Regulation of mitochondrial biogenesis in brown adipose tissue: nuclear respiratory factor-2/GA-binding protein is responsible for the transcriptional regulation of the gene for the mitochondrial ATP synthase β subunit

Josep A. VILLENA, Octavi VIÑAS, Teresa MAMPEL, Roser IGLESIAS, Marta GIRALT and Francesc VILLARROYA¹

Department of Biochemistry and Molecular Biology, University of Barcelona, Diagonal 645, 08028-Barcelona, Spain

The regulation of transcription of the gene for the β subunit of the FoF1 ATP synthase (ATPsyn β) in brown adipose tissue has been studied as a model to determine the molecular mechanisms for mitochondrial biogenesis associated with brown adipocyte differentiation. The expression of the ATPsyn β mRNA is induced during the brown adipocyte differentiation that occurs during murine prenatal development or when brown adipocytes differentiate in culture. This induction occurs in parallel with enhanced gene expression for other nuclear and mitochondrially-encoded components of the respiratory chain/oxidative phosphorylation system (OXPHOS). Transient transfection assays indicated that the expression of the ATPsyn β gene promoter is higher in differentiated HIB-1B brown adipocytes than in non-dif-

ferentiated HIB-1B cells. A major transcriptional regulatory site was identified between nt –306 and –266 in the ATPsyn β promoter. This element has a higher enhancer capacity in differentiated brown adipocyte HIB-1B cells than in non-differentiated cells. Electrophoretic shift analysis indicated that Sp1 and nuclear respiratory factor-2/GA-binding protein (NRF2/GABP) were the main nuclear proteins present in brown adipose tissue that bind this site. Double-point mutant analysis indicated a major role for the NRF2/GABP site in the enhancer capacity of this element in brown fat cells. It is proposed that NRF2/GABP plays a pivotal role in the co-ordinated enhancement of *OXPHOS* gene expression associated with mitochondrial biogenesis in brown adipocyte differentiation.

INTRODUCTION

Most mammalian cells rely on the ATP produced by oxidative phosphorylation in mitochondria for survival and function. The enzymic machinery for aerobic production of ATP consists of enzyme complexes in the mitochondrial inner membrane that constitute the respiratory chain/oxidative phosphorylation system (OXPHOS). In mammals, these enzyme complexes are large protein oligomers most of which are encoded in the nuclear genome, except for thirteen subunits that are encoded by mitochondrial DNA. The expression of genes for components of the OXPHOS system is regulated in relation to the cell type and mitochondrial content and also to physiological adaptations to demands of ATP (for a review see [1]).

The ATP synthase β -subunit (ATPsyn β) is a major component of the catalytic site of mitochondrial FoF1 ATP synthase. It is encoded by a single copy gene that is ubiquitously expressed in mammalian cells and is regulated similarly to other nuclear *OXPHOS* genes [2–4]. The expression of the ATPsyn β gene is regulated by both transcriptional and post-transcriptional mechanisms in relation to cell type or development. Studies performed in developing liver indicate that transcriptional regulation of ATPsyn β correlates with mitochondrial biogenesis whereas the mitochondrial differentiation in liver cells is mostly regulated by post-transcriptional events [5]. We and others have characterized gene elements for transcriptional regulation of the ATPsyn β gene in cell lines such as HepG2, HeLa or C2C12 [4,6–8]. This allowed identification of a proximal CCAAT box element essential for basal expression [6,8], a close upstream

element able to bind and confer ETS-dependent trans-activation [6], a far-upstream enhancer [7] and an OXBOX/REBOX binding element involved in the preferential expression of ATPsyn β in myogenic cells [8].

The brown adipocyte is a cell type specialized in thermogenesis. The capacity for heat production in brown fat is determined by its high content of mitochondria that are naturally uncoupled due to the presence of the uncoupling protein 1 (UCP1) [9]. Probably because of this high content of mitochondria, *OXPHOS* genes are highly expressed in brown fat, which also shows very high levels of expression of ATPsyn β mRNA despite the fact that brown fat mitochondria are not especially enriched in ATP synthase [10]. Moreover, brown fat is probably the mammalian tissue in which the mitochondrial content is most modified in response to different environmental and physiological changes determining adaptative thermogenesis, such as exposure to different ambient temperatures [11,12], changes in dietary caloric intake [13,14] or pregnancy and lactation [15,16]. The determination of the mechanisms for regulation of mitochondrial biogenesis in brown adipocytes is essential to understand the differentiation process of this cell type and, even more importantly, the brown adipocyte appears to be a very useful model for understanding the molecular mechanisms involved in the regulation of mammalian mitochondrial biogenesis. In the present work we have analysed the regulation of transcription of the gene for ATPsyn β in brown adipocytes as a means to identify the mechanisms for regulation of *OXPHOS* gene expression in brown adipose tissue. We have identified nuclear respiratory factor-2/GA-binding protein (NRF2/GABP) as the transcrip-

Abbreviations used: OXPHOS, respiratory chain/oxidative phosphorylation system; ATPsyn β , ATP synthase β -subunit; NRF2/GABP, nuclear respiratory factor-2/GA-binding protein; FCS, fetal-calf serum; UCP1, uncoupling protein 1; DMEM/F12, Dulbecco's modified Eagle's medium/Ham's F12 medium; CAT, chloramphenicol acetyltransferase; COII, cytochrome oxidase subunit II; COIV, cytochrome oxidase subunit IV; RSV, Rous sarcoma virus; MEF2, myocyte enhancer-specific factor 2; TK, thymidine kinase.

¹ To whom correspondence should be addressed.

tion factor which plays the major role in the regulation of *ATPsyn β* gene expression in association with the brown adipocyte differentiation process.

MATERIALS AND METHODS

Materials

All reagents and enzymes used were of the highest grade available. Tissue culture media, fetal calf serum (FCS) and supplements were from BioWhittaker and Sigma. DNA-modifying enzymes and poly (dI).(dC) were purchased from Boehringer Mannheim or Promega. [α - 32 P]dCTP and D-threo-[1,2- 14 C]chloramphenicol were from Amersham Inc. Oligonucleotides were from Oligos Inc. and Pharmacia.

Animals

Female Swiss mice were mated with adult males and the day of pregnancy was determined by the presence of spermatozoa in vaginal smears. When prenatal mRNA expression in brown adipose tissue was studied, Caesarean sections were performed on days 15 and 18 of pregnancy. When the postnatal period was studied, pups remained with their mother after spontaneous delivery and were killed just before they started suckling (birth) or 15 days later. Adult controls were two-month-old mice. Fetuses, pups or adult mice were killed by decapitation and interscapular brown adipose tissue was extracted and immediately frozen in liquid N₂. Samples from each litter of fetuses or pups were pooled.

Cell culture

Isolation and culture of primary brown pre-adipocytes was performed as described previously [17,18]. Three-week-old Swiss mice were killed and interscapular, cervical and axillary depots of brown adipose tissue were removed. Precursor cells were isolated, plated on 60-mm diam. Petri dishes (7500 cells/cm²) and grown in 5 ml of Dulbecco's modified Eagle's medium:Ham's F12 medium (DMEM/F12) (1:1, v/v) supplemented with 10% (v/v) FCS, 20 nM insulin, 2 nM triiodothyronine and 100 μ M ascorbate (differentiating medium). Where indicated, cells were grown in a hormone-depleted (non-differentiating) medium containing 10% (v/v) charcoal-treated fetal calf serum [18,19]. The HIB-1B brown adipocyte cell line, a gift from B. Spiegelman (Dana Farber Cancer Institute, Boston, U.S.A.) was cultured and differentiated as described previously [20]. HIB-1B brown pre-adipocytes were cultured in DMEM/F12 containing 10% (v/v) heat-inactivated FCS and differentiated HIB-1B brown adipocytes were cultured in the same medium containing only 5% (v/v) FCS, 20 nM insulin and 1 nM T3. Differentiation was induced by incubating confluent pre-adipocytes for 24 h in DMEM/F12 (1:1, v/v) containing 5% (v/v) FCS, 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μ M hydrocortisone and 0.125 mM indomethacin. The cells were typically 90–95% differentiated (as determined by morphology) 5 to 7 days after confluence.

RNA isolation, Northern-blot analysis and estimation of mRNA decay rates

Total RNA from brown adipose tissue and from cultured cells was extracted using a guanidine thiocyanate method [21]. For Northern-blot analysis, 10 μ g of total RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels, and transferred to positively charged nylon membranes (N+, Boehringer

Mannheim). Ethidium bromide (0.2 μ g) was added to RNA samples in order to check equal loading of the gels and transfer efficiency [22]. Pre-hybridization and hybridization were carried out at 55 °C in 0.25 M Na₂HPO₄ (pH 7.2)/1 mM EDTA/20% (w/v) SDS/0.5% (w/v) blocking reagent (Boehringer Mannheim) solution [23]. Blots were hybridized using the following DNA probes: a human genomic probe for *ATPsyn β* [24], the rat cDNA for UCP1 [25], the cDNA of human cytochrome oxidase subunit IV (COIV) (A.T.C.C. Rockville, MD, U.S.A.), a 0.5 kb fragment of the murine cDNA for cytochrome oxidase subunit II (COII) [26] and a genomic DNA probe for rat β -actin [27]. Hybridization signals were quantified using the Molecular Image System GS-525 (Bio-Rad). *ATPsyn β* mRNA half-life was determined as reported previously for other mRNAs in brown adipocytes in culture [28]. Hybridization signals in Northern-blot assays of equal amounts of RNA isolated from cells were determined after 4, 8 and 24 h treatment with actinomycin D at a concentration (1 μ g/ml) that inhibited transcription in different cell types, including brown adipocytes, by > 90% [28].

Plasmids and transfection assays

The plasmid 786*ATPsyn β* -CAT, in which the region –786 to +89 of the human *ATPsyn β* gene drives the promoterless chloramphenicol acetyltransferase (CAT) gene as well as the derived plasmids containing 5'-deletion mutations of this 786*ATPsyn β* -CAT, have been described previously [6]. The plasmid in which the –306/–266 sequence of the *ATPsyn β* gene is placed upstream the herpes simplex virus thymidine kinase (TK) promoter driving CAT (–306/–266-TK-CAT) was obtained by cloning a synthetic double stranded oligonucleotide with the following sense strand sequence: 5'-CAACAGGAAC-TCGGCCCTTTCCTAAACGTTAGTTCCTCTGA-3' into the *Xba*I site of PBLCAT2. Two mutated versions of this plasmid were obtained by cloning the same oligonucleotide except for TT instead of GG at sites –301 and –300 (Mut NRF2/GABP –306/–266-TK-CAT) or TT instead of CC at sites –291 and –290 (Mut Sp1 –306/–266-TK-CAT). HIB-1B brown adipocytes were transiently transfected by a modified calcium phosphate precipitation method [29]. Each transfection contained 10 μ g of the CAT expression plasmids. CAT activity was determined as described previously [30]. In order to assess the efficiency of separate transfections, 5 μ g of pRSV (Rous sarcoma virus) β -galactosidase was added to each transfection and the β -galactosidase activity was determined as an internal control. To establish that a particular regulation of the RSV promoter did not preclude its usefulness as an internal control to allow comparison of the efficiency of transfection between cells at different stages of differentiation, direct assessment of the efficiency in transfection was performed by standard Southern-blot analysis of DNA from transfected cells using the cDNA of CAT as a probe. Data indicated that the β -galactosidase activity driven by pRSV- β -galactosidase was a reliable index of efficiency of transfection in different cell cultures. Independent DNA preparations for each construct were always tested in the transfection assays.

DNA-binding experiments

For electrophoretic gel mobility-shift assays, the double-stranded synthetic oligonucleotide corresponding to –306/–266 and containing 5' extruding ends was end-labelled using [α - 32 P]dCTP and the Klenow enzyme. Labelled probe (20000–30000 c.p.m.) was incubated in each single assay with 5 μ g of rat brown adipose tissue nuclei protein [31]. The binding reactions were performed

in 20 mM Hepes (pH 7.6)/0.1 mM EDTA/1 mM dithiothreitol/10 % glycerol/50 mM NaCl containing 1 μ g poly(dI-dC) in a final volume of 20 μ l and after 20 min at 22 °C samples were loaded on to 5 % polyacrylamide gels in 0.5 $\times$ TBE (44.5 mM Tris/44.5 mM borate/1 mM EDTA). Electrophoresis was performed at 250 V for 45–60 min at 4 °C. In the competition experiments various molar excesses of unlabelled oligonucleotides were included in the binding reaction. Sense-strand sequences of the duplexed synthetic oligonucleotides used for competition were: 5'-GAGGAAGCATTTCCTGG-3', corresponding to the ETS site of the rat stromelysin promoter [32]; 5'-CCGCTCTTCGGTCCGCG-3', corresponding to the NRF2/GABP binding site in the rat cytochrome oxidase subunit IV promoter [33]; 5'-ATTCGTCGGGGCGGGGCGAG-3', consensus binding site for Sp1 [34]; 5'-CTAGACTCTAAAATA-ACCCTA-3', consensus oligonucleotide for myocyte enhancer-specific factor-2 (MEF2) [35] used as a negative control, and the two oligonucleotides corresponding to the double-point mutant forms of -306/-266 sequence of the *ATPsyn β* gene promoter depicted above. When antibodies were used, 1 μ l of anti-sera against GA-binding protein (GABP) β 1 or Sp3, gifts from Dr. S. McKnight (Tularik Inc., San Francisco, U.S.A.) and Dr. G. Suske (University of Marburg, Germany) respectively, or 1 μ l of anti-bodies against Sp1 or retinoic acid receptor α (Santa Cruz Inc.) were included in the incubation mixture. Gels were analysed by autoradiography.

RESULTS

The expression of *ATPsyn β* mRNA is up-regulated during brown adipocyte differentiation *in vivo* and *in vitro*, in association with enhanced nuclear and mitochondrial gene expression for components of the OXPHOS system

The expression of *ATPsyn β* mRNA and other OXPHOS gene mRNAs was studied during the ontogeny of brown adipose tissue and is depicted in Figure 1. The expression of UCP1 mRNA, the molecular marker of brown adipocyte phenotype, was almost negligible at day 15 of mouse fetal development and maximum levels were attained at day 18, just before birth. This key period of brown fat differentiation *in vivo* was associated with a rise in expression of *ATPsyn β* mRNA levels, which attained maximum levels just before birth. This increase appeared to be part of an overall augmentation in gene expression for components of the OXPHOS system. Thus both COII mRNA and COIV mRNAs, taken as examples of mitochondrial and nuclear genome-encoded components, respectively, of the OXPHOS system, increased during this prenatal period. The pattern was not due to an overall profile of gene expression in brown fat during this period, as β -actin mRNA levels followed an opposite pattern.

Figure 2 depicts a parallel approach using brown pre-adipocytes differentiating in culture. Precursor cells from murine brown adipose tissue showed non-adipocyte morphology on day 4 (Figure 2A). At confluence, morphology became typically adipocytic with the appearance of multilocular lipid depots (day 7) with a maximum of lipid accumulation after further culture (day 10). When precursor cells at day 4 were cultured in hormone-depleted medium, adipocyte differentiation was stunted and cells remained undifferentiated at day 7. The expression of UCP1 mRNA correlated strictly with the morphological observation of brown fat cell differentiation in culture (Figure 2B). Again, the expression of *ATPsyn β* mRNA, COII mRNA and COIV mRNA paralleled adipocyte differentiation, with a marked rise between days 4 and 7, which was impaired when differentiation was prevented (day 7, non-differentiated). These findings indicate

Figure 1 Changes in the expression of *ATPsyn β* mRNA and other OXPHOS genes during brown adipose tissue development

Left: total RNA (10 μ g) extracted from the interscapular brown adipose tissue of mice at the indicated stage of development was analysed by Northern blot as described in the Materials and methods section. For fetuses and mice at birth, RNA was extracted from pooled tissues of a single litter. Results are expressed as percentages relative to the mean value at the developmental time of maximum levels of expression, which was set to 100 (arbitrary units). Results are the mean $\pm$ S.E.M. of at least three independent analyses of RNA from different samples. Right: examples of Northern-blot analyses of RNA from fetuses at days 15 and 18 showing the sizes of the detected mRNAs.

that high expression of the *ATPsyn β* gene is a feature of the brown adipocyte and is associated with the acquisition of the differentiated phenotype, and is representative of a general enhancement of gene expression for OXPHOS components.

Expression of *ATPsyn β* mRNA and other OXPHOS gene mRNAs in differentiating HIB-1B brown adipocyte cells

To assess the molecular mechanisms for enhanced *ATPsyn β* gene expression in brown adipocytes, the brown adipocyte cell line

Figure 2 Changes in the expression of ATPsyn β mRNA and other OXPHOS genes during brown adipocyte differentiation in primary cultures

(A) Photomicrographs of pre-adipocytes isolated from brown adipose tissue in primary culture at days 4, 7 (Day 7. Differentiated) and 10 (Day 10. Differentiated) of culture, and at day 7 of culture after replacement of regular medium with depleted medium at day 4 (Day 7. Non differentiated). Magnification $\times 100$. (B) Total RNA (10 μ g) extracted from cells at the indicated stage of development (●, days 4, 7 and 10 in regular medium; ○, day 7 in depleted medium) was analysed by Northern blot as described in the Materials and methods section. The results are expressed as percentages relative to the mean value at the developmental time of maximum levels of expression, which was set to 100 (arbitrary units). Results are the mean $\pm$ S.E.M. of at least three independent analyses of RNA from different culture experiments, each single sample of RNA was a pool of at least three culture plates.

Table 1 Changes in the expression of the mRNAs for ATPsyn β and other OXPHOS genes in HIB-1B cells at different stages of differentiation

Northern-blot analyses of RNA (10 μ g) from non-differentiated or differentiated HIB-1B cells were used for quantification. Northern blots were probed for mRNA expression of the mitochondrial proteins ATPsyn β , COIV and COII and the non-mitochondrial control β -actin. The results are expressed as percentages of the non-differentiated cells and are means $\pm$ S.E.M. of three independent cultures.

	ATPsyn β	COIV	COII	β -actin
Non-differentiated	100 $\pm$ 9	100 $\pm$ 12	100 $\pm$ 10	100 $\pm$ 13
Differentiated	306 $\pm$ 19	352 $\pm$ 16	278 $\pm$ 31	107 $\pm$ 70

HIB-1B was used. These brown fat tumour-derived cells can be induced to differentiate to brown adipocytes when cells reach confluence and are further cultured under specific conditions

Figure 3 ATPsyn β mRNA half-life in HIB-1B cells at different stages of brown adipocyte differentiation

Representation of the log of ATPsyn β mRNA levels (arbitrary units/ μ g of total RNA) at various times after exposure to actinomycin D (1 μ g/ml) in proliferating non-differentiated (▲) or differentiated (●) HIB-1B cells. ATPsyn β mRNA levels were assessed by Northern-blot analysis.

[20]. Identical with primary cultures, differentiated HIB-1B cells (5 days post-confluence) showed higher expression of ATPsyn β mRNA, COII mRNA and COIV mRNA when compared with non-differentiated cells (Table 1). UCP1 mRNA expression was detectable only in noradrenaline-treated differentiated HIB-1B cells (results not shown), as reported previously [20]. To establish whether an increase in ATPsyn β mRNA stability could be involved in the rise of ATPsyn β mRNA observed in differentiated brown adipocytes, the decay in ATPsyn β mRNA levels was determined in non-differentiated and differentiated HIB-1B cells after treatment with the transcription inhibitor actinomycin D. Figure 3 shows that ATPsyn β mRNA was slightly more stable in non-differentiated cells than in differentiated brown adipocytes. The half life for ATPsyn β mRNA was 39 h for non-differentiated cells and 26 h for differentiated brown adipocytes, which is within the range of values for mRNAs of other nuclear-encoded OXPHOS genes in different cell types [36]. It was concluded that brown adipocyte differentiation is not associated with any stabilization of ATPsyn β mRNA.

ATPsyn β promoter activity in HIB-1B brown adipocytes: mutant deletion analysis

Non-differentiated and differentiated HIB-1B cells were transiently transfected with an expression vector in which the $-786/+89$ region of the gene drives the CAT reporter gene. The results showed a three-fold higher activity in differentiated cells with respect to non-differentiated ones (Figure 4). This indicated that the ATPsyn β promoter contains elements involved in the preferential expression of this gene in the differentiated brown adipocyte. Progressive deletion mutant analysis indicated that deletions from -786 to -381 did not cause any reduction of promoter activity either in differentiated or non-differentiated cells. Only when the region between -381 and -246 was deleted, did the promoter activity fall to low levels. This deletion caused the elimination of the E region, a site between -306 and -266 previously reported to be a major regulatory element for the ATPsyn β in other cell lines and which includes an ETS-response element [6]. Basal promoter activity required a minimal region containing a CCAAT box between nt $-246/-220$.

Figure 4 ATPsyn β promoter activity in differentiated and non-differentiated HIB-1B brown adipocytes: effect of progressive 5' deletions

Proliferating non-differentiated and differentiated HIB-1B cells were transiently transfected with 10 μ g of the chimaeric ATPsyn β gene-CAT constructs depicted on the left and 5 μ g of RSV β -galactosidase. After 36 h the CAT activity in cell extracts was measured (see the Materials and methods section). The main *cis*-acting elements described so far for the ATPsyn β promoter [6,8] are shown above the left panel. Results are expressed as percentages of the activity obtained with the 786ATPsyn β -CAT construct when transfected to differentiated HIB-1B cells (100%) and means $\pm$ S.E.M. of three to four independent transient transfection experiments, performed in duplicate, for each construct.

NRF2/GABP and Sp1 are the transcription factors in brown adipose tissue nuclei that bind the -306/-266-regulatory region of the ATPsyn β promoter

The identity of the protein factors from brown adipose tissue interacting with the E site of the ATPsyn β gene was assessed by electrophoretic shift assays using protein extracts from brown adipose tissue nuclei. Incubation of the DNA fragment from -306 to -266 with these extracts resulted in the appearance of two main retarded bands (Figure 5), which were named A and B. Previous analyses of this regulatory site of the ATPsyn β promoter

in hepatic cells revealed that this region was able to bind ETS-related proteins because of the AGGAA site at -302/-298 [6]. Moreover, this region can bind Sp1-related proteins due to the GC-rich region at -295/-289 [8]. Here we performed competition gel-shift analysis by incubating the DNA probe and brown fat nuclei protein extracts with an excess of oligonucleotides corresponding to the DNA-binding sites for Sp1 [34] or ETS proteins [32]. As shown in Figure 5(A), the B band disappeared only with an excess of the GC-rich oligonucleotide, indicating that a member of the Sp family of transcription factors present in brown fat nuclei was responsible for the interaction with the -306/-266 region of the ATPsyn β promoter leading to the B band. Conversely, competition analysis using the ETS oligonucleotide demonstrated that the A band was caused by the interaction of an ETS protein present in brown fat nuclei. Further competition gel-shift assays indicated that lower amounts of NRF2/GABP oligonucleotide were able to suppress the appearance of the A band, whereas higher amounts of an ETS2 oligonucleotide were required for full competition (Figure 5B). The identity of NRF2/GABP as the ETS protein binding the E site was established by incubating the brown fat extract and the DNA probe with a specific antibody against the β 1 subunit of NRF2/GABP. As shown in Figure 5(C), this resulted in the specific disappearance of band A in accordance with the requirement of the β 1 subunit for the optimal stability of the NRF2/GABP complex when bound to DNA [37]. Incubation with an Sp1 antibody resulted in the appearance of a higher-mobility band, typical of super-shifted complexes for the Sp1 protein when bound to DNA. Moreover, there was a reduction in the intensity of the B band (see Figure 5C) and of the A band. Incubation with an antibody against Sp3 was without effect (results not shown). The antibody against the retinoic acid receptor α (RAR), used as a negative control, was also without effect. It is concluded that the -306/-266 region of the ATPsyn β gene binds the NRF2/GABP and Sp1 transcription factors present in brown fat nuclei.

Figure 5 Electrophoretic mobility-shift analysis of the interaction of the -306/-266 region of the ATPsyn β promoter with nuclear proteins from brown adipose tissue

A double-stranded synthetic oligonucleotide corresponding to -306/-266 was end-labelled and incubated with 5 μ g of nuclear protein extracts from brown adipose tissue and subjected to PAGE (5% polyacrylamide). (A) Competition analysis with a 25-fold (+) or 100-fold (++) excess of unlabelled oligonucleotides, optimum for binding to Sp1 [34] or ETS2 [32]. (B) Competition analysis with increasing (10-, 25- or 100-fold) excesses of unlabelled oligonucleotides, optimum for binding NRF2/GABP [32], ETS2 or the negative control MEF2 [35]. (C) Effects of including 1 μ l of anti-serum against Sp1, NRF2/GABP/ β 1 or retinoic acid receptor α (RAR).

Figure 6 Expression of chimaeric constructs containing the $-306/-266$ region upstream of the basal TK promoter in non-differentiated and differentiated HIB-1B brown adipocytes: effects of two double-point mutations suppressing the binding to NRF2/GABP or Sp1

Two mutated versions of the $-306/-266$ were designed in which the GG at -301 and -300 were replaced by TT (Mut NRF2/GABP) or the CC at -291 and -290 were replaced by TT (Mut Sp1). (A) Competition electrophoretic mobility-shift assay. The wild-type $-306/-266$ oligonucleotide was labelled and incubated with $5 \mu\text{g}$ of brown adipose tissue nuclear protein extract. A 100-fold excess of each of the two mutated versions of the oligonucleotide was included in the incubation mixture. (B) Left: proliferating non-differentiated and differentiated HIB-1B cells were transiently transfected with $10 \mu\text{g}$ of the chimaeric CAT constructs and $5 \mu\text{g}$ of RSV β -galactosidase. Right: after 36 h CAT activity was measured in cell extracts (see the Materials and methods section). Results are expressed as the fold-induction of activity with respect to the basal activity of the basal TK-CAT construct when transfected to differentiated HIB-1B cells and are the means $\pm$ S.E.M. of three to four independent transient transfection experiments, performed in duplicate, for each construct.

Enhancer activity of the $-306/-266$ region of the ATPsyn β promoter in differentiating HIB-1B cells: double-point mutation analysis of the function of NRF2/GABP and Sp1 sites

A plasmid construct in which a double stranded synthetic oligonucleotide spanning from -306 to -266 was placed upstream of the TK promoter driving CAT was transfected into HIB-1B cells either non-differentiated or differentiated to brown adipocytes. The presence of the E region resulted in a two-fold enhancement of the basal promoter in non-differentiated cells, but led to a much higher increase in differentiated brown adipocytes (Figure 6B). This indicated that this element is more

active in differentiated cells and therefore involved in the enhancement of ATPsyn β promoter activity in association with brown adipocyte differentiation. Plasmid constructs were designed in which the E region, placed upstream of the TK promoter, was substituted by a mutant version in which the GG base pair at the NRF2/GABP site (-301 and -300) was replaced with TT (Mut NRF2/GABP) or the CC base pair at the Sp1 site (-291 , -290) was replaced with TT (Mut Sp1). Competition gel-shift assays showed that these changes resulted in a loss of the binding capacities for NRF2/GABP (no competition of the first mutant form for the A band) and for the B band (no competition of the second mutant form for the B band) respectively (Figure 6A). Direct labelling of the two mutant oligonucleotides confirmed the lack of binding of the NRF2/GABP and Sp1 bands for Mut NRF2/GABP and Mut Sp1 respectively (results not shown). The mutation which blocked NRF2/GABP binding completely suppressed the enhancer properties of the E region (Figure 6B). Conversely, the mutations suppressing the Sp1 site were without effect. This indicated that NRF2/GABP binding to the E site was a major requirement for the involvement of the E site in the activation of transcription in the brown adipocyte.

DISCUSSION

The present data indicate an overall enhancement of the mRNA expression for *OXPHOS* genes, including ATPsyn β mRNA, in the late prenatal period of brown adipose tissue development. This correlates with previous morphological observations of brown fat, which shows a sudden rise in mitochondrial content before birth [38]. When the present data are compared with previous studies on the ontogeny of *OXPHOS* gene expression, it is evident that full expression of the *OXPHOS* system in brown adipose tissue is attained, at least at the mRNA level, much earlier than in other tissues such as heart or brain, in which a progressive rise in *OXPHOS* mRNAs occurs postnatally [39,40]. This suggests that specific mechanisms must operate in brown adipose tissue to elicit this early rise in *OXPHOS* mRNA expression associated with tissue maturation *in utero*.

Data on the pattern of *OXPHOS* mRNA expression when brown adipocytes differentiate in culture lead to a clear conclusion, analogous to that arising from studies in brown adipose tissue prenatal ontogeny *in vivo*: the acquisition of the differentiated brown adipocyte phenotype (lipid accumulation, induction of *UCP1* gene expression) is associated with induction of ATPsyn β mRNA expression, which is part of a co-ordinated induction of nuclear and mitochondrial genome-encoded *OXPHOS* genes. Thus, there must be some molecular mechanism that establishes a link between the co-ordination of *OXPHOS* gene expression and the programme of adipocyte differentiation in the brown fat cell.

The enhancement of ATPsyn β mRNA when HIB-1B cells differentiate to brown adipocytes is associated with a higher activity of the ATPsyn β promoter. Preferential stabilisation of ATPsyn β mRNA, as observed in post-natal liver development [5], does not appear to occur in brown adipose tissue differentiation. The mutant-deletion analysis indicated that, in contrast to analogous studies performed in muscle cells, the enhanced expression of the ATPsyn β promoter in differentiated brown fat cells did not depend on a tissue-specific element such as the OXBOX site [8], but on a proximal region previously identified as an ETS-responsive region.

NRF2/GABP and Sp1 present in brown fat nuclei were identified as the two major transcription factors in brown fat nuclei that bind this regulatory region of the ATPsyn β promoter. This region can bind purified ETS1 and can be trans-activated by

ETS expression vectors in HepG2 cells [6]. The present results indicate that in brown adipose tissue NRF2/GABP is the major, if not the only, ETS transcription factor that binds this site. Although the recognition sequences for ETS1 and NRF2/GABP are very close [41], minor differences in the nucleotide sequences adjacent to the AGGAA core are likely to lead to the observed preferential affinity for NRF2/GABP. On the other hand, the relative abundance of NRF2/GABP and other ETS proteins in brown adipocytes with respect to other cell types awaits further studies.

Point mutant studies established that the regulatory region of ATPsyn β promoter lost its differentiation-dependent enhancer activity in brown adipocytes only when the NRF2/GABP binding site was suppressed. This finding is in contrast with observations in transformed cell lines of different origin, such as the hepatoma-derived HepG2 cells or the breast cancer MCF-7 cells, in which this site behaves also as an enhancer, and only suppression of Sp1 binding blocks the enhancer activity (J. Villena and F. Villarroya, unpublished work). Therefore it appears that this composite NRF2/GABP and Sp1 binding site is a transcriptional regulatory element in which NRF2/GABP or Sp1 action have a different relevance depending on which cell type and which stimulus lead to a high ATPsyn β expression. High levels of cell proliferation or cell transformation, known to induce high ATPsyn β gene expression [42,43], might rely on a major action of Sp1, whose expression is high in non-differentiated cells, whereas the high expression associated with the acquisition of the brown adipocyte phenotype may have NRF2/GABP as its major effector.

Previous reports have described NRF2/GABP elements in the promoters of the *COIV*, *COVb* and *COVIIaL* genes [33,44,45]. The present results are the first to point to NRF2/GABP as a mechanism for differentiation-dependent gene expression, in this case for the enhancement of ATPsyn β as a part of *OXPHOS* gene induction associated with the acquisition of the brown adipocyte phenotype. To date, the transcription factors CCAAT/Enhancer binding protein α and peroxisomal proliferator activated receptor γ are considered the master regulators of the differentiation programme of white adipocytes, and most probably also of brown adipocytes, in terms of lipid accumulation and expression of gene markers of the adipocyte phenotype [46]. However, the mechanisms by which mitochondrial biogenesis and *OXPHOS* gene expression are activated during brown adipocyte differentiation had not been established. We propose that NRF2/GABP constitutes a molecular mechanism for the enhanced mitochondrial biogenesis associated with the acquisition of the brown adipocyte phenotype. Further research will be needed to identify the links and interactions between the molecular mechanisms eliciting the adipogenic and the mitochondrial components of the differentiation process of the brown adipocyte.

We thank Dr. D. Ricquier (CEREMOD, CNRS, Meudon, France) and Dr. N. Glaichenhaus (University of Nice, Nice, France) for the UCP1 and COII cDNA probes, Dr. S. McKnight (Tularik Inc., San Francisco, U.S.A.) and Dr. G. Suske (University of Marburg, Germany) for the NRF2/GABP β 1 and the Sp3 anti-sera and Dr. B. Spiegelman (Dana Farber Cancer Institute, Boston, U.S.A.) for the HIB-1B cells. This research was supported by grant PB95.0969 from DGICYT, Ministerio de Educación y Cultura, Spain; by grant 1995SGR 00096 from the Generalitat de Catalunya and by the Fundació August Pi i Sunyer, Marató de TV3, Spain.

REFERENCES

- Poyton, R. O. and McEwen, J. E. (1996) *Annu. Rev. Biochem.* **65**, 563–607
- Kagawa, Y. and Ohta, S. (1990) *Int. J. Biochem.* **22**, 219–229
- Neckelmann, N., Warner, C. K., Chung, A., Kudoh, J., Minooshima, S., Fukuyama, R., Maekawa, M., Shimizu, Y., Shimizu, N., Liu, J. D. and Wallace, D. C. (1989) *Genomics* **5**, 829–843
- Ohta, S., Tomura, H., Matsuda, H. and Kagawa, Y. (1988) *J. Biol. Chem.* **263**, 11257–11262
- Izquierdo, J. M., Ricart, J., Ostronoff, L. K., Egea, G. and Cuezva, J. M. (1995) *J. Biol. Chem.* **270**, 10342–10350
- Villena, J. A., Martin, I., Vinas, O., Cormand, B., Iglesias, R., Mampel, T., Giral, M. and Villarroya, F. (1994) *J. Biol. Chem.* **269**, 32649–32654
- Tomura, H., Endo, H., Kagawa, Y. and Ohta, S. (1990) *J. Biol. Chem.* **265**, 6525–6527
- Haraguchi, Y., Chung, A. B., Neill, S. and Wallace, D. C. (1994) *J. Biol. Chem.* **269**, 9330–9334
- Nicholls, D., Cunningham, S. A. and Rial, E. (1986) in *Brown Adipose Tissue*, (Trayhurn, P. and Nicholls, D. G., eds.), pp. 52–85, Edward Arnold, London
- Houstek, J., Tvrdik, P., Pavelka, S. and Baudysova, M. (1991) *FEBS Lett.* **294**, 191–194
- Mory, G., Bouillaud, F., Combes-George, M. and Ricquier, D. (1984) *FEBS Lett.* **166**, 393–396
- Desautels, M. and Himms-Hagen, J. (1980) *Can. J. Biochem.* **58**, 1057–1068
- Desautels, M. (1985) *Am. J. Physiol.* **249**, E99–E106
- Villarroya, F., Felipe, A. and Mampel, T. (1986) *Biosci. Rep.* **6**, 669–675
- Villarroya, F., Felipe, A. and Mampel, T. (1986) *Biochim. Biophys. Acta* **882**, 187–191
- Trayhurn, P., Douglas, J. B. and McGuckin, M. M. (1982) *Nature (London)* **298**, 59–60
- Rehmark, S., Nechad, M., Herron, D., Cannon, B. and Nedergaard, J. (1990) *J. Biol. Chem.* **265**, 16464–16471
- Alvarez, R., Deandres, J., Yubero, P., Vinas, O., Mampel, T., Iglesias, P., Giral, M. and Villarroya, F. (1995) *J. Biol. Chem.* **270**, 5666–5673
- Samuels, H. H., Stanley, F. and Casanova, J. (1979) *Endocrinology* **105**, 80–85
- Ross, S. R., Choy, L., Graves, R. A., Fox, N., Solevjeva, V., Klaus, S., Ricquier, D. and Spiegelman, B. M. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7561–7565
- Chomczynski, P. and Sachi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Pappu, H. R. and Hiruki, C. (1989) *Focus* **11**, 156–157
- Engler-Blum, G., Meier, M., Frank, J. and Müller, G. A. (1993) *Anal. Biochem.* **210**, 235–244
- Martin, I., Giral, M., Viñas, O., Mampel, T., Iglesias, R. and Villarroya, F. (1995) *Biochem. J.* **308**, 749–752
- Bouillaud, F., Ricquier, D., Thibault, J. and Weissenbach, J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 445–448
- Glaichenhaus, N., Leopold, P. and Cuzin, P. (1986) *EMBO J.* **5**, 1262–1265
- Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z. and Yaffe, D. (1983) *Nucleic Acid Res.* **11**, 1759–1771
- Pico, C., Herron, D., Palou, A., Jacobsson, A., Cannon, B. and Nedergaard, J. (1994) *Biochem. J.* **302**, 81–86
- Graves, R. A., Tontonoz, P., Ross, S. and Spiegelman, B. M. (1991) *Genes Dev.* **5**, 428–437
- Pothier, F., Ouellet, M., Julien, J.-P. and Guérin, S. L. (1992) *DNA Cell Biol.* **11**, 83–90
- Yubero, P., Manchado, C., Cassard-Doucier, A. M., Mampel, T., Viñas, O., Iglesias, R., Giral, M. and Villarroya, F. (1994) *Biochem. Biophys. Res. Commun.* **198**, 653–659
- Wasylyk, C., Gutman, A., Nicholson, R. and Wasylyk, B. (1991) *EMBO J.* **10**, 1127–1134
- Virbasius, J. V. and Scarpulla, R. C. (1991) *Mol. Cell. Biol.* **11**, 5631–5638
- Kadonaga, J. T., Courey, A. J., Ladika, J. and Tjian, R. (1988) in *The Control of Human Retrovirus Gene Expression* (Franza, Jr., B. R., Cullen, B. R. and Wong-Staal, F., eds.), pp. 239–250, Cold Spring Harbor Laboratory
- Cserjesi, P. and Olson, N. E. (1991) *Mol. Cell. Biol.* **11**, 4854–4862
- Chrzanowska-Lightowler, Z. M., Preiss, T. and Lightowler, R. N. (1994) *J. Biol. Chem.* **269**, 27322–27328
- La Marco, K., Thompson, C. C., Byers, B. P., Walton, F. M. and McKnight, S. L. (1991) *Science* **253**, 789–792
- Barnard, T. and Lindberg, O. (1969) *J. Ultrastruct. Res.* **29**, 293–310
- Kim, K., Lecordier, A. and Bowman, L. H. (1995) *Biochem. J.* **306**, 353–358
- Vega-Nunez, E., Menéndez-Hurtado, A., Garesse, R., Santos, A. and Perez-Castillo, A. (1995) *J. Clin. Invest.* **96**, 893–899
- Brown, T. A. and McKnight, S. L. (1992) *Genes Dev.* **6**, 2502–2512
- Torroni, A., Stepien, G., Hodge, J. A. and Wallace, D. C. (1990) *J. Biol. Chem.* **265**, 20589–20593
- Luciakova, K., Li, R. G. and Nelson, B. D. (1992) *Eur. J. Biochem.* **207**, 253–257
- Carter, R. S. and Avadhani, N. G. (1994) *J. Biol. Chem.* **269**, 4381–4386
- Seelan, R. S., Gopalakrishnan, L., Scarpulla, R. C. and Grossman, L. I. (1996) *J. Biol. Chem.* **271**, 2112–2120
- Brun, R. P., Kim, J. B., Hu, E., Altio, S. and Spiegelman, B. M. (1996) *Curr. Opin. Cell Biol.* **8**, 826–832