CCAAT/enhancer-binding proteins α and β in brown adipose tissue: evidence for a tissue-specific pattern of expression during development

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CCAAT/enhancer-binding protein (C/EBP) α mRNA and its protein products C/EBPα and 30 kDa C/EBPα are expressed in rat brown-adipose tissue. Results also demonstrate the expression of C/EBPβ mRNA and its protein products C/EBPβ and liver inhibitory protein (LIP) in the tissue. The abundance of C/EBPα and C/EBPβ proteins in adult brown fat is similar to that found in adult liver. However, the expression of C/EBPα and C/EBPβ is specifically regulated in brown fat during development. C/EBPα, 30 kDa C/EBPα, C/EBPβ and LIP content is several-fold higher in fetal brown fat than in the adult tissue, or liver at any stage of development. Peak values are attained in late fetal life, in concurrence with the onset of transcription of the uncoupling protein (UCP) gene, the molecular marker of terminal brown-adipocyte differentiation. When adult rats are exposed to a cold environment, which is a physiological stimulus of brown-adipose tissue hyperplasia and UCP gene expression, a specific rise in C/EBPβ expression with respect to C/EBPα, 30 kDa C/EBPα and LIP is observed. Present data suggest that the C/EBP family of transcription factors has an important role in the development and terminal differentiation of brown-adipose tissue.

INTRODUCTION

Brown-adipose tissue (BAT) is the main site for facultative thermogenesis in mammals. Particular relevance of BAT thermogenesis during the neonatal period has been recognized (Nedergaard et al., 1986). The thermogenic activity of this tissue relies on a proton-conductance pathway generated by the presence of the uncoupling protein (UCP) in the inner mitochondrial membrane (Nicholls et al., 1986). UCP is uniquely expressed in BAT. Hence, it is an unequivocal marker of brown adipocytes with respect to other cell types, including white adipocytes (Bouillaud et al., 1985; Ricquier et al., 1992). In rat, as in most mammalian species, differentiated BAT develops prenatally and UCP gene expression starts on day 19 of fetal life (Giralt et al., 1990). After birth, the BAT thermogenic activity is strictly regulated according to the heat needs. Thus exposure of adult rodents to a cold environment triggers two main phenomena: a rapid increase in UCP gene transcription in mature brown adipocytes and the proliferation and differentiation of precursor cells, which do not express UCP, into UCP-expressing brown adipocytes (Bouillaud et al., 1984; Bukowiecki et al., 1986). Both phenomena are caused by activation of the sympathetic nervous system in the tissue. Even though cyclic AMP and thyroid hormone [3,3′,5-L-tri-iodothyronine (T_3)] are involved in the regulation of UCP gene expression (Ricquier et al., 1986; Bianco et al., 1988), the molecular mechanisms leading to brown-adipocyte differentiation during ontogenesis or thermogenic activation are basically unknown.

The CCAAT/enhancer-binding proteins (C/EBP) are transcription factors belonging to the class termed basic zipper (bZIP) proteins (Landschulz et al., 1988a). The bZIP domain consists of both a basic DNA-binding region and a dimer-forming region called the leucine zipper. Since the isolation of the first C/EBP (C/EBPα), different members of this family of transcription factors have been isolated, termed C/EBPβ, C/EBPδ, C/EBPγ and others (Cao et al., 1991; Williams et al., 1991; Ron and Habener, 1992). The nomenclature proposed by Cao et al. (1991) is used to name the different members of the C/EBP family. C/EBPβ has also been referred to as LAP (Descombes et al., 1990), IL6-DBP (Poli et al., 1990), AGP/EBP (Chang et al., 1990), NF-IL6 (Akira et al., 1990) and crp2 (Williams et al., 1991). Most of these proteins act as transcriptional activators (Lamb and McKnight, 1991). However, there is a translational variant of C/EBPβ, named liver inhibitory protein (LIP), which has been reported to act as a transcriptional inhibitor (Descombes and Schibler, 1991). More recently, a smaller (30 kDa) fragment of full-length C/EBPα (42 kDa) (previously described as a proteolytic form) has also been shown to be a translation product of C/EBPα mRNA (Lin et al., 1993; Ossipow et al., 1993), but with a much lower transcriptional activation potential (Ossipow et al., 1993). As most members of the C/EBP family of proteins are able to form heterodimers when interacting with DNA sequences, a complex interplay of effects upon transcription of target genes is expected, which may depend on the relative concentration of the different C/EBP isoforms (Cao et al., 1991; Williams et al., 1991).

The C/EBP proteins are believed to be essential for the establishment of the differentiated phenotype of certain cell types. C/EBPα and C/EBPβ are preferentially expressed in a limited number of tissues, such as white-adipose tissue and liver (Birkenmeier et al., 1989; Descombes et al., 1990). Furthermore, C/EBPα and β isoforms activate transcription of several genes that mark the differentiated state of white adipocytes and hepatocytes (Christy et al., 1989; Friedman et al., 1989; Park et al., 1990). When C/EBPα expression is blocked in 3T3 pre-adipocytes, the acquisition of the white-adipocyte pattern of gene expression is suppressed (Samuelsson et al., 1991; Lin and Lane, 1992). C/EBPα has also been shown to be involved in the induction of the growth-arrested state typical of differentiated cells (Umek et al., 1991). Studies on C/EBPα and β expression in developing liver agree with this postulated role in terminal differentiation, as both transcription factors show high levels of

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; LIP, liver inhibitory protein; BAT, brown adipose tissue; UCP, uncoupling protein; PEPCK, phosphoenolpyruvate carboxykinase; T_3, 3,3′,5-L-tri-iodothyronine (thyroid hormone); bZIP, basic zipper.

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expression after birth, in parallel with the sudden rise in the expression of molecular markers of differentiated hepatocyte function (Birkenmeier et al., 1989; Descombes and Schibler, 1991; Ossipow et al., 1993).

The aim of the present study is to characterize the expression of two C/EBP genes, C/EBPα and C/EBPβ, and their translational products, in rat BAT. We report their pattern of expression during development and in the physiological response to cold exposure. Our results suggest the involvement of these transcription factors in the differentiation process of brown adipocytes.

MATERIALS AND METHODS

Experimental procedures

Female Wistar rats weighing 180–210 g were mated with adult male rats. The day of conception was determined by the presence of spermatozoa in vaginal smears. Fetuses were obtained by Caesarian section on days 18, 20 and 21.5 of gestation. When the postnatal period was studied, pups remained with their mother after spontaneous delivery and were killed at 0 h (when all pups had been born but none had started suckling) and 24 h after birth. Adult male rats (200–240 g) were also used. All the animals were maintained in a controlled environment (21 °C, 12 h light/dark cycles) with free access to water and food (stock diet A03 type, Panlab, Barcelona, Spain). Cold-exposed rats were placed at 4 °C for 1, 6, 12 or 24 h.

Animals were killed by decapitation. Liver and interscapular BAT were extracted and immediately processed or frozen in liquid N_2.

Preparation of nuclear protein extracts and Western-blot analysis

Nuclear proteins were isolated from adult rat liver and BAT according to the procedure of Gorski et al. (1986) with the modifications of Landschulz et al. (1988b). In the developmental studies, a modified method was used to prepare crude protein extracts from isolated nuclei because of the small size of the samples. This method was as follows: tissue was homogenized in buffer A (10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride, 2.5 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml leupeptin 1 μg/ml pepstatin) containing 0.2 M sucrose. The homogenates were centrifuged for 10 min at 1500 *g*. Pellets were resuspended in 2 ml of homogenization buffer and centrifuged for 10 min at 5000 *g*. Nuclei were lysed and resuspended in buffer A containing 10% (v/v) glycerol. All the steps were performed at 4 °C. When adult liver and BAT nuclear protein extracts, obtained by either the original or the modified method, were compared by Western-blot analysis, the relative concentrations of all polypeptides studied were found to be equivalent (results not shown). Nuclear extracts were stored at −80 °C. Protein concentration was determined by the micromethod of Bio-Rad using BSA as standard.

For Western-blot analysis, samples containing equal amounts of protein were mixed with equal volumes of 2 × SDS loading buffer, incubated at 90 °C for 5 min and electrophoresed on SDS/10% polyacrylamide gels (Laemmli, 1970). Coomassie Blue staining of gels was performed systematically and showed similar patterns of major nuclear proteins in the different extracts, thus indicating similar overall quality. Proteins were then transferred to nitrocellulose membranes (Hybond C, Amersham) and probed using specific antisera for either C/EBPα (Landschulz et al., 1988b) or C/EBPβ (Cao et al., 1991), kindly provided by Dr. S. L. McKnight. Immunoreactive material was detected using ^{125}I-labelled Protein A and visualized by autoradiography. Quantification of autoradiographs was performed by scanning densitometry (LKB).

RNA isolation and Northern-blot analysis

Total RNA was extracted using the guanidine isothiocyanate method (Chomczynsky and Sacchi, 1987). For Northern-blot analysis, 24 μg of total RNA containing 0.2 μg/μl ethidium bromide was denatured at 65 °C in the presence of formamide and formaldehyde, electrophoresed on formaldehyde/1.5% agarose gels and transferred to nylon membranes (Hybond N, Amersham). Transfer efficiency and equivalent amounts of ribosomal RNA in the samples were checked by ethidium bromide u.v. visualization (Pappu and Hiruki, 1989). Hybridization procedures were carried out as reported (Giralt et al., 1990). Blots were hybridized to DNA probes corresponding to the full-length cDNA for either rat C/EBPα (Birkenmeier et al., 1989), mouse C/EBPβ (Cao et al., 1991) or rat UCP (Bouillaud et al., 1985). The cDNA probes were labelled using [α-^{32}P]dCTP by the random oligonucleotide-priming method. Filters were washed under stringent conditions (30 mM NaCl/3 mM sodium citrate, 0.1% SDS; 65 °C, 30 min). Autoradiographs were quantified by densitometry.

Statistical analysis

Where appropriate, statistical analysis was performed by the Student's *t* test and significance is indicated in the figures.

RESULTS

C/EBPα and C/EBPβ are expressed in rat BAT

The expression of C/EBPα and C/EBPβ in rat BAT was characterized by Northern- and Western-blot analysis. As depicted in Figure 1(a), Northern blots revealed a single C/EBPα mRNA species of 2.7 kb in both liver and BAT, in agreement with previous data (Birkenmeier et al., 1989). A major mRNA

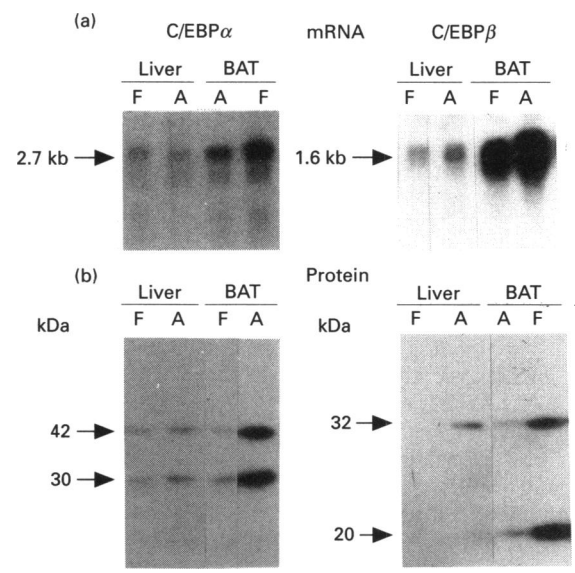

Figure 1 C/EBPα and C/EBPβ expression in BAT

(**a**) Northern-blot analysis of C/EBPα mRNA and C/EBPβ mRNA (24 μg of total RNA/lane). (**b**) Western-blot analysis of C/EBPα and C/EBPβ (100 μg of nuclear protein/lane). Lanes F, samples from 20-day-old fetal tissues; lanes A, samples from adult tissues.

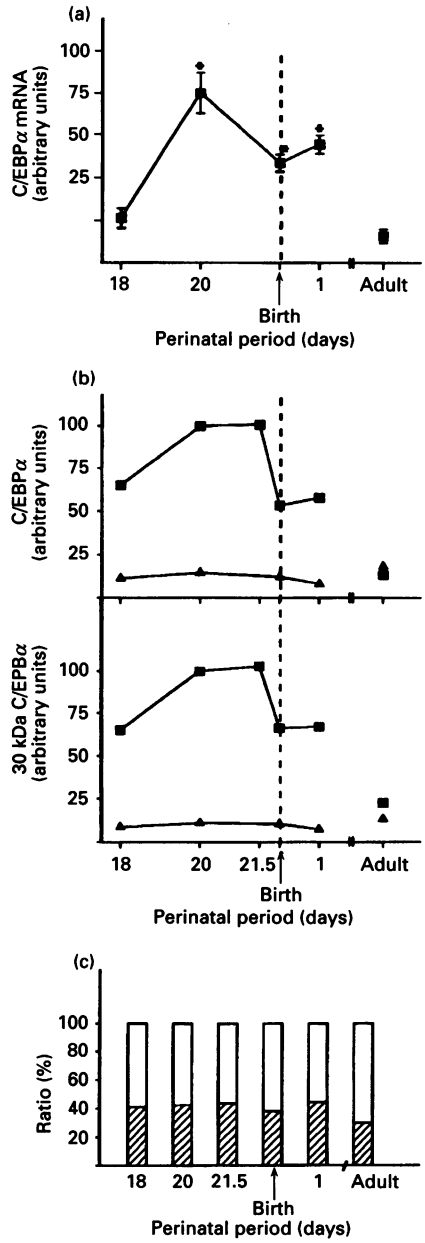

Figure 2 C/EBPα expression in BAT during development

(**a**) Levels of C/EBPα mRNA in interscapular BAT from fetuses, newborn and adult rats. Values are means ± S.E.M. of densitometric data from at least three different samples analysed in independent Northern blots of 24 μg of total RNA. Statistical significance of comparisons between different groups is stated. $^*P \leqslant 0.05$ versus adult. (**b**) Levels of C/EBPα and 30 kDa C/EBPα proteins in interscapular BAT (■) and liver (▲) from fetuses, newborn and adult rats. Values are obtained from densitometric data of one Western-blot analysis of 100 μg of nuclear protein from pooled tissues from at least three different litters (fetuses and neonates) or individuals (adults). The data are representative of three independent Western blots analysing different pools. (**c**) C/EBPα:30 kDa C/EBPα ratio in BAT. Ratio is expressed as a percentage of individual values for each different experimental group. ▨, C/EBPα; □, 30 kDa C/EBPα.

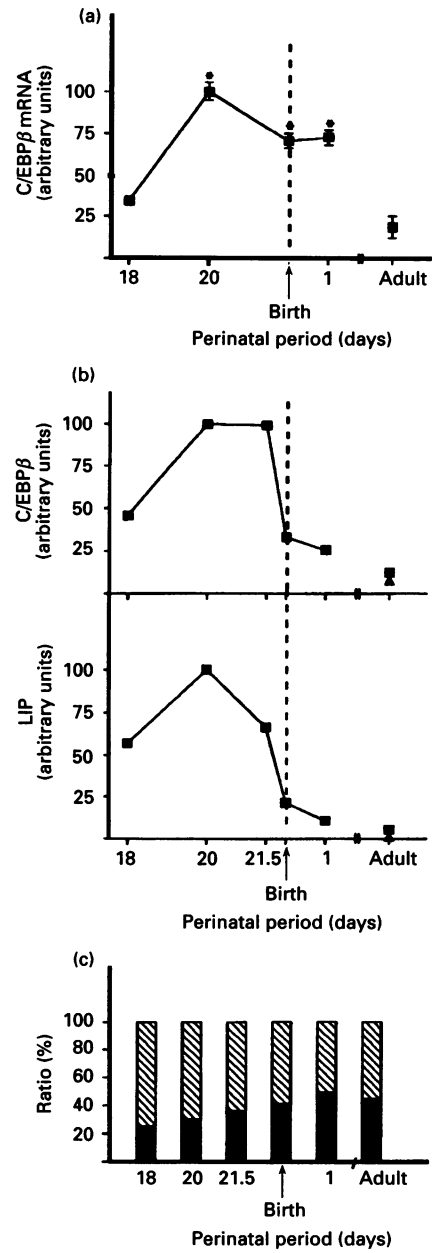

Figure 3 C/EBPβ expression in BAT during development

(**a**) C/EBPβ mRNA abundance in interscapular BAT from perinatal and adult rats. (**b**) C/EBPβ and LIP protein content in interscapular BAT (■) from fetuses, newborn and adult rats. The protein content was below the detection limit for densitometry quantification in the perinatal liver. Adult liver (▲) values are shown in the graph. (**c**) Ratio of C/EBPβ:LIP proteins in BAT. For experimental and statistical details, see the legend to Figure 2. ▧, LIP; ■, C/EBPβ.

species of 1.6 kb was detected for C/EBPβ in both fetal and adult BAT. This finding is similar to that described for other tissues, including liver (Cao et al., 1991). The abundance of both C/EBPα mRNA and C/EBPβ mRNA in fetal and adult BAT were higher than that in liver.

Figure 1(b) (left-hand panel) shows a Western blot of nuclear protein from BAT and liver using a specific anti-C/EBPα serum. Two major polypeptide species were detected in the protein extracts from rat liver nuclei, a 42-kDa form corresponding to full-length C/EBPα and a 30-kDa form corresponding to a shorter translation product, in agreement with previous data (Ossipow et al., 1993; Lin et al., 1993). Comparable cross-reacting polypeptides were detected in the protein extracts from BAT nuclei, although the C/EBPα:30-kDa form ratio was somewhat lower than in the liver samples. As also shown in Figure 1(b) (right-hand panel), both C/EBPβ and LIP proteins

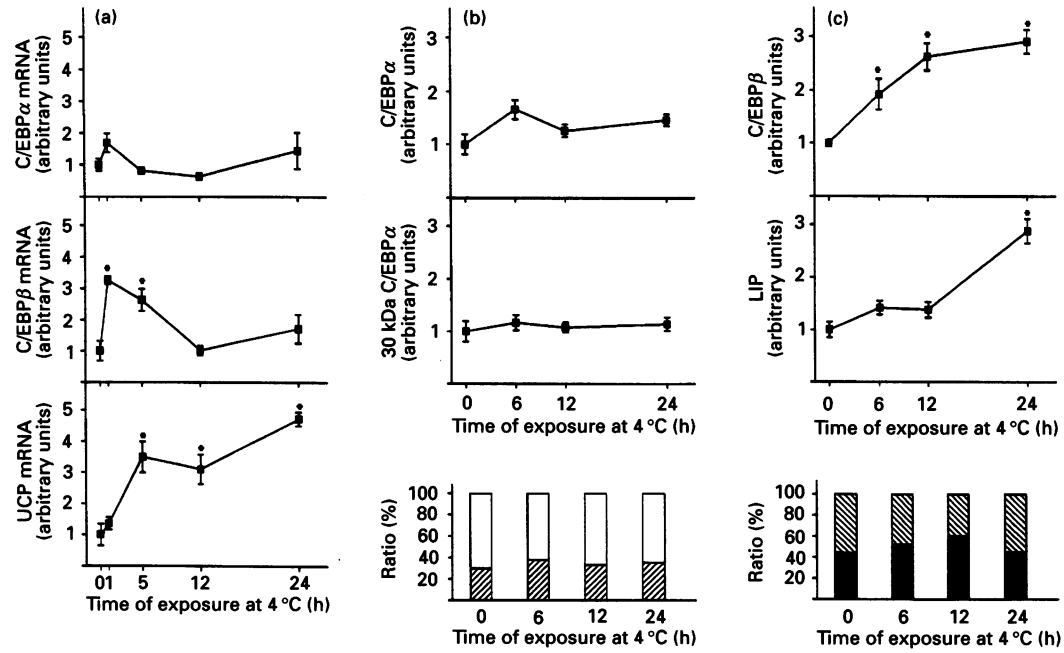

Figure 4 Effects of cold exposure on C/EBPα and C/EBPβ expression in BAT of adult rats

(**a**) Levels of C/EBPα mRNA, C/EBPβ mRNA and UCP mRNA in interscapular BAT from rats exposed to 4 °C for 0, 1, 5, 12 and 24 h. Values are means ± S.E.M. of densitometric data from independent Northern-blot analyses of 24 μg of total RNA from at least three animals at each point. (**b**) Levels of C/EBPα and 30 kDa C/EBPα proteins in interscapular BAT from rats exposed to 4 °C for 0, 6, 12 and 24 h. The ratio of C/EBPα (▨) and 30 kDa C/EBPα (□) proteins is also depicted. (**c**) C/EBPβ and LIP protein content and C/EBPβ (■):LIP (▧) ratio in interscapular BAT from control or cold-exposed rats. Protein values in (**b**) and (**c**) are means ± S.E.M. of densitometric data from independent Western-blot analyses of 100 μg of nuclear protein from at least three animals at each point. The protein ratio is expressed as a percentage of individual values for each different experimental group. Statistical significance of comparisons between different groups of cold-exposed rats versus non-cold-exposed rats (0 h) is stated; $^*P \leq 0.05$.

were also detected in liver and BAT nuclear extracts. The apparent molecular masses of the polypeptides recognized, 32 kDa (C/EBPβ) and 20 kDa (LIP), were consistent with previous data for the liver (Descombes and Schibler, 1991). The relative abundances of C/EBPβ in adult BAT and liver were similar. However, the C/EBPβ:LIP ratio was slightly higher in liver than in BAT (Figure 1b). On the other hand, the large amounts of C/EBPβ found in fetal BAT were in contrast with the barely detectable levels in fetal liver.

C/EBPα and C/EBPβ expression is specifically regulated in BAT during early development

The patterns of C/EBPα and C/EBPβ expression during development in rat BAT are depicted in Figures 2 and 3 respectively. As shown in Figure 2(a), C/EBPα mRNA was already detected in 18-day-old fetuses at similar levels to those in adult BAT. Significantly higher levels were observed in late fetal and early postnatal periods. Abundance of C/EBPα protein in BAT from 18-day-old fetuses was higher than that in the adult (Figure 2b). An increase was also observed in the C/EBPα content during late fetal stages, followed by a decrease immediately before birth. However, postnatal C/EBPα levels were still higher than those in adult BAT. A similar profile of expression during development was found for the 30 kDa C/EBPα polypeptide in BAT. In contrast, the relative abundance of both C/EBPα and 30-kDa proteins in developing liver was much lower than BAT values, whereas a similar content of C/EBPα was found in the adult tissues (Figure 2b). As seen in Figure 2(c), the ratio of C/EBPα to 30-kDa proteins did not change significantly during early development, whereas it was slightly lower in adult BAT. The level of these proteins in liver samples was approx. 50% of each form and remained unchanged throughout development and in adults (results not shown).

C/EBPβ mRNA expression was higher in perinatal than in adult BAT (Figure 3a), a developmental profile paralleling that described for the C/EBPα mRNA. Adult liver and BAT showed similar C/EBPβ protein contents (Figure 3b). However, levels of C/EBPβ protein were hardly detectable in liver during the perinatal period. In contrast, C/EBPβ content in fetal BAT was higher than that in the adult tissue. There was a decrease in the abundance of C/EBPβ just around birth. The profile of LIP content during BAT development was somewhat different from that described for C/EBPβ. LIP abundance decreased on day 21.5 of fetal life and reached levels similar to those in the adult at 1 day of extra-uterine life (Figure 3b). Thus there was a progressive increase in the C/EBPβ:LIP ratio during BAT development (Figure 3c).

Northern-blot hybridization with the UCP probe was also performed (results not shown). Results were in agreement with our previously reported data (Giralt et al., 1990; Tuca et al., 1993). No specific signal for UCP mRNA was detectable in the RNA samples from 18-day-old fetuses. On day 20, the levels of UCP mRNA were higher than those in adults and similar to those at birth. There was a 2–3-fold increase in the UCP mRNA levels 24 h after birth.

Expression of C/EBPβ, but not C/EBPα, is increased in rat BAT after cold exposure

Cold exposure led to a significant increase in C/EBPβ mRNA in rat BAT, whereas it did not significantly change C/EBPα mRNA

expression (Figure 4a). The increase in C/EBPβ mRNA was evident after 1 h of cold exposure, still present at 5 h, but no longer evident by 12 h. The levels of UCP mRNA increased significantly at 5, 12 and 24 h of cold exposure.

There was no change in the abundance of either C/EBPα or 30 kDa C/EBPα protein in BAT during cold exposure (Figure 4b). Therefore, the C/EBPα:30 kDa C/EBPα ratio remained essentially unchanged. In contrast, C/EBPβ protein content increased significantly after 6, 12 and 24 h of cold exposure (Figure 4c). However, LIP abundance remained unchanged for the first 12 h of cold exposure and increased at 24 h. Thus the C/EBPβ:LIP ratio increased during early stages of cold-exposure returning to control values at 24 h, although the total amount of both proteins was 3-fold higher than the control levels.

DISCUSSION

C/EBPα and C/EBPβ genes are expressed in BAT from adult rats at similar levels to those in liver. Whereas the presence of mRNA for C/EBPα and C/EBPβ had been reported in BAT (Birkenmeier et al., 1989; Rehnmark et al., 1993), the present study indicates that their respective protein products, full-length C/EBPα and 30 kDa C/EBPα, and C/EBPβ and LIP, are expressed in this tissue. The expression of the C/EBPβ and LIP proteins in BAT is a noteworthy finding. Although a variety of rat tissues express the C/EBPβ transcript, substantial amounts of its protein products have been described only in liver (Descombes et al., 1990; Descombes and Schibler, 1991). In addition, we describe changes in the C/EBPβ:LIP ratio in BAT during development and cold-exposure, the functional significance of which is discussed below.

C/EBPα and C/EBPβ expression show a specific developmental regulation in BAT in comparison with other C/EBP-expressing tissues such as liver (Birkenmeier et al., 1989; Descombes and Schibler, 1991; and present results) or intestine (Birkenmeier et al., 1989). Two main differential features are evident. First, the levels of expression of these proteins in BAT from late fetal rats are more than 10-fold those found at any stage of liver development. Secondly, maximum levels of C/EBPα and C/EBPβ in BAT occur earlier (late fetal life) than in other C/EBP-expressing tissues, whose higher levels of expression are always attained after birth (Birkenmeier et al., 1989; Descombes and Schibler, 1991; Ossipow et al., 1993). Peak values of C/EBP proteins in BAT occur at day 20 of fetal life, concurrently with the achievement of BAT differentiation *in vivo*. At this time, UCP gene expression is switched on (Giralt et al., 1990) and other putative regulatory events, such as T_3 accumulation in BAT nuclei, occur (Tuca et al., 1993).

A particular characteristic of C/EBPβ expression in developing BAT is the lower C/EBPβ:LIP ratio compared with that in liver and its progressive increase before birth. An increase in this ratio has also been reported in liver development after birth (Descombes and Schibler, 1991). C/EBPβ activates the transcription of several genes expressed in liver, such as the albumin and phosphoenolpyruvate carboxykinase (PEPCK) genes (Descombes et al., 1990; Park et al., 1993). In contrast, LIP has been reported to act as a transcription inhibitor when interacting with a C/EBP-binding site on the albumin promoter (Descombes and Schibler, 1991). However, the relevance of the C/EBPβ:LIP ratio and/or the absolute amounts of each transcription factor for activation or inhibition of target genes remains to be determined. Similarly, little is known about the functional consequences of a given C/EBPα:30 kDa C/EBPα ratio. In fact, it is still controversial whether the 30 kDa C/EBPα form efficiently activates transcription (Lin et al., 1993) or not (Ossipow et al., 1993).

Cold environment is a known physiological stimulus of BAT thermogenesis. Sudden exposure of adult rats to a cold environment results in an increase in UCP gene transcription in mature brown adipocytes together with the proliferation and progressive differentiation of precursor cells into UCP-expressing brown adipocytes, thus leading to BAT depots hyperplasia and UCP mRNA accumulation in the tissue (Bouillaud et al., 1984; Ricquier et al., 1986; Bukowiecki et al., 1986). These events are triggered by the adrenergic stimulation caused by the release of noradrenaline from the sympathetic terminals innervating BAT (Mory et al., 1984). Cyclic AMP and T_3 have been proposed as the main intracellular mediators of this adrenergic stimulus (Ricquier et al., 1986; Bianco et al., 1988, 1992). In this study, we demonstrate that cold stress is also a physiological inducer of C/EBPβ expression in rat BAT, at both the mRNA and protein levels. Our results are essentially in agreement with a recent report (Rehnmark et al., 1993) on changes in the mRNA levels for C/EBPα and C/EBPβ in mice BAT in response to cold exposure. An analogous specific increase in C/EBPβ with respect to other C/EBP isoforms has been observed in response to the cyclic AMP stimulus of 3T3 preadipocytes in culture (Cao et al., 1991) and rat liver *in vivo* (Park et al., 1993). In 3T3 preadipocytes, cyclic AMP is a positive mediator in the acquisition of the differentiated white-adipocyte pattern of gene expression, and one of the first events induced by cyclic AMP is the specific rise in C/EBPβ expression (Cao et al., 1991). Our present findings in BAT from cold-exposed rats are compatible with a similar role for C/EBPβ in the differentiation of brown preadipocytes *in vivo*. Moreover, the increase in the C/EBPβ:LIP ratio observed in the first hours of cold exposure is also remarkable.

BAT thermogenesis is specially active in the neonatal period. Postnatal changes in BAT have been mainly interpreted as a response to the cold environment experienced after birth, but the involvement of ontogenic factors cannot be ruled out. We have previously demonstrated that the rise in UCP mRNA expression that occurs after birth is dependent on thermic stress (Giralt et al., 1990). However, our present study indicates that C/EBPβ expression does not significantly increase in BAT during the first hours of postnatal life, in contrast with the rise observed in BAT from cold-exposed adults. The reasons for this discrepancy are unclear. Nevertheless, other differences have been observed in BAT from cold-exposed adults and newborns. For example, in contrast with the adult animal the iodothyronine 5′-deiodinase activity, an adrenergic-regulated key parameter of BAT functionality, is not linked to the thermogenic activity of the tissue after birth (Giralt et al., 1988). Moreover, unlike in adults, the thermogenic stimulus after birth only comprises the recruitment of the tissue, as shown by the increase in UCP gene expression (Giralt et al., 1990), but does not involve hyperplasia of BAT depots (Nedergaard et al., 1986).

C/EBP proteins may determine the differentiated phenotype of liver and white-adipose-tissue cells through their action as transactivators of genes whose expression marks the terminal differentiated state of these cell types. The presence of C/EBPα and C/EBPβ in BAT, their high expression in physiological situations when UCP gene transcription is increased and the characteristics of UCP as restricted to the terminally differentiated brown adipocyte (Ricquier et al., 1992; Klaus et al., 1991) suggest that C/EBP proteins could be involved in the regulation of UCP gene transcription, either that related to brown-adipocyte differentiation or that related to hormonal regulation in the mature brown adipocyte. Moreover, we have recently demonstrated that transcription from the UCP gene

promoter is indeed regulated by both C/EBPα and C/EBPβ proteins (Yubero et al., 1994). Hence, the presence of large amounts of C/EBP proteins in the BAT nuclei concomitant with the first induction of UCP gene transcription strongly suggests a relevant role for C/EBP proteins in the onset of UCP gene expression during ontogenesis. Likewise, the burst of C/EBPβ expression in BAT from cold-exposed rats suggests a specific role for this isoform in the adrenergic regulation of UCP gene transcription in the adult tissue. C/EBPβ might be involved in the differentiation of pre-adipocytes into UCP-expressing brown adipocytes which is known to occur in the rat BAT depots in response to cold. However, it cannot be ruled out that C/EBPβ might also be involved in the cyclic AMP- and T_3-mediated adrenergic stimulus of UCP gene transcription in mature brown adipocytes. It is interesting to point out that C/EBP-binding sites are necessary for cyclic AMP and T_3 response in the PEPCK promoter (Giralt et al., 1991) and that C/EBPβ is capable of mediating a greater cyclic AMP-responsiveness to that promoter than C/EBPα (Park et al., 1993).

On the other hand, a possible role for C/EBPα and C/EBPβ in the regulation of other genes involved in BAT energy metabolism cannot be ruled out. BAT contains substantial amounts of proteins such as GLUT4 glucose transporter (James et al., 1989) or PEPCK (Zimmer and Magnuson, 1990), whose gene expression is activated by C/EBP proteins in 3T3 white adipocytes (Kaestner et al., 1990) or liver (Park et al., 1990) respectively. The pattern of expression of these genes in BAT during development or cold exposure (Frohlich et al., 1976; Santalucía et al., 1992; Nikami et al., 1992), together with present data, suggest that C/EBP proteins may also regulate them in BAT.

In conclusion, the present study shows a specific regulation of the expression of C/EBPα and C/EBPβ genes in rat BAT during ontogenesis and in the physiological response to cold. As C/EBP isoforms may form heterodimers, changes in their abundance and in the C/EBPβ:LIP ratio provide further support for a functional significance of accumulation *in vivo* of these transcriptional variants in regulating BAT gene expression. Considering the expression of the UCP gene as the unequivocal molecular marker of mature brown adipocyte, our recent identification of the UCP gene as a target for C/EBP transcription factors (Yubero et al., 1994) fully supports this proposal. Taken together, our results lead us to propose that the C/EBP family of transcription factors are crucial for the establishment of the terminally differentiated brown-adipocyte phenotype.

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REFERENCES

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirnao, T. and Kishimoto, T. (1990) EMBO J. **9**, 1897–1906
Bianco, A. C., Sheng, X. and Silva, J. E. (1988) J. Biol. Chem. **263**, 18168–18175
Bianco, A. C., Kieffer, J. D. and Silva, J. E. (1992) Endocrinology **130**, 2625–2633
Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H. and McKnight, S. L. (1989) Genes Dev. **3**, 1146–1156
Bouillaud, F., Ricquier, D., Mory, G. and Thibault, J. (1984) J. Biol. Chem. **259**, 11583–11586
Bouillaud, F., Ricquier, D., Thibault, J. and Weissenbach, J. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 445–448
Bukowiecki, L., Geloen, A. and Collet, A. J. (1986) Am. J. Physiol. **250**, C880–C887
Cao, Z., Umek, R. M. and McKnight, S. L. (1991) Genes Dev. **5**, 1538–1552
Chang, C.-J., Chen, T.-T., Lei, H.-Y., Chen, D.-S. and Lee, S.-C. (1990) Mol. Cell. Biol. **10**, 6642–6653
Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J. and Lane, M. D. (1989) Genes Dev. **3**, 1323–1335
Descombes, P. and Schibler, U. (1991) Cell **67**, 569–579
Descombes, P., Chojkier, M., Lichsteiner, S., Falvey, E. and Schibler, U. (1990) Genes Dev. **4**, 1541–1551
Friedman, A. D., Landschulz, W. H. and McKnight, S. L. (1989) Genes Dev. **3**, 1314–1322
Frohlich, J., Hahn, P., Kirby, L. and Webber, W. (1976) Biol. Neonate **30**, 40–48
Giralt, M., Martin, I., Mampel, T., Villarroya, F., Iglesias, R. and Viñas, O. (1988) Biochem. Biophys. Res. Commun. **156**, 493–499
Giralt, M., Martin, I., Iglesias, R., Viñas, O., Villarroya, F. and Mampel, T. (1990) Eur. J. Biochem. **193**, 297–302
Giralt, M., Park, E. A., Gurney, A. L., Liu, J., Hakimi, P. and Hanson, R. W. (1991) J. Biol. Chem. **266**, 21991–21996
Gorski, K., Carnieiro, M. and Schibler, U. (1986) Cell **47**, 767–776
James, D. E., Strube, M. and Mueckler M. (1989) Nature (London) **338**, 83–87
Kaestner, K. H., Christy, R. J. and Lane, M. D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 251–255
Klaus, S., Cassard-Doulcier, A. M. and Ricquier, D. (1991) J. Cell. Biol. **11**, 4147–4156
Laemmli, U. D. (1970) Nature (London) **227**, 680–685
Lamb, P. and McKnight, S. L. (1991) Trends Biochem. Sci. **16**, 417–422
Landschulz, W. H., Johnson, P. F. and McKnight, S. L. (1988a) Science **240**, 1759–1764
Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J. and McKnight, S. L. (1988b) Genes Dev. **2**, 786–800
Lin, F. T. and Lane, M. D. (1992) Genes Dev. **6**, 533–544
Lin, F. T., MacDouglas, O. A., Diehl, A. M. and Lane, M. D. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 9606–9610
Mory, G., Bouillaud, F., Combes-George, M. and Ricquier, D. (1984) FEBS Lett. **166**, 393–395
Nedergaard, J., Connolly, E. and Cannon, B. (1986) in Brown Adipose Tissue (Trayhurn, P. and Nicholls, D. G., eds.), pp. 152–213, Edward Arnold, London
Nicholls, D. G., Cunningham, S. A. and Rial, E. (1986) in Brown Adipose Tissue (Trayhurn, P. and Nicholls, D. G., eds.), pp. 52–85, Edward Arnold, London
Nikami, H., Shimizu, Y., Endoh, D., Yano, H. and Saito, M. (1992) Biochem. Biophys. Res. Commun. **185**, 1078–1082
Ossipow, V., Descombes, P. and Schibler, U. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8219–8223
Pappu, H. R. and Hiruki, C. (1989) Focus **11**, 6–7
Park, E. A., Roesler, W. J., Liu, J., Klemm, D. J., Gurney, A. L., Thatcher, J. D., Shuman, J. D., Friedman, A. and Hanson, R. W. (1990) Mol. Cell. Biol. **10**, 6264–6272
Park, E. A., Gurney, A. L., Nizielski, S. E., Hakimi, P., Cao, Z. and Hanson, R. W. (1993) J. Biol. Chem. **268**, 613–619
Poli, V., Mancini, F. P. and Cortese, R. (1990) Cell **63**, 643–653
Rehnmark, S., Antonson, P., Xanthopoulos, K. G. and Jacobsson, A. (1993) FEBS Lett. **303**, 235–241
Ricquier, D., Bouillaud, F., Toumelin, P., Mory, G., Bazin, R., Arch, J. and Pénicaud, L. (1986) J. Biol. Chem. **261**, 13905–13910
Ricquier, D., Raimbault, S., Champigny, O., Miroux, B. and Bouillaud, F. (1992) FEBS Lett. **303**, 103–106
Ron, D. and Habener, J. F. (1992) Genes Dev. **6**, 439–453
Samuelsson, L., Stromberg, K., Vikman, K., Bjursell, G. and Enerback, S. (1991) EMBO J. **12**, 3787–3793
Santalucía, T., Camps, M., Castelló, A., Muñoz, P., Nuel, A., Testar, X., Palacín, M. and Zorzano, A. (1992) Endocrinology **130**, 837–846
Tuca, A., Giralt, M., Villarroya, F., Viñas, O., Mampel, T. and Iglesias, R. (1993) Endocrinology **132**, 1913–1920
Umek, R. M., Friedman, A. D. and McKnight, S. L. (1991) Science **251**, 288–292
Williams, S. C., Cantwell, C. A. and Johnson, P. F. (1991) Genes Dev. **5**, 1553–1567
Yubero, P., Manchado, C., Cassard-Doulcier, A. M., Mampel, T., Viñas, O., Iglesias, R., Giralt, M. and Villarroya, F. (1994) Biochem. Biophys. Res. Commun. **198**, 653–659
Zimmer, D. B. and Magnuson, M. A. (1990) J. Histochem. Cytochem. **38**, 171–178

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