. Hormones are among the extensively studied stimuli affecting mRNA half-life. The role of hormone-responsive regions in gene regulation has received considerable attention in recent years (reviewed by Roesler *et al.*, 1988; Beato, 1989), and the molecular mechanisms for differential stabilities of mRNAs are now being elucidated.

This study reports the effect of dexamethasone, insulin and dibutyl cyclic AMP (Bt₂cAMP) on the stability of GS transcripts in cultured adipocytes. Our findings show that GS mRNA has a half-life of about 95–110 min. Bt₂cAMP increases GS-mRNA degradation by more than 2-fold, whereas insulin or dexamethasone have little effect on GS-mRNA stability.

MATERIALS AND METHODS

Materials

Calf serum and fetal-calf serum were obtained from Flow Laboratories. Pig crystalline zinc insulin was obtained from Eli

Lilly Co. Bt₂cAMP was from Boehringer Mannheim, Indianapolis, IN, U.S.A. [5,6-³H]Uridine was from New England Nuclear, Boston, MA, U.S.A. Most of the other chemicals were from either Sigma, St. Louis, MO, U.S.A., or other standard suppliers, and were of the highest purity available.

Cell culture and RNA isolation

Starter cultures of 3T3-L1 cells were obtained from American Type Culture Collection, Rockville, MD, U.S.A. Unless indicated otherwise, 3T3-L1 cells were grown and maintained as previously described (Bhandari & Miller, 1985). Adipocyte differentiation were achieved either (i) by the dexamethasone/isobutylmethylxanthine/insulin method or (ii) by the dexamethasone/isobutylmethylxanthine method (Miller *et al.*, 1983). RNA was isolated from adipocytes by the guanidinium isothiocyanate method and repeated ethanol precipitation (Chirgwin *et al.*, 1979).

RNA labelling and determination of GS-mRNA half-life

To radiolabel RNA, >90%-confluent 3T3-L1 adipocytes were incubated for the indicated times in fresh medium containing [³H]uridine (100 µCi/dish; 50 Ci/mmol). After this, the cultures were washed twice with phosphate-buffered saline (0.15 M-NaCl/0.1 M-sodium phosphate, pH 7.4), and total RNA was isolated by the guanidinium isothiocyanate method (Chirgwin *et al.*, 1979). ³H-labelled GS-mRNA was quantified as described under 'DNA-excess filter hybridization'.

The half-life of GS transcripts were determined by the [³H]uridine pulse-decay method (Levis & Penman, 1977; Hod & Hanson, 1988). Confluent (>90%) 3T3-L1 adipocytes were incubated for 4 h (short-term treatment) or 48 h (long-term treatment) in fresh culture medium containing no added hormone (control), or in medium containing 1 µM-dexamethasone, 1 µg of insulin/ml, 0.5 mM-Bt₂cAMP plus 1.0 mM-theophylline or their various combinations. [³H]Uridine (100 µCi/dish, 50 Ci/mmol) was added to radiolabel newly synthesized RNA. Thereafter, the [³H]uridine-containing medium was removed, cultures were washed three times with phosphate-buffered saline, and a 'chase' period was initiated after the addition of fresh medium containing the respective hormone treatment(s) and 5 mM each of cytidine and uridine to dilute the radioactive label released from the

Abbreviations used: GS, glutamine synthetase; cAMP, cyclic AMP; Bt₂cAMP, dibutyl cyclic AMP; kb, kilobases.

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breakdown of RNA. Samples of the cells were harvested for RNA isolation at 0, 1, 2 and 4 h after the start of the 'chase' period. The half-life of GS mRNA was calculated from the disappearance of ^3H -labelled GS-specific transcripts as described below.

DNA-excess filter hybridization

A recombinant pBR322 clone containing a GS cDNA (GS-89) from a rat liver cDNA library was obtained as follows: two synthetic oligonucleotide primers were prepared, one primer in the forward direction corresponding to nucleotides 1252–1275 and the other primer complementary to nucleotides 1336–1356 of the previously published GS coding sequence (Bhandari *et al.*, 1988). A radiolabelled DNA probe was generated with the polymerase chain reaction (Schowalter & Sommer, 1989) with 3T3-L1 genomic DNA as the template. The radiolabelled probe was used to screen a rat liver cDNA library (generously given by Dr. Robert Hill, Department of Biochemistry, Duke University Medical Center, Durham, NC, U.S.A.) by using the procedures described in Maniatis *et al.* (1982). Among the various positive GS-cDNA clones selected, the clone with the strongest positive signal was isolated and designated as GS-89. The GS-89 plasmid is composed of pBR-322 and contains a GS-cDNA insert of approx. 1.6 kb, compared with the 1.3–1.4 kb inserts cloned for the human GS cDNA (Gibbs *et al.*, 1987). The growth, amplification and purification of pBR322 and GS-89 were performed by standard protocols (Maniatis *et al.*, 1982). Plasmid DNA (10 $\mu\text{g}/\text{filter}$) was immobilized on 7 mm-diam. nitrocellulose filters (grade BA 85; Schleicher and Schuell, Keene, NH, U.S.A.) by the method of Parnes *et al.* (1981) as modified by Maniatis *et al.* (1982). The filters were prehybridized for 16–18 h at 45 °C in hybridization buffer [20 mM-Pipes, pH 6.4, 50% (v/v) formamide, 0.5 M-NaCl, 0.5% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA and 200 μg of denatured salmon sperm DNA/ml]. In preliminary experiments, the amount of plasmid DNA loaded on to each filter to form an excess was found by hybridizing different amounts of GS-89 with various amounts of cytoplasmic ^3H -labelled RNA from 3T3-L1 adipocytes. ^3H -labelled RNA [containing $(2-5) \times 10^6$ c.p.m./dish; 200 μg of RNA per dish] was hybridized in triplicate to GS-89, β -actin and pBR322 filters at 45 °C for 70–72 h in the hybridization buffer described above.

After hybridization, the filters were washed as described by McKnight & Palmiter (1979). For elution of hybridized RNA, filters were incubated with 200 μl of 0.05 M-NaOH for 30 min at 65 °C. This was followed by neutralization with 100 μl of 0.1 M-acetic acid and addition of 5 ml of Aquasol (New England Nuclear). ^3H radioactivity was counted in a liquid-scintillation counter (LKB, 1217 Rackbeta). The values for GS mRNA were calculated after correcting for machine background (< 10 c.p.m.) and non-specific binding to pBR322. The radioactivity (c.p.m.) bound to pBR322 was $< 10\%$ of that hybridized to GS-89. Typically, 40–60 counts/ 10^6 ^3H counts were hybridized with filters containing β -actin probe, and the difference between specific and non-specific binding was < 25 c.p.m. Together, this contributed to $< 5\%$ variability among various treatments with respect to the β -actin mRNA.

RESULTS AND DISCUSSION

Post-transcriptional control of mRNA processing and turnover determines the steady-state level of a given mRNA in the cytoplasm. An increase in GS-mRNA abundance can result from decreased mRNA degradation, increased mRNA production or both. Increased (mRNA production) rates normally result from increased gene transcription. The efficiency of processing of the

primary transcript and the transport of mRNA to the cytoplasm also play an important role. Nutrition, hormones, physiological and pharmacological stimuli are the main factors known to control mRNA turnover in eukaryotes. The regulation of mRNA turnover by hormones is important, and has received considerable attention in recent years (reviewed by Beato, 1989; Saini *et al.*, 1990). However, the molecular mechanisms underlying the regulation of mRNA decay by hormones are still poorly understood.

Northern hybridization analysis

Northern hybridization analysis (with GS-89 as a radiolabelled probe; Thomas, 1980; Feinberg & Vogelstein, 1983) of total RNA from 3T3-L1 adipocytes yielded two GS-mRNAs, of sizes 3.2 and 1.6 kb (results not shown). These sizes are identical with those reported previously (Sanders & Wilson, 1984; Bhandari *et al.*, 1986). In addition, the effects of dexamethasone, insulin and Bt_2cAMP were comparable with those reported previously (Bhandari *et al.*, 1986, 1988).

Cytoplasmic GS-mRNA appearance rate

Studies shown in Fig. 1 (inset) indicate that it is possible to quantify the GS-mRNA appearance rate in fully differentiated 3T3-L1 adipocytes. The rate of appearance of GS-specific transcripts was directly proportional to the incubation time of the untreated adipocytes with the labelled uridine (Fig. 1, inset). We have employed a combination of cDNA-excess filter hybridization and a dot-blot assay to quantify the concentration of GS mRNA. The use of these two techniques to determine half-life

Fig. 1. Effect of dexamethasone, insulin and Bt_2cAMP on GS-mRNA stability in cultured adipocytes

Untreated (control) and treated (with various hormones) 3T3-L1 adipocytes ($> 90\%$ confluent) were incubated in the presence of [^3H]uridine (100 $\mu\text{Ci}/\text{dish}$, 50 Ci/mmol) for 4 h. After three washings with phosphate-buffered saline, the cells were incubated with their respective medium, which also included 5 mM-cytidine and -uridine. At the indicated times, the cells were harvested for RNA isolation. GS-mRNA quantification and half-life determination were done as described in the Materials and methods section. Each point represents the mean $\pm$ S.E.M. for three independent experiments. Symbols (half-life in parentheses): $\circ$, control (110 min); $\bullet$, Bt_2cAMP (50 min); $\square$, insulin (110 min); $\triangle$, dexamethasone (105 min); $\diamond$, insulin plus dexamethasone (90 min). The inset shows the rate of appearance of GS transcripts, without any added hormone, in $> 90\%$ -differentiated adipocytes.

Table 1. Effect of time of exposure to hormone(s) on the glutamine synthetase mRNA half-life

Confluent (> 90%) 3T3-L1 adipocytes were either untreated (control) or treated with various hormone(s) for 4 h (short-term) or 48 h (long-term) as described in the Materials and methods section. Thereafter, 'chase' was initiated and samples were harvested for RNA at 0, 1, 2 and 4 h after chase. GS-mRNA quantification and half-life measurements were as described in the Materials and methods section. The results are means $\pm$ S.E.M. of three experiments, with each treatment in triplicate: ND, not determined.

Treatment	Short-term		Long-term	
	Half-life (min)	% of control	Half-life (min)	% of control
Control	110 $\pm$ 3.6	100	95 $\pm$ 5.9	100
Dexamethasone	105 $\pm$ 7.5	95	95 $\pm$ 9.0	100
Insulin	110 $\pm$ 10.4	100	115 $\pm$ 8.1	121
Insulin + dexamethasone	90 $\pm$ 6.2	82	ND	ND
cAMP	50 $\pm$ 4.3	45	ND	ND
Insulin + cAMP	ND	ND	35 $\pm$ 3.3	37

measurements for mRNAs is described in detail by Rosen and colleagues (Rodgers *et al.*, 1985).

GS-mRNA half-life and stability

It is now well established that the regulation of mRNA turnover plays a significant role in the overall control of gene expression. Among eukaryotes, mRNA life-expectancy seems to vary from minutes to days. mRNAs with short half-lives would provide a burst of new proteins in response to external or internal stimuli. Recent evidence suggest that a number of mammalian genes require a co-ordinate response at the level of both transcription and mRNA stability for their expression.

In 3T3-L1 adipocytes incubated in the presence of a specific inhibitor of mRNA synthesis, dichlororibofuranosylbenzimidazole, glycerol-3-phosphate dehydrogenase mRNA decayed with a half-life of about 2 h (Cook *et al.*, 1985). However, in the present work on cultured adipocytes, cytoplasmic RNA production rate and GS-mRNA stability were analysed directly by [³H]uridine labelling (synthesis rate) and pulse/chase analyses (stability). In addition, we have directly measured the half-life of GS-mRNA and also examined the effects of dexamethasone, insulin and Bt₂cAMP on GS-mRNA stability. The use of RNA-synthesis inhibitors was avoided, as they have been reported to affect the normal turnover of specific mRNAs (Cavaliere *et al.*, 1977; Hod & Hanson, 1988). Long-term exposure (48 h) compared with short-term exposure (4 h) to dexamethasone or insulin caused little effect on the GS-mRNA half-life (Table 1). Using the pulse-decay technique (Levis & Penman, 1977; Hod & Hanson, 1988), we determined the half-life of GS mRNA to be 95–110 min (Fig. 1, Table 1). Recently, using similar techniques, Hod & Hanson (1988) have demonstrated that the life-expectancy of phosphoenolpyruvate carboxykinase mRNA varies between 30 and 40 min. Since dexamethasone caused > 10-fold increase in GS-mRNA abundance (Bhandari & Miller, 1985) and about 2-fold increase in GS-gene transcription (Bhandari & Miller, 1987), additional regulation must occur at post-transcriptional step(s) before the appearance of GS-mRNA in the cytoplasm. It appears likely that the regulation by dexamethasone occurs at a step after the GS gene is transcribed and before GS-mRNA is transported to the cytoplasm. Dexamethasone caused negligible effect on the stability of mature GS transcript once it reaches the cytoplasm.

Molecular mechanisms by which cAMP re-programs the gene expression in mammalian cells are now being elucidated. A recent review has highlighted the progress on the regulation of gene expression by cAMP in eukaryotes (Roesler *et al.*, 1988).

For example, cAMP stabilizes the mRNA for phosphoenolpyruvate carboxykinase by > 5-fold, whereas insulin caused an opposite effect on the turnover of this mRNA (Hod & Hanson, 1988). In rat liver, cAMP has also been shown to increase the levels of mRNAs for five urea-cycle enzymes (Morris *et al.*, 1987) and for ornithine aminotransferase (Merrill *et al.*, 1985). These diverse effects of cAMP are pleiotropic in nature, usually involving simultaneous alterations for a number of steps in a metabolic pathway, as reported for the urea-cycle enzymes. In the present study, cAMP enhanced GS-mRNA degradation by > 2-fold, whereas insulin or dexamethasone had a negligible effect (Table 1). Bhandari & Miller (1987) have shown that in 3T3-L1 adipocytes Bt₂cAMP caused a 2-fold decrease in GS-gene transcription, and here we have shown more than a 2-fold increase in GS-mRNA degradation by cAMP (Fig. 1). Together, these studies suggest that the down-regulation of GS-gene expression by cAMP appears to involve a co-ordinate effort at the level of transcription and mRNA stability. These results are in agreement with the GS cellular content and the enzyme-activity data (Miller *et al.*, 1978, 1983; Miller & Carrino, 1980).

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