RESEARCH COMMUNICATION

A 211-bp enhancer of the rat uncoupling protein-1 (UCP-1) gene controls specific and regulated expression in brown adipose tissue

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The uncoupling protein-1 gene is uniquely expressed in brown adipose tissue (BAT) and is positively regulated by cold exposure of animals and the sympathetic nervous system. To analyse the importance of a previously identified 211-bp enhancer [Cassard-Doulcier, Gelly, Fox, Schrementi, Raimbault, Klaus, Forest, Bouillaud and Ricquier (1993) *Mol. Endocrinol.* 7, 497–506] in the tissue-specific expression of this gene, transgenic mice were generated using the chloramphenicol acetyltransferase (CAT) gene as a reporter gene. One out of fourteen lines of the control transgenic mice bearing the *Herpes simplex* thymidine kinase (TK) promoter expressed weakly the CAT reporter gene in several tissues, whereas the other lines did not express CAT. Eight founders bearing the 211-bp enhancer-TK transgene were

obtained. In six lines, no expression of CAT was detected. In one line, the expression of CAT was restricted to BAT. In another line, the expression of CAT was found in BAT and, to a lesser extent, in testis. Moreover, in these lines a marked and specific increase in the expression of the reporter gene in BAT was observed either after exposure of mice to the cold or by treating them with a β -adrenoceptor agonist drug. These results demonstrate that the 211-bp enhancer alone is sufficient to both direct and restrict expression to BAT. This enhancer also mediates the transcriptional response of the gene to β -adrenergic stimulation, although it does not contain conserved cAMP response element.

INTRODUCTION

The function of brown adipose tissue (BAT) is adaptive thermogenesis. The brown adipocytes function specifically to dissipate energy as heat in response to a cold environment or overfeeding [1,2]. Heat production by brown adipocytes results from a regulated uncoupling of respiration [1]. The mitochondrial uncoupling protein (UCP) unique to brown adipocytes, and referred to as UCP-1 since the discovery of the widely expressed UCP-2 [3], is responsible for the dissipation of oxidation energy as heat in these cells [1,2]. During exposure to the cold, the mitochondrial UCP-1 is acutely activated by free fatty acids [1]. In addition to UCP-1 activation, cold rapidly and markedly activates UCP-1 gene transcription, a phenomenon mediated by noradrenaline (norepinephrine) acting on adrenergic receptors and the cAMP pathway [4–9]. Thyroid hormones [10–12] and retinoic acid [7,13–16] also activate UCP-1 gene transcription. More recently, a role for ligands of peroxisome-proliferator-activated receptor (PPAR) was proposed [17–19]. The 5'-flanking region of rat or mouse UCP-1 gene contains *cis*-acting elements involved in tissue-specific expression and hormonal regulation [6,14–16,20,21]. A search for these regulating regions identified a 211-bp enhancer located at positions bp –2494 to –2283 upstream of the transcription-start point in the rat gene [6]. Analysis of mouse UCP-1 gene revealed the existence of a similar enhancer between bp –2530 and bp –2310 [21]. A role for the enhancer in the specific expression of UCP-1 in brown adipocytes was suggested by cell-transfection and transgenic-mice experiments [6,14,18,21]. The present study demonstrates that the 211-bp enhancer alone is sufficient to specifically drive the expression of a reporter gene in BAT. Moreover, this enhancer also mediates

the positive regulation induced by either exposure to the cold or catecholamine injection.

EXPERIMENTAL

Transgene construction

The 211-bp *AatII*/*ApaI* enhancer fragment was isolated, repaired and cloned into the unique *XbaI* restriction site of pBLCAT5 plasmid [22]. The thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT) or 211-TK-CAT transgenes were excised by *SalI*/*KpnI* digestion and prepared as described previously [14].

Generation of transgenic mice and CAT assay

DNA fragments from the constructs were cleaved with appropriate enzymes to release the transgene from the vector. Purified fragments were obtained by electroelution from agarose gels. The DNA was microinjected into pronuclei of single-cell embryos obtained from B6D2 F1 parents and injected embryos were transplanted into the oviducts of pseudo-pregnant foster mothers [23]. After preparation of tail DNA, transgenic offspring were identified by PCR amplification of the CAT gene. The integrity of the transgene was examined by Southern blot analysis. For stimulation analysis, mice were either exposed to 4 °C for a period of 24 h or kept at room temperature and injected with a β_3 -adrenoceptor agonist (CL-316,243) at 1 mg/kg [24]. Experiments with mice were performed in accordance with official guidelines for animal experimentation and legal requirements of

Abbreviations used: BAT, brown adipose tissue; CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; PPAR, peroxisome-proliferator-activated receptor; TK, thymidine kinase; UCP, uncoupling protein.

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the French authorities. CAT activity was determined by TLC analysis or by using the phase-extraction assay [25,26]. Cellular extracts from cells transfected with CAT constructs (obtained as described in [14]) were used as internal controls during the assay of CAT activity in tissues from transgenic mice.

RESULTS AND DISCUSSION

Transgenic mice containing the TK promoter fused to the CAT reporter gene were generated (TK-CAT construct). Fourteen lines having this TK-CAT construct in their DNA were obtained. The level of transgenic expression was estimated and correlated with activity of the CAT enzyme. No trace of CAT activity was measured in nine tissues (heart, lung, liver, kidney, testis, white adipose tissue, skeletal muscle, brain and BAT) in thirteen lines out of fourteen. Only one out of the fourteen TK-CAT lines expressed the CAT gene. In this line (TK/90 line), weak CAT activity was measured in heart, lung, liver, skeletal muscle, BAT or white adipose tissue, whereas higher CAT activity was detected in the kidney and brain (see Table 1); an example of the data is given in Figure 1(A). Moreover, no stimulation of CAT activity in these tissues was observed in several experiments in which these animals were exposed to the cold or treated by a β_3 -adrenoceptor agonist (results not shown).

Eight founders bearing the 211-bp enhancer of the rat UCP-1 gene upstream of the heterologous TK promoter (211-TK-CAT construct) were obtained. Two independent 211-TK-CAT transgenic lines expressed the CAT gene; these two lines were referred to as the 211/TK26 and 211/TK50 lines respectively. In these two lines, the CAT activity was uniquely detected in BAT, except for a weak CAT activity measured in testis of mice belonging to the 211/TK 50 line (Table 1). Figure 1(B) shows an example of data obtained with mice belonging to this line. Using long CAT constructs, Kozak et al. [21] originally demonstrated that a region between -3 kb and -1.2 kb was required for controlling mouse UCP-1 expression *in vivo*, whereas Cassard-Doulcier et al. [6] demonstrated that 4.5 kb of DNA upstream of the transcriptional-start site of the rat gene was controlling the tissue-specific expression of the rat UCP-1 gene *in vivo*. More recently, specific expression of a reporter gene driven by the rat enhancer attached to 400 bp of the proximal region of the promoter has been described [14]. The results presented here are in agreement with these previous data. Moreover, they establish

that the 211-bp enhancer alone is the genomic region controlling the specific expression of the UCP-1 gene in BAT.

To test the capacity of the 211-TK-CAT transgene to respond to stimulation, we either exposed transgenic animals to the cold or treated them with an adrenergic compound, because it is known that the effect of cold is mediated by the sympathetic innervation of brown adipocytes [2]. The adrenergic agonist used was the compound CL-316,243, which is a specific agonist of β_3 -adrenoceptors present on the plasma membrane of brown adipocytes [24]. A pronounced stimulation of expression by either cold exposure or the administration of a β_3 -adrenoceptor agonist was obtained for the 211/TK26 mice (Figure 2 shows an example of data corresponding to this line of mice). In these animals, a 3.7-fold or 10.7-fold stimulation by cold exposure or CL-316,243 treatment respectively was measured (means for three or four experiments; see Table 1). Mice from the 211/TK50 cell line also possessed a significantly higher activity after stimulation. Results obtained for stimulated mice from this line are not shown. Although the role of other regions in hormonal stimulation cannot be excluded, it was concluded that the 211-bp domain is involved in the transcriptional activation of the UCP-1 gene provoked by cold exposure or adrenergic stimulation.

Kozak et al. [21] identified cAMP response element (CRE) in both the basal promoter and the enhancer of the mouse UCP-1 gene. The regulation of rat UCP-1 gene by cAMP seems to be different in some way because mutations of a CRE consensus site in the proximal region of the rat gene (bp -147 to -120) only induced a small decrease of CAT expression in cell-transfection experiments (M. Larose, A. M. Cassard-Doulcier and D. Ricquier, unpublished work). Recent data from Silva and co-workers [18] agree with a non-requirement of the proximal region of the promoter for the β -adrenergic stimulation of rat UCP-1. The same group also reported a role for the upstream moiety of the rat enhancer in response to cAMP, although this region does not contain the CRE consensus sequence [18]. In this particular region of the rat gene, we previously described a domain of 20 bp, referred to as UAR, and made of an inverted repeat of type 2 adjacent to an activator protein (AP)-1-related binding site [14]. Mutations of the UAR domain strongly decreased the transcriptional stimulation by retinoids or cAMP in cell-transfection experiments ([14], and M. Larose and D. Ricquier, unpublished work). Both these results and the present study of transgenic mice treated by an adrenergic compound

Table 1 CAT activity in tissues from TK-CAT or 211-TK-CAT transgenic mice

TK/90 refers to a line of transgenic mice bearing the TK promoter attached to the CAT gene. This line, out of 14 lines that did not express CAT activity, expressed this activity in several tissues. TK211/50 and TK211/26 are two lines of transgenic mice bearing the 211-bp enhancer of the UCP-1 gene attached to the TK promoter and the CAT gene. These two lines (out of eight) expressed the CAT gene in BAT. The data correspond to CAT activity expressed as percentages of chloramphenicol acetylation in different tissues of mice kept at room temperature (RT) or stimulated by cold exposure or treatment by the β_3 -adrenoceptor agonist, CL-316,243. The fold-stimulation after exposure to the cold (Fold st. 4 °C) or treatment with the β_3 -adrenoceptor agonist CL-316,243 (Fold st. CL) relative to the values measured in animals at RT is indicated. The data represent the means $\pm$ S.E.M. The number of repetitions for each experiment is indicated (*n*). n.d., non-detectable CAT activity; WAT, white adipose tissue.

		BAT	Brain	WAT	Testis	Muscle	Liver	Heart	Kidney	Lung
TK/90	RT	0.3 $\pm$ 0.1	4.5 $\pm$ 0.9	0.3 $\pm$ 0.2	n.d.	0.6 $\pm$ 0.1	n.d.	0.2 $\pm$ 0.1	1.8 $\pm$ 0.4	0.5 $\pm$ 0.2
	<i>n</i>	5	7	4	2	6	4	4	6	4
TK211/50	RT	0.9 $\pm$ 0.1	n.d.	n.d.	0.4 $\pm$ 0.2	n.d.	n.d.	n.d.	n.d.	n.d.
	<i>n</i>	8	2	2	3	3	2	2	2	2
	Fold st. 4 °C	3.7 $\pm$ 1	n.d.	n.d.	0.8 $\pm$ 0.2	n.d.	n.d.	n.d.	n.d.	n.d.
	<i>n</i>	3	2	2	3	3	2	2	2	2
	Fold st. CL	10.7 $\pm$ 2.1	n.d.	n.d.	1	n.d.	n.d.	n.d.	n.d.	n.d.
	<i>n</i>	4	1	1	1	1	1	1	1	1
TK211/26	Mean RT	14.9 $\pm$ 4.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	<i>n</i>	7	5	2	4	5	3	3	5	3

Figure 1 CAT activity in tissues from TK-CAT or 211-TK-CAT transgenic mice

(A) TK-CAT transgenic mice (TK/90 line) bearing the TK promoter (bp -115 to +54) upstream of the CAT gene. One line of mice (the TK/90 line) out of fourteen expressed CAT activity in several tissues; in the 13 other lines, no trace of CAT activity was detected. (B) 211-TK-CAT mice bearing the 211-bp enhancer of the rat UCP-1 gene upstream of TK-CAT. The 211-bp enhancer is between positions bp -2494 (*AatII* site) and bp -2283 (*Apal* site) of the rat gene relative to the transcriptional-start site [6]. Two lines of mice (TK211/50 and TK211/26 lines) out of eight exhibited CAT activity in BAT; in the six other lines, no CAT activity was detected in BAT or in other tissues. This Figure is an example of data corresponding to TK211/50 line. The same data were obtained with several mice from the two independent lines. Br, brain; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; WAT, white adipose tissue. — and + are negative and positive CAT controls corresponding to cells transfected with CAT constructs. The CAT assay was carried out using 100 μ g of protein extracted from the different tissues.

demonstrate that the stimulation of the rat gene by cAMP is mediated by mechanisms not requiring canonical CRE in the 211-bp enhancer.

The upstream moiety of UCP-1 enhancer also contains a peroxisome-proliferator-response element responding to thiazolidinediones [17–19]. Therefore this enhancer has a complex organization, because it contributes to the stimulation of transcription by different types of hormones, including thyroid hormones, retinoids and catecholamines. The very recent discovery of PGC-1, a new co-activator, by Puigserver et al. [27] is especially interesting, because it explains how a particular protein increases the transcriptional activity of PPAR and thyroid-hormone receptor on the UCP-1 enhancer. Undoubtedly, PGC-1 is interacting with transcriptional factors that bind to the 211-bp enhancer. The molecular mechanism of this interaction remains to be determined.

In conclusion, the creation of transgenic mice containing the 211-bp enhancer upstream of a heterologous promoter demonstrates conclusively that this enhancer alone is sufficient to drive the expression of a reporter gene specifically in BAT. The rat UCP-1 enhancer also contains sequences, distinct from canonical CRE, controlling stimulation mediated by β -adrenergic agonists.

Figure 2 Effect of exposure to the cold or dosing with an adrenergic agonist on CAT activity in tissues of 211-TK-CAT mice

Mice from a 211-TK-CAT line expressing CAT activity in their BAT were exposed to the cold or injected with an adrenergic drug. This Figure represents an example of data corresponding to the TK211/26 line. RT, animal at room temperature; 4 °C, mice exposed to 4 °C for 24 h; CL: mice injected with CL-316,423 compound. Br, brain; H, heart; K, kidney; Li, liver; Lu, lung; M, muscle; WAT, white adipose tissue. A similar effect of cold exposure was observed with the TK211/50 line. The CAT assay was performed with 100 μ g of tissue protein.

The proximal promoter of the rat UCP-1 gene is not necessary for the specific expression of the gene in BAT.

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