Multiple Regulation of S_{14} Gene Expression during Brown Fat Differentiation*

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ABSTRACT

S_{14} is a gene known to be under thyroid hormone control. Its mRNA concentration is very high in lipogenic tissues, and although the precise function of the protein is still unknown, indirect data suggest its implication in triglyceride synthesis. S_{14} gene expression is up-regulated by thyroid hormone in liver, white adipose tissue, and lactating mammary gland. However, in brown fat, the level of this sequence is increased 3-fold in the hypothyroid animal. We have used primary cultures of brown preadipocytes differentiated to fully mature brown adipocytes to investigate the influence of cellular differentiation and hormonal stimulation on S_{14} gene expression. Steady state levels of S_{14} mRNA rose from nondetectable levels in preadipocytes to reach a maximum in fully mature adipocytes. Treatment of brown adipocytes

cultures with T_3 did induce S_{14} gene expression. This induction reflects in part a posttranscriptional stabilization of the messenger by T_3. Insulin, insulin-like growth factor-I, and inositol phosphate-glycan also increase the level of S_{14} mRNA. Norepinephrine (NE) plays a major role in the regulation of S_{14} gene, and 24 h after its addition, NE elicited a 20-fold decrease in mRNA S_{14} concentrations. An elevated intracellular concentration of cAMP is a strong negative effector of S_{14} gene expression, and neither NE nor cAMP action is totally overcome by T_3. As happens *in vivo*, glucose is a potent stimulator of S_{14} mRNA; however, there is a lag time of several hours before its effects can be detected. The increase in S_{14} gene expression with the maturation stage of the cell suggests an important role for S_{14} in adipocyte differentiation. (*Endocrinology* **133**: 545–552, 1993)

THE rat hepatic gene S_{14} has been one of the most carefully studied thyroid hormone-responsive genes. Initially described in 1981 (1), the mRNA for S_{14} is induced within 20 min in hypothyroid rat liver after the injection of a saturating dose of T_3 (2). The nuclear precursor for this mRNA begins to rise only 10 min after administration of the hormone (3). This rapid induction suggested that this gene could represent a direct target for T_3 action. In fact, in spite of several contradictory reports (4, 5), it has been shown that thyroid hormones act directly on the transcriptional machinery by mechanisms involving direct interactions of the T_3-receptor complex with *cis*-regulatory elements in the S_{14} promoter (6, 7). Besides thyroid hormone regulation, the S_{14} gene is subjected to a complex tissue-specific developmental, hormonal, and nutritional regulation (8, 9). The S_{14} gene codes for an acidic protein (pI ~4.9) with a mol wt of 17,010. Although the function of this protein is unknown, a series of functional correlations suggests its involvement in lipid metabolism (10, 11)

The response characteristics to T_3 of mRNA S_{14} depends on the tissue studied. In brown adipose tissue (BAT), the concentration of S_{14} mRNA is very high, twice that in white adipose tissue and 20-fold the level in euthyroid rat liver. In contrast with that in liver and white adipose tissue, T_3 does

not induce S_{14} gene in BAT; moreover, S_{14} mRNA levels are 3-fold higher in BAT from hypothyroid animals (12).

Previous studies have also shown that S_{14} gene expression is lacking in the liver of fasting and diabetic animals (13). However, definitive studies of hormone action in the whole animal are difficult, especially for the opposite effects of hormones such as glucagon that are secreted in response to the insulin-induced hypoglycemia. For instance, the increase in insulin concentrations after a meal is accompanied by a concomitant drop in plasma glucagon levels, and both hormones have been shown to be, respectively, positive and negative effectors of S_{14} gene expression. Moreover, it has been shown that the diabetic animal has very low T_3 levels (14). This situation is even more complicated because this gene is regulated by many other metabolites and hormones.

Clearly, tissue culture cell lines or primary cultures are the best systems to define the cellular environment. With this in mind, we have undertaken studies of S_{14} gene regulation by nutrients, hormones, and cell differentiation in primary cultures of brown preadipocytes with the capacity to differentiate in culture into mature adipocytes. Brown adipocytes constitute an excellent model to study the molecular mechanisms implicated in the regulation of many enzymes related to the synthesis and degradation of fatty acids. Before reaching confluence, these cells exhibit the morphological characteristics of fibroblasts; however, confluent brown preadipocytes are capable of differentiation into adipocytes, and cells become highly responsive to hormones that regulate lipid metabolism. We show here that S_{14} gene expression increases concomitantly with brown adipocyte differentiation, and in contrast with their effects *in vivo*, thyroid hor-

mones greatly induce S_{14} gene expression in brown adipocytes. At least a part of the up-regulation of S_{14} gene expression by thyroid hormone is mediated by an increase in messenger stability. There is also a striking induction of S_{14} mRNA levels by insulin and insulin-like growth factor-I (IGF-I) in the early phases of adipocytes differentiation as well as in totally differentiated brown adipocytes. When added to the cells, the insulin-induced inositol phosphate-glycan (IPG) closely mimicked the effects of insulin, supporting the idea that IPG could be a mediator of insulin action. In addition to the regulation exerted by thyroid hormones, insulin, and differentiation, S_{14} gene expression exhibits a multifactorial control in this system.

Materials and Methods

Materials

Collagenase, bovine insulin, ascorbic acid, guanidinium HCl, 3-(N-morpholino)propanesulfonic acid (MOPS), agarose, norepinephrine (NE), T_3, 8-bromo-cAMP, forskolin, and retinoic acid were obtained from Sigma (St. Louis, MO). Crystalline bovine glucagon was obtained from Eli Lilly Co. (Indianapolis, IN). Human recombinant IGF-I and IGF-II were purchased from Bachem, Inc. (Torrance, CA). Formamide was obtained from Fluka (Buchs, Switzerland). Dulbecco's Modified Eagle's Medium (DMEM) and DMEM low in glucose (D medium; 5 mM) were purchased from Gibco (Grand Island, NY), and newborn calf serum was obtained from Flow Laboratories (Rockville, MD). Radiolabeled [α-^{32}P]deoxy-CTP (3000 Ci/mmol) and DNA labeling system were obtained from Amersham Corp. (Arlington Heights, IL) and Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ), respectively. Nytran membranes (NY 13N) were purchased from Renner GmBH (Dannstadt, Germany). Cycloheximide, α-amanitin, and restriction enzymes were purchased from Boehringer Mannheim (St. Louis, MO). All other chemicals were reagent grade or molecular biology grade. IPG [also known as phosphooligosaccharide (POS); isolated from rat liver] was a gift from Dr. J. M. Ruiz (Department of Biochemistry and Molecular Biology, School of Medicine, Complutense University, Madrid, Spain).

Cell cultures

Precursor cells were obtained from the interscapular brown adipose tissue of 20-day-old rats (Sprague-Dawley), isolated according to the method of Nèchad et al. (15), seeded, and grown in DMEM supplemented with 10% newborn calf serum, 4 nM insulin, 10 mM HEPES, 50 IU penicillin and 50 μg streptomycin/ml, and 15 μM ascorbic acid. Insulin was omitted in the experiments with insulin, IPG (POS), and IGFs. Cells reached confluence 4–5 days after seeding and by day 8 were fully differentiated into mature brown adipocytes. Studies were performed during the period of differentiation (5–8 days of culture). The day indicated in the figures represent the day of harvesting. The various groups were: 1) controls, DMEM plus 10% calf serum (C); 2) DMEM medium plus 10% calf serum, made hypothyroid by standard procedures (16) (Tx); and 3) DMEM medium with 5 mM (20% of normal DMEM medium) glucose plus 10% calf serum (D). To obtain a totally glucose-depleted medium, cells were maintained in D medium from day 3 without changing the medium. By days 7–8, glucose levels were undetectable. All experiments were performed three to five times in duplicate plates.

RNA preparation and Northern blot analysis

Total cellular RNA was extracted in guanidinium-HCl, as described previously (17). Total RNA from selected samples (10 μg) was denatured and electrophoresed on a 2.2 M formaldehyde-1% agarose gel in 1 × MOPS buffer (20 mM, pH 7) and transferred to nylon membranes (Nytran, NY 13N, Renner GmbH). Filters were hybridized for 20 h at

42 C [50% formamide, 3 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1 × Denhardt's, and 0.2% sodium dodecyl sulfate (SDS)], and washed once in 2 × SSC-0.5% SDS at 65 C for 30 min and then twice in 0.2 × SSC-0.5% SDS at 65 C for 20 min. Autoradiograms were obtained from the filters and quantified by computer-assisted videodensitometry. The 660-basepair fragment of an S_{14} clone (kindly provided by Dr. H. Towle), corresponding to most of the translated region of the mRNA, was used as template for [α-^{32}P]deoxy-CTP-labeled probes, using random primers (>10^8 cpm/μg DNA). Results in the text are expressed as the mean ± SD of at least three different experiments using duplicate plates.

Results

Effect of adipogenic differentiation on S_{14} gene expression

After 4–5 days of culture, brown preadipocytes became visually confluent and started to express the brown fat-specific thermogenin (UCP) mRNA after adrenergic stimulation (18). They also began to accumulate drop lipids, as observed by microscopy (data not shown), 4–5 days after plating and were fully differentiated by days 7–8.

Since in vivo studies with BAT showed a good correlation between S_{14} mRNA levels and the degree of lipogenesis, we examined whether in our system the differentiation from preadipocytes to adipocytes, followed by increases in fat accumulation, influences S_{14} gene expression. As shown in Fig. 1A, differentiation into adipocytes is accompanied by the onset of S_{14} gene expression. Nondetectable levels of the mRNA for S_{14} were found in brown preadipocytes on day 4 after seeding. A low, but significant, concentration of S_{14} mRNA was found on day 5, when cells are already confluent, and during differentiation to adipocytes, the relative level of mRNA for S_{14} increased dramatically (80 ± 10-fold from days 5 to 7) in parallel with lipid accumulation.

Thyroid hormone induction of S_{14} gene expression

S_{14} mRNA has been reported to be increased in the BAT of the hypothyroid animal. However, in this model of cultured brown preadipocytes, T_3 is a strong inductor of S_{14} gene expression (Fig. 1C). Maximal stimulation of mRNA S_{14} by T_3 was achieved in cells before reaching maturity. A 22-fold increment was observed on day 6, while the increase in the level of S_{14} transcripts in totally differentiated cells exposed for 48 h to 2 nM T_3 was only 3-fold (days 7 and 8; Fig. 1C). These results probably reflect the fact that the levels of S_{14} mRNA expression in postconfluent cells are already extremely high, and T_3 treatment can only result in a further 3-fold stimulation. Retinoic acid seems to have a very moderate effect on this gene, and this effect appears to be dependent on the differentiation stage of the cells. After 48 h in the presence of 1 μM retinoic acid, the cells showed a moderate, but reproducible, increase in S_{14} mRNA on day 6 (2.5-fold) and a 2-fold decrease in mature adipocytes (days 7 and 8; Fig. 1C).

Addition of T_3 (50 nM) to 6-day-old cultures induced accumulation of mRNA S_{14} in the adipocytes in a time-dependent manner (Fig. 2). Increased concentrations of mRNA S_{14} were detectable by 3 h (4 ± 0.5-fold) and reached a maximum 24 h after administration of the hormone (3.5 ±

FIG. 1. S$_{14}$ gene expression during brown preadipocyte differentiation: effects of thyroid hormone and retinoic acid. A, Brown adipocytes were grown in control medium, and total cellular RNA was isolated at various stages of differentiation. B, Methylene blue staining of the membranes after transfer. C, Brown adipocytes were grown in T$_3$-free medium (Tx) or in T$_3$-free medium containing T$_3$ (2 nM) and/or retinoic acid (RA; 1 μM) for 48 h. Cells were harvested at specified times after addition of the hormones, and total cellular RNA was isolated.
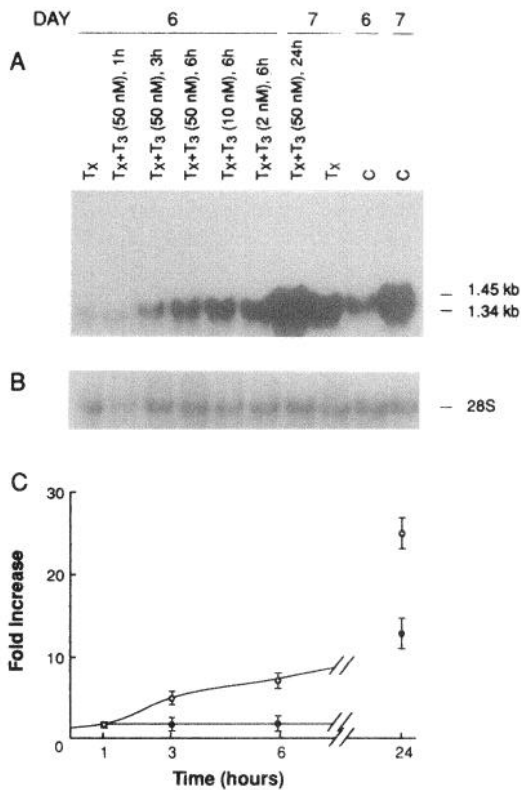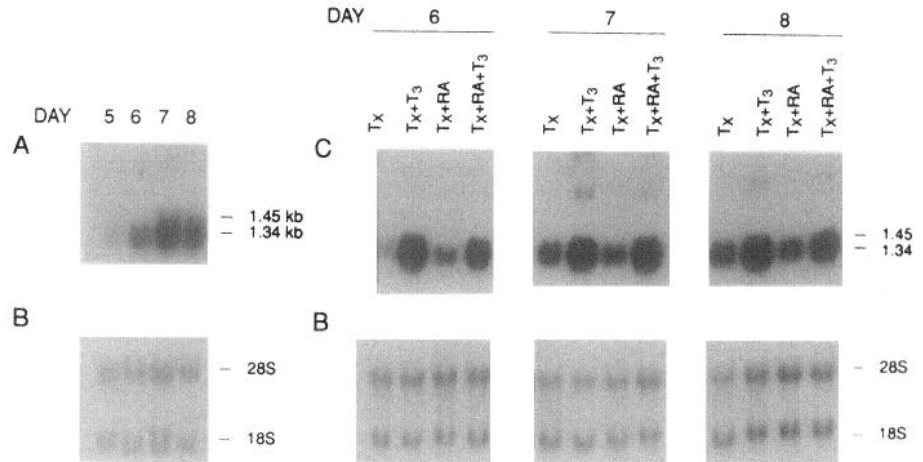

FIG. 2. Time course of induction of S$_{14}$ mRNA by T$_3$. A, Brown adipocytes were grown in T$_3$-free medium (Tx) for 24 h before the addition of T$_3$ to the medium. Cells were harvested at specified times after T$_3$ addition, and total cellular RNA was isolated. B, Same blot probed with 28S rRNA. C, Quantitation of the autoradiograms by computer-assisted densitometry. The mRNA level was expressed as the fold increase relative to the lowest value at zero time. ○, Effect of thyroid hormone on S$_{14}$ gene expression; ●, differentiation effect on S$_{14}$ gene expression. kb, Kilobases.

0.8-fold). In the absence of T$_3$, no increase in the S$_{14}$ mRNA concentration was observed at 3 or 6 h compared with the control value at 0 h (Fig. 2C), although a significant increase was observed between 12–24 h. This increment corresponds to the effect of differentiation, which was observed even in the absence of the hormone. The inductive response was

also observed at lower concentrations of T$_3$ (2 nM; this range encompasses physiological levels of thyroid hormone).

It has been suggested that modulation of gene expression by thyroid hormone can be mediated by stabilization of nuclear or mature transcripts (19). Therefore, we determined the degradation rate of S$_{14}$ mRNA by measuring the remaining message at various times after treatment of the cells with α-amanitin, a specific inhibitor of polymerase-II transcription. On day 6, half of the cultures were treated with 10 nM T$_3$. α-Amanitin (2 μg/ml) was added 24 h later to all cultures. At different times, cells, stimulated or not with T$_3$, were harvested, and S$_{14}$ transcripts were measured by Northern blot analysis. As shown in Fig. 3, there was little or no degradation in T$_3$-treated cells, but degradation occurred at a faster rate in control cultures not treated with T$_3$ (half-life, ~90 min).

Insulin induction of S$_{14}$ gene

The level of S$_{14}$ mRNA was measured in postconfluent brown adipocytes after the addition of insulin. Figure 4 shows a dose-response curve for the induction of S$_{14}$ mRNA in 6-day-old cultures of brown adipocytes treated for 24 h with insulin. Insulin treatment increased the level of S$_{14}$
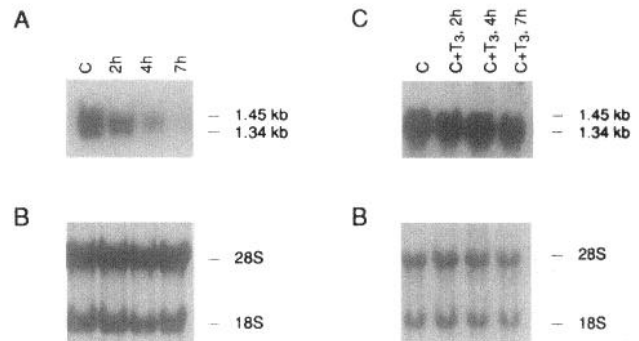

FIG. 3. Effect of T$_3$ on S$_{14}$ message stability. A and C, Brown adipocytes were grown until day 6, then half of the cultures were pretreated with 10 nM T$_3$. Twenty-four hours later, α-amanitin (2 μg/ml) was added to all cultures. The cells were incubated for the times indicated, RNA was isolated, and Northern blots were performed. B, Methylene blue staining of the membranes after transfer. C, Control; kb, kilobases.
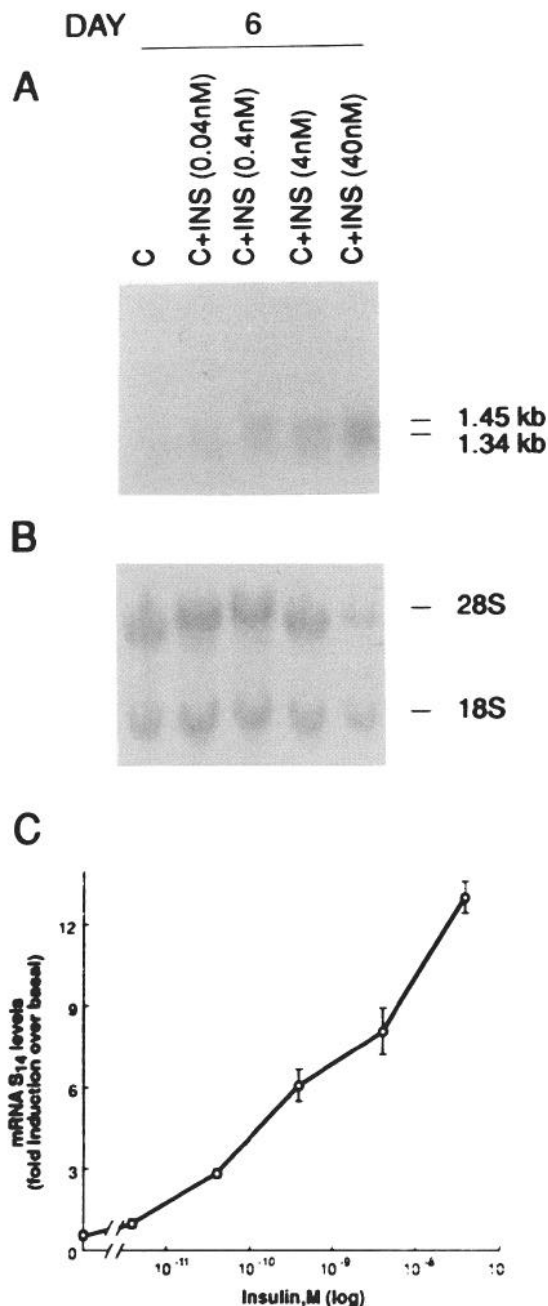
FIG. 4. Dose-response of insulin (INS) action on the induction of S$_{14}$ mRNA. Brown adipocytes were grown in DMEM medium without any insulin added for 24 h before the addition of insulin at the indicated concentrations. Cells were harvested 24 h after insulin addition, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. C, Control.

transcripts in a dose-dependent manner. Half-maximal stimulation was reached at a physiological concentration of insulin (0.4 nM), and maximal stimulation (13-fold) at 40 nM insulin. No bigger induction was observed with higher doses of insulin (data not shown). The effect of insulin was also detectable at later differentiation stages and as early as 1 h after addition of the hormone (data not shown).

The effect of IPG on S$_{14}$ mRNA levels was also tested.

Seven-day-old cultures were grown for 24 h in the absence of insulin before adding IPG (4 μM), and the cells were harvested 1 h later. We found that IPG was able to produce an 8-fold increase in S$_{14}$ mRNA levels as early as 1 h after its addition (Fig. 5).

Insulin, IGF-I, and IGF-II are members of a family of related peptide hormones. Since both IGF-I and IGF-II receptors show considerable similarities to those corresponding to insulin, and it has been shown that there is some kind of cross-affinity between them, we further tested the effect of recombinant IGF-I and IGF-II on S$_{14}$ mRNA levels. In these experiments, cells were incubated in insulin-free medium for 24 h with either IGF-I or IGF-II. Figure 6 shows that the lower dose of IGF-I tested (1 nM) was sufficient to elicit maximal induction of S$_{14}$ mRNA levels. Higher doses were not effective at inducing further increases in S$_{14}$ levels. On the contrary, IGF-II had little or no effect on the level of S$_{14}$ mRNA.

Effects of NE and cAMP on basal and stimulated S$_{14}$ gene expression

The effects on S$_{14}$ gene expression of stimuli such as NE, cAMP, or glucagon, known to be strong negative effectors of lipogenesis, were also tested. NE, a lipolytic agent that plays a central role in BAT metabolism, has a marked effect on S$_{14}$ mRNA levels (Fig. 7). Treatment of 8-day-old cultures with 10 μM NE for 24 h elicited a 20 ± 3-fold decrease in mRNA S$_{14}$ abundance. T$_3$ and insulin were not able to completely prevent this effect. S$_{14}$ mRNA levels in cells pretreated with T$_3$ or insulin before adding NE were 25%
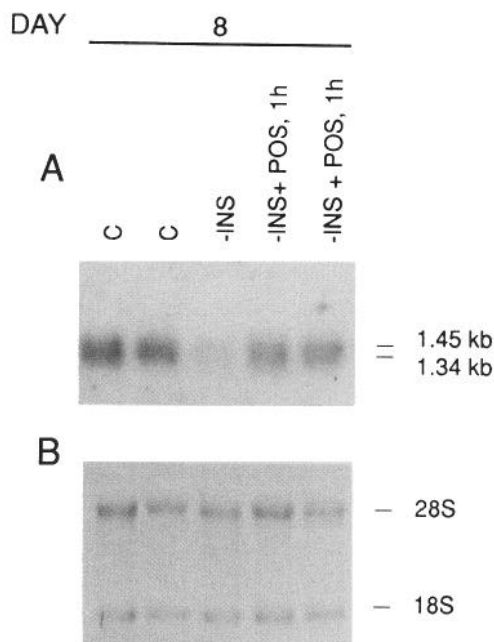

FIG. 5. Induction of S$_{14}$ mRNA levels by the insulin (INS)-induced IPG. Seven-day-old brown adipocytes were grown for 24 h in DMEM medium with (C) or without (−INS and IPG) insulin before the addition of 4 μM IPG (POS). Cells were harvested 1 h later, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. kb, Kilobases.
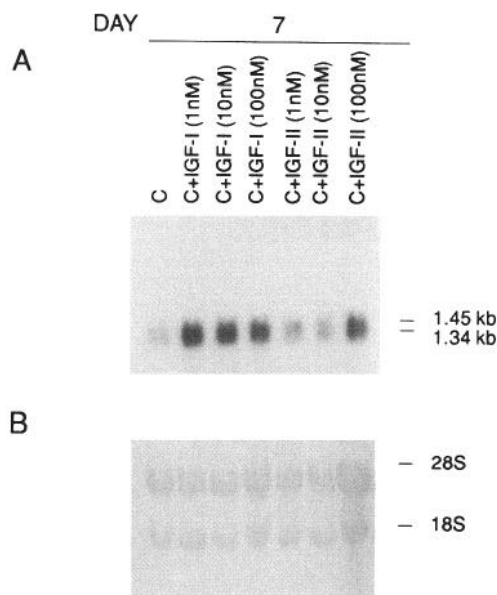
FIG. 6. Dose response of the actions of IGF-I and IGF-II on the induction of S$_{14}$ mRNA. Brown adipocytes were grown in DMEM without any insulin. IGF-I and IGF-II were added at the indicated concentrations. Cells were harvested 24 h after addition of the growth factors, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. C, Control; kb, kilobases.
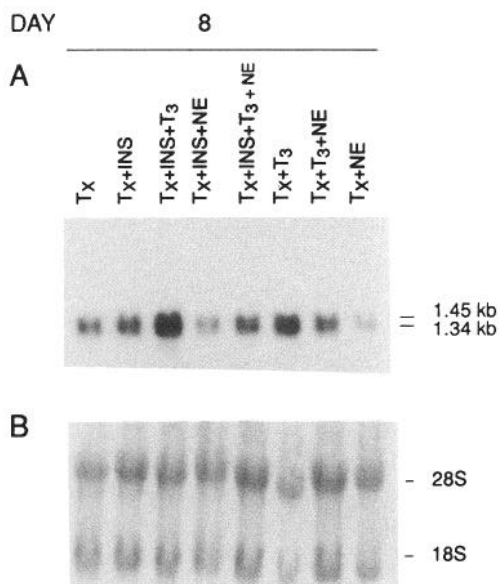

FIG. 7. Inhibition of S$_{14}$ gene expression by NE. A, Brown adipocytes were grown in T$_3$-free medium (Tx) or in T$_3$-free medium containing NE (10 μM), T$_3$ (10 nM), insulin (INS; 4 nM), or a combination of the hormones for 24 h. Cells were harvested on day 8, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. kb, Kilobases.

and 20%, respectively, of the value in control pretreated cells. The effect of NE was also observed in cells grown in control medium (data not shown).

Treatment of brown adipocytes with glucagon (100 nM), 8-Br-cAMP (1 mM), or forskolin (1 μM) caused a significant decrease in S$_{14}$ mRNA levels. When 8-Br-cAMP was added

to 6-day-old cultures and the level of S$_{14}$ mRNA was determined 24 h later, a reduction by 90% of the control value was observed (Fig. 8). A moderate effect of forskolin and glucagon on S$_{14}$ mRNA levels was observed 4 h after its addition to 7-day-old cultures (50% of the control value). Thyroid hormone, when added 1 h after the addition of glucagon, was not able to override the glucagon effect; however, if the adipocytes were preincubated with T$_3$ for 24 h before the addition of glucagon, T$_3$ behaved as a dominant positive effector of S$_{14}$ gene expression, preventing the negative signal generated by glucagon action.

Effect of carbohydrate on S$_{14}$ expression

Since it has been shown that S$_{14}$ gene expression is induced in the livers of rats fed a high carbohydrate fat-free diet and by glucose in hepatocytes in culture (13, 20), we further examined the mode of regulation of the S$_{14}$ gene by carbohydrates.

In this experiment, cells were incubated in control and low glucose medium (D) for 4 days (days 3–7), using 4 nM insulin (after 48 h, cells had disposed of the majority of the available glucose). On day 7, glucose and/or T$_3$ were also added to some cultures, and cells were harvested 5 or 24 h later.

Compared to control cultures (7 day-old; 25 mM glucose), S$_{14}$ mRNA levels in adipocytes grown in low glucose medium (D) for 4 days fell to extremely low values (~20% of the controls; Fig. 9). In contrast with the rapid induction of S$_{14}$ mRNA by glucose in rat liver *in vivo*, no induction of expression was observed 5 h after the addition of glucose (50 mM). The addition of T$_3$ was also ineffective at inducing S$_{14}$ gene expression above glucose-depleted levels. However, incubation for 24 h with glucose or T$_3$ induced a significant increase

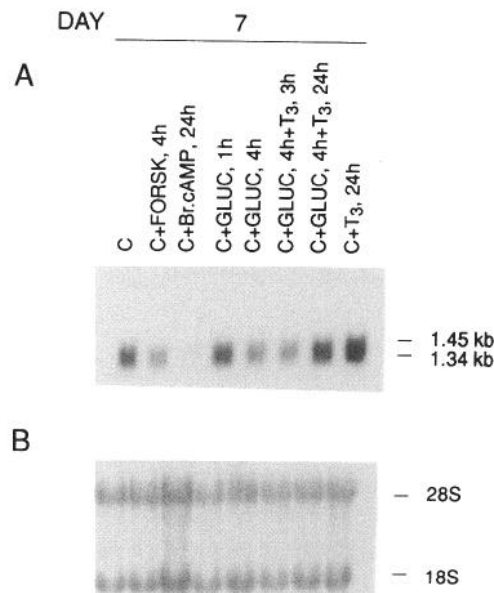FIG. 8. Inhibition of S$_{14}$ gene expression by glucagon (GLUC), cAMP, and forskolin (FORS). A, Brown adipocytes were grown in control medium (C) or in control medium containing glucagon (GLUC; 100 nM), forskolin (FORSK; 1 μM), 8-Br-cAMP (1 mM), or T$_3$ (10 nM). Cells were harvested on day 7, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. kb, Kilobases.
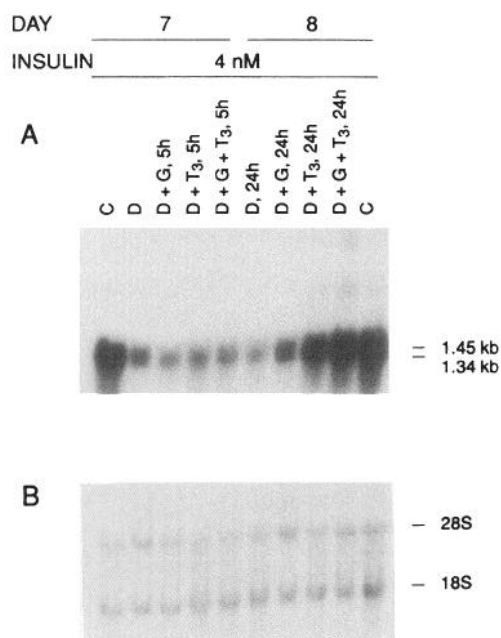
FIG. 9. Additive response to T_3 and carbohydrates of mRNA S_{14}. A, Brown adipocytes were grown in control medium (C) or DMEM low in glucose (D), containing 4 nM insulin, from day 3 without changing the medium. Glucose (G; 50 mM), T_3 (10 nM), or a combination of both was added on day 7 for 5 or 24 h. Cells were harvested at different times, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. kb, Kilobases.

FIG. 10. Effects of cycloheximide on basal and T_3- and insulin (INS)-induced expression of S_{14} mRNA. A, Brown adipocytes were grown in T_3-free medium (Tx) or in control medium (C) without insulin, and cycloheximide (CHX; 25 μM) was added to the cells 30 min before the addition of T_3 (10 nM) or insulin (40 nM). Cells were harvested at the indicated time, and total cellular RNA was isolated. B, Methylene blue staining of the membranes after transfer. kb, Kilobases.

(6 ± 1.5- and 15 ± 3-fold, respectively) in mRNA S_{14} abundance. The addition of a combination of the two treatments further induced mRNA S_{14} levels to those found in control cultures.

Effect of cycloheximide on S_{14} gene expression

Previous studies *in vivo* have shown a requirement for ongoing protein synthesis for S_{14} stimulation by T_3 in liver. To determine whether this requirement was tissue specific or a general one for this gene, we further tested the effect of cycloheximide on the T_3- and insulin-induced rise of mRNA S_{14} activity in brown adipocytes (Fig. 10). When cycloheximide (25 μM) was added 30 min before addition of the hormones, the expected rise in mRNA S_{14} was completely blocked. Cycloheximide alone was added to some cultures to determine whether inhibition of protein synthesis could affect baseline levels of this messenger. As shown in Fig. 10, expression of mRNA S_{14} in the absence of T_3 or insulin was also completely blocked (<10% of the control values), suggesting that the expression of this gene is dependent on short-lived proteins even for its expression in the absence of stimuli. The effect of cycloheximide is not a general one due to the lability of the cells, as expression of the actin gene in these cells was not modified by cycloheximide treatment (data not shown).

Discussion

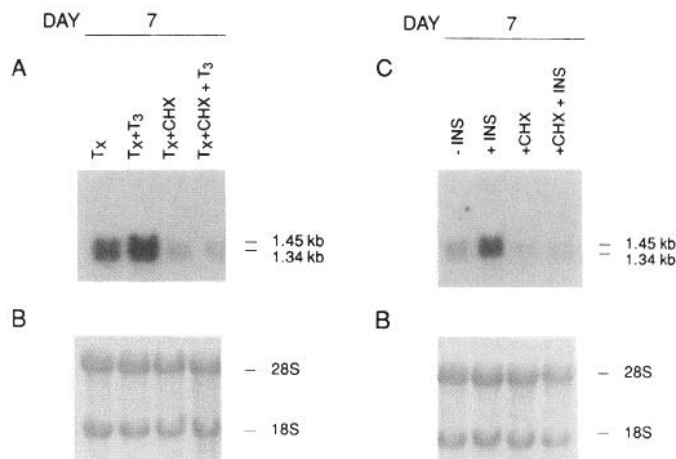In the present study we have shown that S_{14} gene expression is strongly regulated during adipogenic differentiation of brown fat cells. mRNA S_{14} concentrations were undetectable in preadipocytes (day 4) before reaching confluence and increased dramatically (80-fold) from days 5–7 when the cells were differentiated into mature brown adipocytes. This dependence of S_{14} gene expression on the differentiation stage of the cells suggests an involvement of S_{14} protein in fatty acid metabolism, because a huge increase in lipid accumulation occurs during this period due to increased lipogenesis (21).

Brown preadipocytes and adipocytes in culture respond to thyroid hormone by increasing expression of the S_{14} gene. The response to T_3 is higher on earlier days of differentiation than in totally differentiated cells, in which a moderate response is observed. This is somewhat in contrast to earlier data *in vivo*, which showed an increase in S_{14} mRNA levels in BAT of hypothyroid animals (12). This apparent discrepancy could be due to the known resistance of the BAT from hypothyroid animals to the action of β-adrenergic agents (22), which will favor the accumulation of S_{14} mRNA in this tissue, as NE is a potent agent that decreases the expression of this gene. In brown adipocytes in culture, the action of NE was the same in cells grown in serum with or without T_3 (data not shown), and no apparent resistance to this agent was observed. Besides, this discrepancy may reflect a more complex regulation of fatty acid synthesis and thermogenesis in brown fat *in vivo* than in our model of brown cells in culture.

The induction of S_{14} transcripts after stimulation by thyroid hormone may be accounted for by a combination of several processes (4, 6). We have observed that the stability of S_{14} mRNA, measured after α-amanitin addition, is greater in T_3-treated cultures than in control cells. The half-life of S_{14} mature transcripts in the absence of T_3 in brown adipocytes appears to be very similar to that reported in hepatocytes in culture (~90 min) (23). This posttranscriptional regulation of S_{14} by thyroid hormone can be caused by the existence of

one or several as yet unidentified regulatory factors expressed after T_3 stimulation, which bind to S_{14} mRNA in brown adipocytes, enhancing its stability.

The role of insulin in controlling S_{14} gene expression has been previously suggested (24). Jump *et al.* (25) have also presented evidence for transcriptional control of S_{14} levels by insulin *in vivo*. The present study, carried out in a defined controlled primary culture, demonstrates that insulin is required for complete expression of this gene during the early phases of adipocyte differentiation. Moreover, insulin increases the abundance of mRNA S_{14} in terminally differentiated brown fat cells. The addition of T_3 in the presence of insulin causes a 5-fold increase in the S_{14} mRNA concentration, suggesting that both hormones are essential to obtain a complete response. The additive effect between thyroid hormone and insulin has also been observed in fetal brown adipocyte primary cultures with malic enzyme (26). The molecular mechanisms by which insulin controls metabolism and gene expression are still not clearly understood. It has been suggested that phospholipase-C-catalyzed hydrolysis of a glycophospholipid would take part in signaling by insulin (27). Insulin would generate diacylglycerol and an IPG, consisting of inositol monophosphate linked to nonacetylated glucosamine and several residues of galactose, that would act as an insulin mediator (28, 29). It has been shown that IPG is able to mimic some of the short and long term actions of insulin on metabolism (30, 31). A very recent study showed that two hepatic genes, whose expression is regulated by insulin, are under the control of the insulin-induced oligosaccharide (32). We now extend this observation by demonstrating that IPG is involved in the activation of another insulin-inducible gene, such as S_{14}, further supporting the idea that IPG could be mediating some of the actions of insulin, such as those regarding the regulation of gene expression.

In this report we have shown that in addition to the known effect of insulin, IGF-I is a very important regulator of S_{14} gene expression. IGF-I had a significant effect on the level of S_{14} transcripts, and a 10-fold increase over basal values (in the absence of insulin) was achieved at the lower dose tested (1 nM). Furthermore, the maximum effect of IGF-I was achieved at 1/40th of the dose of insulin that gives a maximal response, suggesting that in these cells, at least some of the effects of insulin on S_{14} mRNA abundance might be mediated through the IGF-I receptor. Eventual characterization of insulin regulatory regions in the S_{14} gene will help to better understand the precise mechanisms involved in insulin induction of S_{14} gene expression.

The mechanism by which T_3 and insulin control mRNA S_{14} levels in brown adipocytes appears to be dependent on protein synthesis. This observation coincides with previous studies *in vivo* suggesting an essential role for proteins with an exceedingly rapid $t_{1/2}$ for T_3-stimulated expression of several hepatic genes, including S_{14} (33). However, it should be noted that in contrast with the lack of effect of the drug in the liver in the absence of T_3, basal expression of S_{14} gene in brown adipocytes is also extremely sensitive to the action of the drug. These results suggest that in brown adipose

tissue, in addition to the effect of cycloheximide on some, as yet unidentified, components of the hormonal regulatory machinery, there are some cellular factors sensitive to cycloheximide that are essential for basal expression of the gene.

NE, through sympathetic inervation, is a potent agent for controlling BAT lipid metabolism and thermogenesis (34) *in vivo*. Here we have shown that NE has a profound effect on S_{14} gene expression, and the addition of this agent to fully mature brown adipocytes *in vitro* practically abolishes S_{14} gene expression. This fall in mRNA S_{14} levels is accompanied by a concomitant loss of lipid droplets in the cells. NE is a potent inhibitor of the expression of this gene, as it is also able to suppress the effects of strong adipogenic agents, such as insulin and T_3. The inhibitory effect of NE on the induction of S_{14} mRNA by high doses of insulin and T_3 suggests that adrenergic stimulation plays a dominant negative role in the control of S_{14} gene expression. Paradoxically, *in vivo* cold exposure for a short time, known to be a stimulus for NE release from sympathetic nerve terminals, does not modify S_{14} levels in BAT (12).

In addition to NE, other agents acting through the adenylate cyclase signaling pathway, such as cAMP, forskolin, and glucagon, were able to diminish the level of S_{14} transcripts. Although the addition of 8-Br-cAMP to fully mature brown adipocytes resulted in an almost complete inhibition of S_{14} gene expression, both forskolin and glucagon exerted only moderate effects on S_{14} mRNA levels. This stands in contrast to the pronounced inhibitory effects of glucagon in liver *in vivo* and in hepatocytes in culture (8) and could be due to a reduced level of glucagon receptors in these cells compared with the high levels found in liver cells.

Finally, in accordance with previous studies *in vivo* and *in vitro*, we have shown that S_{14} gene expression is absolutely dependent on glucose concentrations. Normal levels of glucose are also required for maximal stimulatory effects of insulin and thyroid hormone on the S_{14} gene. These results clearly suggest that the concentration of ambient glucose, or a metabolite derived from glucose, is a critical factor for T_3- and insulin-dependent induction of the S_{14} gene.

In summary, the accumulation of S_{14} transcripts when brown adipocytes are starting to differentiate and the responses of this gene to classical regulatory hormones of lipid synthesis strongly suggest an important function of the S_{14} protein in the initial phases of adipocyte differentiation.

Primary cultures of brown preadipocytes provide an *in vitro* system in which the S_{14} gene is strongly regulated by differentiation and by all of the hormones and agents that control its expression *in vivo*. Therefore, adipogenically determined brown preadipocytes constitute an excellent model system in which to explore the cellular and molecular mechanisms through which expression of the S_{14} gene is regulated. Work is underway to identify the regulatory sequences in the S_{14} promoter responsible for this regulation and the precise role of S_{14} protein in brown adipocyte differentiation.

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