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Transcriptional Activation of Type III Inner Ring Deiodinase by Growth Factors in Cultured Rat Brown Adipocytes*

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ABSTRACT

The activity of the type III inner ring deiodinase (DIII), which converts T_4 and T_3 to inactive metabolites, is induced by serum and growth factors in primary cultures of rat brown adipocytes. The contribution of pretranslational mechanisms to this increase in DIII activity was examined in the present studies. DIII mRNA is undetectable in differentiated brown adipocytes when cultured in serum-free medium. However, exposure to epidermal growth factor (EGF), acidic or basic fibroblast growth factors (aFGF or bFGF) increase DIII transcript levels. Lesser inductions are found with platelet-derived growth factor, and insulin-like growth factor I has no effect. Maximal induction of DIII mRNA is obtained after 9 h of exposure to EGF, bFGF, or aFGF at a concentration of 10 ng/ml. The increase in DIII mRNA in response to aFGF, bFGF, and EGF requires gene transcription and protein synthesis, as the inductive effect on mRNA is completely blocked by actinomycin D or cycloheximide. The DIII mRNA half-life is 4 h when stimulated with bFGF and increases to 12 h when 10% serum, EGF, or aFGF is present.

In conclusion, EGF, aFGF, and bFGF increase DIII mRNA expression in differentiated brown adipocytes. This effect appears to be exerted at the level of both enhanced transcription and mRNA stabilization. (*Endocrinology* **139:** 634–639, 1998)

THE IODOTHYRONINE deiodinases are key enzymes regulating thyroid hormone action in peripheral tissues. Three different deiodinases have been described, each of which shows distinctive characteristics, which include substrate specificity and reaction kinetics, tissue expression patterns, and relative sensitivity to the inhibitors 6-*n*-propyl-2-thiouracil and gold thioglucose and to the factors regulating their activity (1). The type I and II deiodinases (DI and DII, respectively) catalyze the conversion of the prohormone T_4 to the biologically active T_3 (1). Therefore, DI and DII facilitate thyroid hormone action. In contrast, the type III deiodinase (DIII) removes iodine from the inner ring of both T_4 and T_3, resulting in the formation of the inactive thyroid hormone analogs rT_3 and 3,3′-diiodothyronine (2). DIII has been postulated to have an important role in protecting various tissues from high T_3 concentrations, especially during the early stages of development (3).

DIII activity has been detected in many rat tissues, including brain (4–6), retina (7), placenta (8), skin (9), intestine, and skeletal muscle (10, 11). Expression of this protein is highest in most of these tissues during development, with levels being low or undetectable in the adult (5, 12). In addition to these development-associated changes, several other factors are known to regulate DIII activity. Thus, hypothyroidism leads to a decrease in both brain and skin DIII activity, whereas hyperthyroidism results in increased DIII activities (9, 13–15). In addition, DIII activity is strongly induced in certain cell culture systems by various growth factors and serum components. For example in rat astroglial cells, several growth factors, including acidic and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), as well as phorbol esters (16) and 8-bromo-cAMP (16, 17) are potent inducers of DIII activity.

DIII activity has not been described to be present in brown adipose tissue (BAT) *in vivo*. Studies in our laboratory have recently demonstrated that DIII activity is markedly induced by serum and several growth factors in primary cultures of rat brown adipocytes (18). This induction of DIII activity by specific growth factors may be relevant to the regulatory control of this enzyme during early development.

Recently, cDNAs coding for the three deiodinases have been isolated from rat (19–22) and other species (22–25). Sequences analysis has demonstrated that all of them contain an in-frame TGA codon that results in the incorporation of the uncommon amino acid selenocysteine into the peptide chain during translation (20, 23, 25). These enzymes are among the few selenocysteine-containing proteins that have been described in mammals and other eukaryotes (26). In the present work we have used a rat DIII cDNA probe to study for the first time the regulation of DIII expression at the mRNA level by growth factors. Using cultured rat brown adipocytes as a model system, we demonstrate that several growth factors induce DIII mRNA levels, an effect most likely exerted by an increase in transcription.

Materials and Methods

Materials

DMEM was obtained from Life Technologies (Uxbridge, UK). Newborn calf serum (NCS) was purchased from Flow (Paisley, Scotland).

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Antibiotics were obtained from the local pharmacy. BSA (in solution at 22%, pH 7.2) was purchased from Ortho Diagnostic Systems, Johnson & Johnson Co. (Raritan, NJ). Collagenase, bovine insulin, ascorbic acid, guanidinium HCl, 3-*N*-morpholino-propanesulfonic acid (MOPS), dithiothreitol, 6-*n*-propyl-2-thiouracil, T_3, norepinephrine, and endothelin-1 were obtained from Sigma Chemical Co. (St. Louis, MO). Agarose was purchased from FMC Bioproducts (Rockland, ME). Cycloheximide, actinomycin D, bFGF, aFGF, insulin-like growth factor I (IGF-I), EGF, and PDGF (B/B homodimer) were obtained from Boehringer Mannheim (Mannheim, Germany). Unless otherwise specified, aFGF was used in the presence of 50 μg/ml heparin (final concentration), as recommended for proper biological activity. Ion exchange resin AG1-X8 was obtained from Bio-Rad (Richmond, CA). Radiolabeled [α-^{32}P]deoxy-CTP (3000 Ci/mmol) was purchased from Amersham International (Aylesbury, UK). Formamide was purchased from Fluka (Buchs, Switzerland) or Merck, and the oligo-labeling system was obtained from Pharmacia (Uppsala, Sweden). Nytran membranes were purchased from Renner (Darmstadt, Germany). Charcoal (Norit-A) to prepare hormone-depleted serum was obtained from Amend (New York, NY).

Cultures of brown adipocytes

Precursor cells were obtained from the interscapular brown adipose tissue of 20-day-old rats (Sprague-Dawley), isolated according to the method described by Néchad *et al.* (27) with modifications (28), using collagenase digestion (0.2%) in DMEM-1.5% BSA at 37 C and filtration through 250-μm pore size silk filters. Mature cells were allowed to float, and the infranatant was filtrated through 25-μm pore size silk filters and centrifuged. A hypoosmotic shock (to remove red blood cells) was not performed. Precursor cells were seeded in 25-cm^2 culture flasks at a density of 1500–2000 cells/cm^2 on day 1 and grown in DMEM supplemented with 10% NCS, 3 nM insulin, 10 mM HEPES, 50 IU penicillin, 50 μg streptomycin/ml, and 15 μM ascorbic acid. Culture medium was changed on day 1 and every second day thereafter until the experiment was performed. Precursor cells proliferate actively under these conditions, reach confluence on the fourth or fifth day after seeding (40,000–60,000 cells/cm^2), and then differentiate into mature brown adipocytes. All studies were performed in fully differentiated brown adipocytes (on the eighth day after seeding).

Both NCS and hypothyroid serum (Hypo serum) were used for culture. The latter was obtained by depleting NCS of thyroid hormones with the anion exchange resin AG1X8, as previously described (29). Hypo serum contained about 10% or less of the original amount of thyroid hormones, as assessed by RIA (30). In NCS, concentrations of T_4 and T_3 were 77 and 1.3 nM, respectively. These levels were decreased to 2.2 nM T_4 and 0.13 nM T_3 in Hypo serum.

RNA preparation and Northern blot analysis

Total cellular RNA was extracted in guanidinium-HCl as previously described (31), using ethanol precipitation. The recovery was 60–90 μg total RNA/25-cm^2 flasks (containing $\sim 5 \times 10^6$ cells). For isolation of polyadenylated [poly(A)$^+$] RNA, cells were collected, and mRNA was isolated using oligo(deoxythymidine) cellulose as previously described (32). For Northern analysis, total RNA (20 μg) or poly(A)$^+$ (5 μg) was denatured and electrophoresed on a 2.2-M formaldehyde/1% agarose gel in 1 $\times$ MOPS buffer and transferred to nylon membranes (Nytran) as previously described (28). A 1100-bp fragment of a rat DIII cDNA clone (21), corresponding to most of the translated region of the DIII mRNA, was used as a probe by labeling with [α-^{32}P]deoxy-CTP using random primers (SA, $>10^8$ cpm/μg DNA). Filters were hybridized for 20 h at 50 C [40% formamide, 5 $\times$ SSC (standard saline citrate), 2 $\times$ Denhardt's, and 0.1% SDS] and washed four times in 2 $\times$ SSC-0.2% SDS at room temperature for 15 min, then twice in 0.1 $\times$ SSC-0.2% SDS at 65 C for 20 min. Autoradiograms were obtained from the filters and quantified by laser computer-assisted densitometry (Molecular Dynamics, Sunnyvale, CA). The filters were hybridized with cyclophilin as a control to correct for differences between lanes in the amount of RNA (33). Some filters were hybridized with the rat uncoupling protein (UCP) cDNA (34).

All of the experiments were repeated at least twice, using duplicates, Northern blot analysis was often performed in both. The more complete and representative experiments are shown in the figures.

Results

We have recently reported that brown adipocytes differentiated in primary culture are able to express DIII activity and that this activity is markedly induced by different growth factors and by serum (18). The increases in DIII activity are dependent on the dose and exposure time used.

Growth factors increase DIII mRNA levels

We have tested the effects of different growth factors on DIII mRNA expression in brown adipocytes. Completely differentiated brown adipocytes were exposed to different growth factors in serum-free medium for a period that ranged from 5–18 h. Northern blot analysis showed hybridization of the DIII probe primarily to a band of approximately 2.2 kilobases (Fig. 1). Basal expression of DIII mRNA was undetectable in the presence of serum-free medium (Fig. 1A, control, lanes 11 and 12). A clear induction of DIII mRNA expression is observed in cells exposed to EGF, aFGF, and bFGF (Fig. 1, A and B). No induction is observed by IGF-I and endothelin-1 at the concentrations used in this study. A smaller induction is observed using transforming growth factor-β1 (TGFβ1; Fig. 2). PDGF did not stimulate DIII mRNA at a dose of 5 ng/ml (Fig. 1A), but a clear induction was observed at a dose of 50 ng/ml (Fig. 2).

Dose-response and time-course experiments of DIII mRNA expression

Figure 3 demonstrates that EGF, aFGF, and bFGF stimulate expression of DIII mRNA in a dose-dependent fashion, with

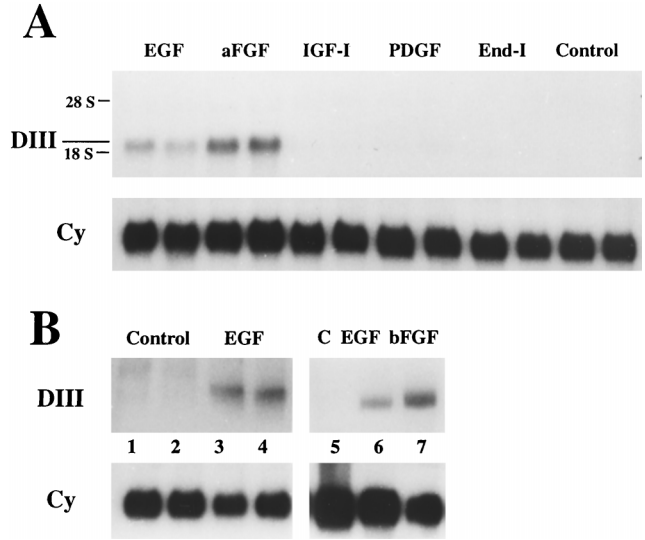

FIG. 1. A, Northern analysis demonstrating induction of DIII mRNA by growth factors in differentiated rat brown adipocytes. Cells were exposed for 12 h to EGF (10 ng/ml), aFGF (4 ng/ml), IGF-I (1 nM), PDGF (5 ng/ml), and endothelin-1 (10 nM) in serum-free medium. Twenty micrograms of total RNA were loaded per lane using duplicates. B, Induction of DIII mRNA by EGF (10 ng/ml for 5 h) in serum-free medium (*left panel*). Induction of DIII mRNA by EGF and bFGF (10 ng/ml and 18 h for both growth factors) in serum-free medium (*right panel*). Five micrograms of poly(A)$^+$ RNA were loaded per lane. A control hybridization with a cyclophilin cDNA probe is also shown.

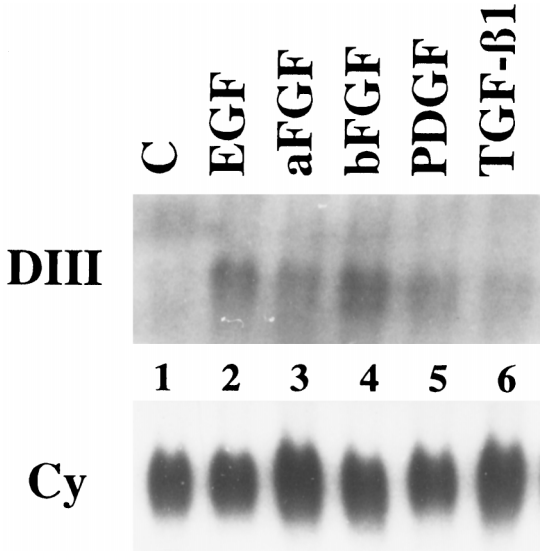

FIG. 2. Induction of DIII mRNA by growth factors. Cultures of differentiated brown adipocytes in serum-free medium were exposed to EGF (10 ng/ml), aFGF (4 ng/ml), bFGF (10 ng/ml), PDGF (50 ng/ml), or TGFβ (10 ng/ml) for 7 h. Twenty micrograms of total RNA were used per lane for Northern analysis. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes.

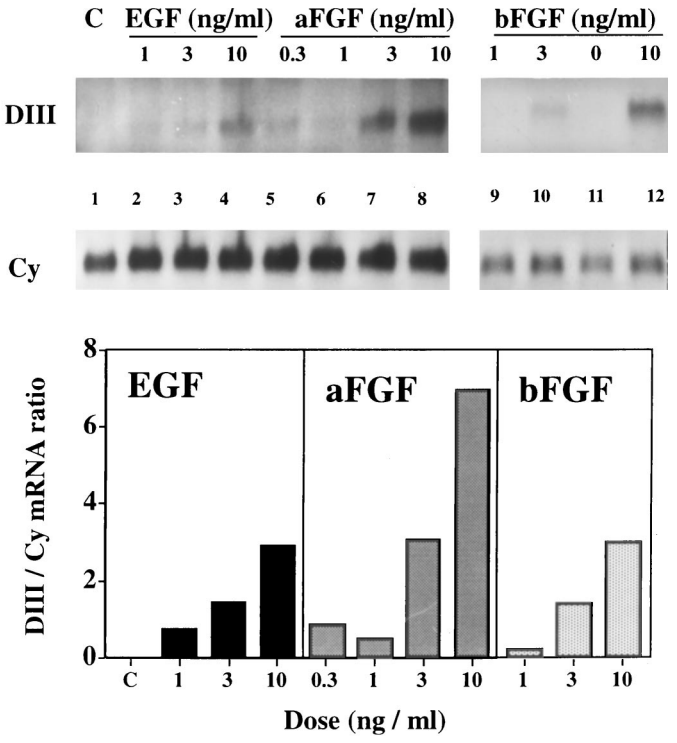

FIG. 3. Dose-dependent induction of DIII mRNA by EGF, aFGF, and bFGF. Cultures of brown adipocytes were exposed for 12 h to different doses of EGF, aFGF, and bFGF in serum-free medium. Twenty micrograms of total RNA were used per lane for Northern analysis. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes.

maximal induction observed at the highest dose tested (10 ng/ml).

To analyze the time dependency of this induction, cells were exposed to EGF (10 ng/ml), aFGF (4 ng/ml), and bFGF (10 ng/ml) for 4, 9, and 24 h. Northern blot analysis (Fig. 4) showed that maximal induction occurred around 9 h of exposure for the three growth factors tested (maximal induction is found between 8–12 h). Densitometric analysis reveals that aFGF is more potent than EGF and bFGF.

DIII mRNA inductions are sensitive to actinomycin D and cycloheximide

Studies with actinomycin D and cycloheximide were performed to determine the dependence of DIII induction on transcription and protein synthesis. Cells were exposed for 6 h to aFGF, bFGF, and EGF in the presence or absence of either actinomycin D or cycloheximide (which were added 15 min before the addition of growth factors). Figure 5 shows that actinomycin D and cycloheximide completely abolish the induction of DIII mRNA produced by both aFGF and bFGF, suggesting that gene transcription and protein synthesis are required for the effects of these agents on DIII mRNA expression.

DIII mRNA stability

Experiments were performed to determine the stability of the DIII mRNA. Mature adipocytes, cultured in the presence of 1% NCS, were exposed to bFGF (10 ng/ml) for 7 h to stimulate DIII mRNA expression. Cells were then switched to either serum-free medium or one containing 10% NCS, Actinomycin D was added, and cells were harvested at varying times for the preparation of RNA. Northern blot analysis (Fig. 6A) shows that the presence of serum plays an important role in increasing the DIII mRNA stability. In cells cultured in serum-free medium, the DIII mRNA level was markedly reduced after 7 h of exposure to actinomycin D. In contrast, only a 20% reduction was observed in cells exposed to 10% serum. Quantitative analysis indicated that the DIII mRNA half-life was approximately 4 h after induction with bFGF in the absence of serum and was longer than 12 h when serum was present.

The effects of EGF and aFGF on DIII mRNA stability were also examined in cells cultured and maintained in 1% NCS (Fig. 6B). Basal levels were undetectable (results not shown). The results demonstrate that, as in the case of serum, EGF and aFGF significantly prolonged the half-life of this transcript.

Expression of DIII and UCP mRNAs

Our previous studies demonstrated before that the expression of UCP, a specific marker for brown fat, is maintained in brown adipocytes even in the presence of high DIII activities (18). To check for the simultaneous expression of UCP and DIII mRNAs, cells were exposed to different growth factors, and at the same time T_3 and norepinephrine were added to stimulate UCP mRNA expression. T_3 was added at 5 nM to compensate for the amounts expected to be degraded by DIII. In Fig. 7 it is shown that after 8 h of exposure both mRNAs were present in the cells. In other words, the presence of these growth factors involved in proliferation did not prevent the induction of UCP mRNA ex-

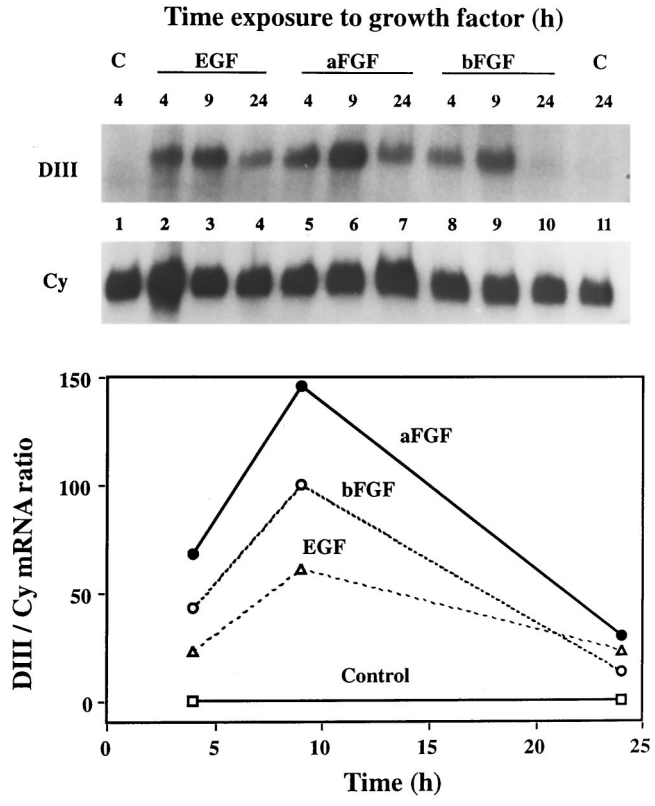

FIG. 4. Time course of induction of DIII mRNA by growth factors. Cultures of brown adipocytes maintained in medium containing 1% hypothyroid serum were exposed for 4, 9, or 24 h to EGF (10 ng/ml), aFGF (4 ng/ml), or bFGF (10 ng/ml). Twenty micrograms of total RNA were used per lane for Northern analysis. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes.

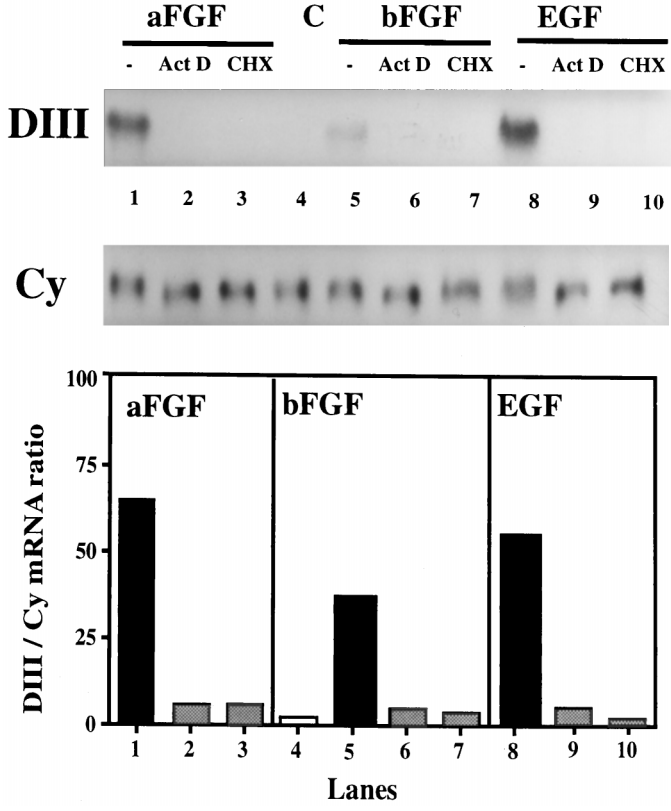

FIG. 5. Effects of actinomycin D and cycloheximide on DIII mRNA. Cultures of brown adipocytes were exposed for 6 h to aFGF (10 ng/ml), bFGF (10 ng/ml), or EGF (10 ng/ml) in medium containing 1% hypothyroid serum and actinomycin D (5 μg/ml) or cycloheximide (25 μM). Twenty micrograms of total RNA were used for Northern analysis. C, Basal expression. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes.

pression by catecholamines; therefore, there are true brown adipocytes.

Discussion

The outer and inner ring deiodinases control thyroid hormone action by providing the appropriate T_3 concentrations required for specific functions in a given tissue. These enzymes are regulated by a number of hormonal factors, including T_3 and T_4. Other factors regulating DIII activity include retinoids, growth factors, and phorbol esters (16, 35).

DIII appears to play an important role during mammalian development, as it is the predominant deiodinase expressed in the placenta and a number of fetal tissues (5, 7, 10). A similar expression pattern has been noted in anuran amphibians, where DIII expression predominates in the early stages of development. Later, DII expression becomes prominent during metamorphic climax in those tissues undergoing the most dramatic anatomical restructuring, such as tail, limbs, and intestine (36). These observations have led to speculation that DIII serves a protective role in limiting early fetal exposure to T_4 and T_3.

We have recently shown that DIII activity can be stimulated in cultured brown adipocytes by serum or a variety of growth factors in a dose- and time-dependent manner (18). In the present studies, we further characterized the mechanisms involved in this stimulatory activity. The predominant 2.2-kilobase DIII mRNA species noted in cultured adipocytes corresponds to that previously reported in neonatal skin, placenta, and cerebral cortex (21). Furthermore, we observed that growth factor-induced increases in DIII mRNA levels accompany the induction of DIII activity in this model cell culture system. This stimulation is relatively short-lived and depends on the growth factor added. As is the case of DIII activity levels (18), EGF and aFGF are the most potent inducers of DIII mRNA (18). For example, aFGF exhibits a stimulatory effect at 3 ng/ml on both of these parameters, and its effect is still present after cells have been exposed for 24 h. In the case of less potent agents, such as bFGF, the stimulatory effects on DIII mRNA wane more quickly. Other investigators (16) have shown that aFGF and bFGF also stimulate DIII activity in a different model system, primary cultures of rat astroglial cell. It thus appears that DIII is upregulated in several cell types by growth factors that act via tyrosine kinase receptors. The specific signaling pathways used by EGF or both fibroblast growth factors to increase DIII transcription are still a matter of speculation, although the *erk* cascade has been recently proposed to regulate the induction of DIII in astroglial cells (37).

The experiments performed in the presence of cycloheximide and actinomycin indicate that DIII mRNA induction

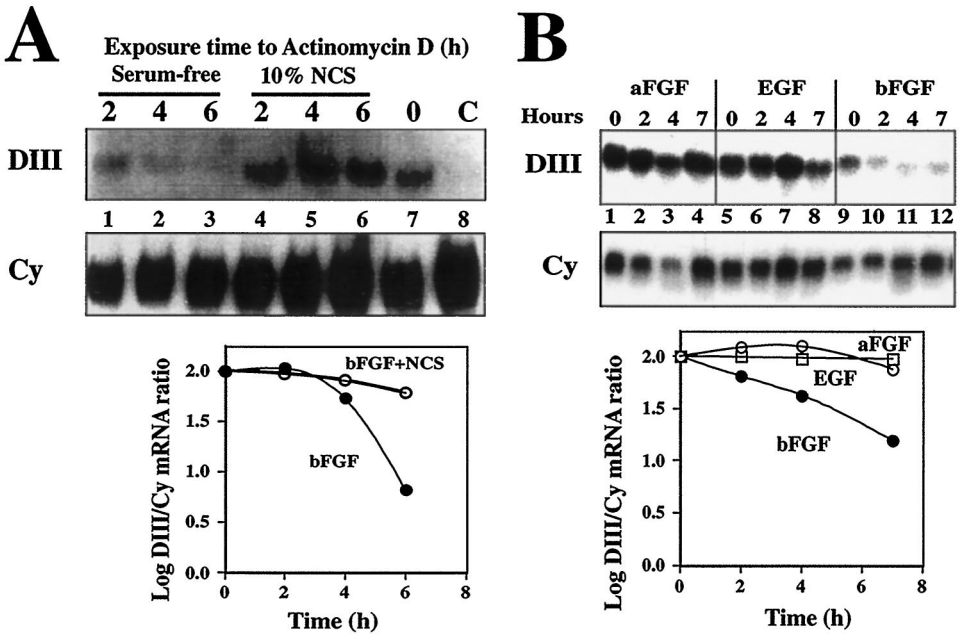

FIG. 6. Determination of half-life of DIII mRNA. A, Eight-day-old cultures of brown adipocytes were exposed to bFGF (10 ng/ml) for 7 h in medium containing 1% NCS. After 7 h, cells were washed and switched to serum-free medium or 10% NCS, and then actinomycin D (5 μg/ml) was added. Cells were harvested at 2, 4, and 6 h. The last two lanes show results from cells with or without induction with bFGF. Twenty micrograms of total RNA were used per lane for Northern analysis. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes. B, Eight-day-old cultures of brown adipocytes were exposed to aFGF, EGF, or bFGF (10 ng/ml for each) for 8 h in medium containing 1% NCS. After 8 h, actinomycin D (5 μg/ml) was added. Cells were collected at 2, 4, and 7 h. Twenty micrograms of total RNA were used per lane for Northern analysis. Hybridization with a cyclophilin cDNA was used to correct for differences between lanes.

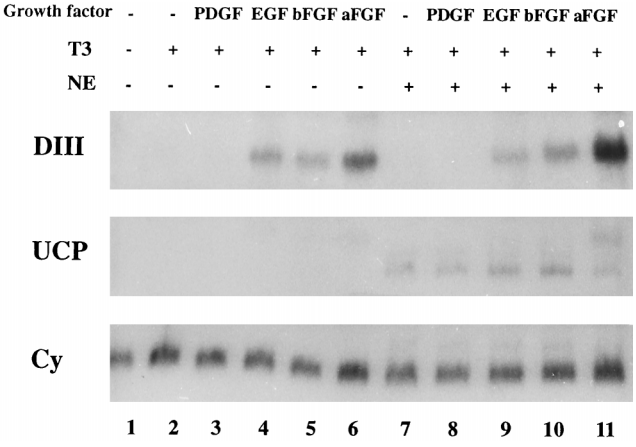

FIG. 7. Effects of growth factors on the adrenergic stimulation of UCP mRNA. Eight-day-old cultures of brown adipocytes were exposed to medium containing 0.5% hypothyroid serum and 5 nM T_3 for 48 h. Norepinephrine (NE; 3 μM) and the respective growth factor were then added during the last 7 h of incubation. Growth factor doses were 5 ng/ml for PDGF, 5 ng/ml for aFGF, and 10 ng/ml for EGF and bFGF. Positive hybridization with UCP cDNA is shown after treatment with NE and T_3, and hybridization with a cyclophilin cDNA was used to correct for differences between lanes.

by growth factors is activated at the transcriptional level and is dependent on *de novo* protein synthesis, suggesting the need for intermediate proteins for increases in mRNA. We also performed studies to determine whether the effects of growth factors on increasing DIII mRNA involve an increase in the DIII mRNA half-life. Our studies suggest that this may indeed be the case, as culturing cells in 10% serum significantly prolongs transcript half-life, suggesting that some factors present in serum may regulate DIII mRNA stability. Although the nature of these factors remains to be established, aFGF or EGF are good candidates, as our results indicate that they both also appear to stabilize DIII mRNA.

The potent effects of growth factors on stimulating DIII in cultured brown adipocytes contrasts sharply with our observations that DIII activity is not detectable in BAT from adult, neonatal, and fetal rats (Hernandez, A., and M. J. Obregon, unpublished data). Several possible explanations can be offered for this discrepancy. Firstly, we do not know whether BAT *in vivo* is exposed to the concentrations of growth factors used in these studies. However, these levels have been commonly used to test for the biological effects of these agents, and they are within the concentration range described in biological fluids. Of note, norepinephrine stimulates the expression of bFGF by brown preadipocytes (38), a effect that may contributes to the hypertrophy of brown fat during cold adaptation (39). It also appears likely that aFGF, a potent angiogenic factor, is present in BAT given the highly vascular nature of this tissue.

Secondly, other factors present in BAT *in vivo* might suppress or counteract the effects of growth factors, preventing expression of DIII. Thirdly, DIII might be expressed in BAT either very early in development or, in more mature animals, only by relatively undifferentiated precursor BAT cells that are present in such small numbers that activity cannot be detected in whole tissue homogenates. Expression of DIII in culture might then result from an enrichment of this population of preadipocyte cells during the initial period of our cell culture protocol. In this sense recent studies from our group show higher DIII expression in preadipocytes during proliferative stages and good correlations with mitogenic activity using the present culture system (40). Finally, induction of DIII expression by growth factors may be associated with cells that keep some proliferative capacity, as has been previously described in cultured hepatocarcinoma cells (41). We now have evidence that the growth factors tested act as mitogens for brown fat cells, stimulating DNA synthesis *in vitro* in proliferation assays (42). Furthermore, this induction of DIII by proliferative agents is noteworthy given that high DIII activities are found early in development when the rates of cellular proliferation are presumably high.

In an attempt to exclude that DIII activity is present in other possible "contaminant" cell types in the cultures, we analyzed DIII activity in primary cultures of aortic endothelial cells and in the fibroblastic cell line 3T3, both stimulated with serum and growth factors, and using similar number of cells, we obtained negative results for the presence of DIII activity. Further studies are needed to localize DIII in brown adipocytes by *in situ* hybridization studies in the primary cultures.

In summary, we have demonstrated that selected growth factors that act as proliferative agents for brown adipocytes are potent inducers of DIII mRNA and activity in this cell type. It will be of interest to determine whether this phenomenon also accompanies mitogenic stimulation for other cells, and what the physiologic consequences of this induction are. Such information may provide important insights into the role of DIII during development.

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