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Commentary

Comment to Shinohara et al. (1991) *FEBS Letters* 293, 173–174
The uncoupling protein is not expressed in rat liver

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Using Northern blot analysis, immunoblotting with purified antibodies and Polymerase Chain Reaction analysis, we were unable to detect the Uncoupling Protein-UCP or its mRNA in liver of control, cold-exposed or newborn rats. The unique expression of this protein in brown adipocytes was confirmed. These data refute the surprising recent report on UCP expression in rat liver (Shinohara (1991) FEBS Lett. 293, 173–174). Moreover we report that the hybridization signal obtained by these authors is probably non-specific and due to the 3′ non-coding domain of the UCP cDNA probe.

Uncoupling protein; Liver mitochondria; Brown adipose tissue; Northern blotting; Immunoblotting; Polymerase chain reaction

1. INTRODUCTION

Brown adipose tissue (BAT) is unique in that its mitochondria have the capacity to uncouple substrate oxidation from ADP phosphorylation. This thermogenic uncoupling mechanism is due to a proton transporter present in the inner membrane of brown adipocyte mitochondria and named Uncoupling Protein or UCP [1–4]. The exclusive occurrence of UCP in BAT was ascertained by many investigators using anti-UCP antibodies and enzyme-linked immunosorbent assay, immunodiffusion, radioimmunoassay or immunoprecipitation [6–11]. These data were confirmed by investigators who were unable to detect UCP mRNA in liver or other tissues [12–16]. Surprisingly, Shinohara et al., using a rat UCP cDNA originating from our laboratory [12], recently claimed they could detect UCP mRNA in newborn rat liver as well as in the liver of cold exposed rats [17]. We present data contradicting such a conclusion and confirming the exclusive expression of UCP in brown adipocytes.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[α-^{32}P]dCTP and nylon (hybond) membranes were purchased from Amersham France. Nitrocellulose membranes were obtained from Sartorius and Affigel 15 and phosphatase alkaline substrates from Biorad. RNAsin was from Pharmacia.

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2.2. *Animals, RNA extraction, isolation of mitochondria*

Adult male Wistar rats (7 weeks old) were kept at 23°C or exposed at 5°C for 8 days. RNA was prepared from their brown adipose tissue, heart, brain and liver as previously described [14]. RNA was also extracted from liver of end-term rat fetus and newborn (0.25, 1 or 3 days old) rat according to Chomczynski and Sacchi [18]. Poly(A)$^+$ RNA was isolated using oligo-dT cellulose. Liver and brown adipose tissue mitochondria were isolated using classical procedure.

2.3. *cDNA probes*

Northern analysis was firstly made as in [14] with the rat UCP cDNA. This cDNA was amplified from a plasmid containing the entire cDNA [12], using polymerase chain reaction technique and oligonucleotides allowing the amplification of the entire coding sequence flanked of short 5′ (28 nucleotides) and 3′ (57 nucleotides) non-coding sequences. For other experiments, a short probe corresponding mainly to the 3′ non-coding region was obtained by *Bam*HI digestion of a pTZ ZU 55 plasmid containing the entire rat UCP cDNA. This ZU 55 plasmid was sent to Dr. Terada and used by Shinohara et al. [17]. The short *Bam*HI probe was made of the C-terminal 28 codons, 112 nucleotides of the 3′ non-coding region (comprising a strecht of 21 A), a stretch of 16 C and 24 nucleotides of the polylinker domain of pTZ plasmid. The fragment was then self-ligated with DNA ligase prior to ^{32}P-labelling.

2.4. *Western analysis of UCP*

UCP was immunodetected by antibodies; these antibodies had been purified by chromatography of total anti-UCP antiserum [9] on affinity column made of the 54 C-terminal aminoacids of UCP attached to Affigel 15 activated beads [19]. The mitochondrial proteins were electrically transferred onto nitrocellulose membrane. Anti-sheep immunoglobulins linked to alkaline phosphatase were used.

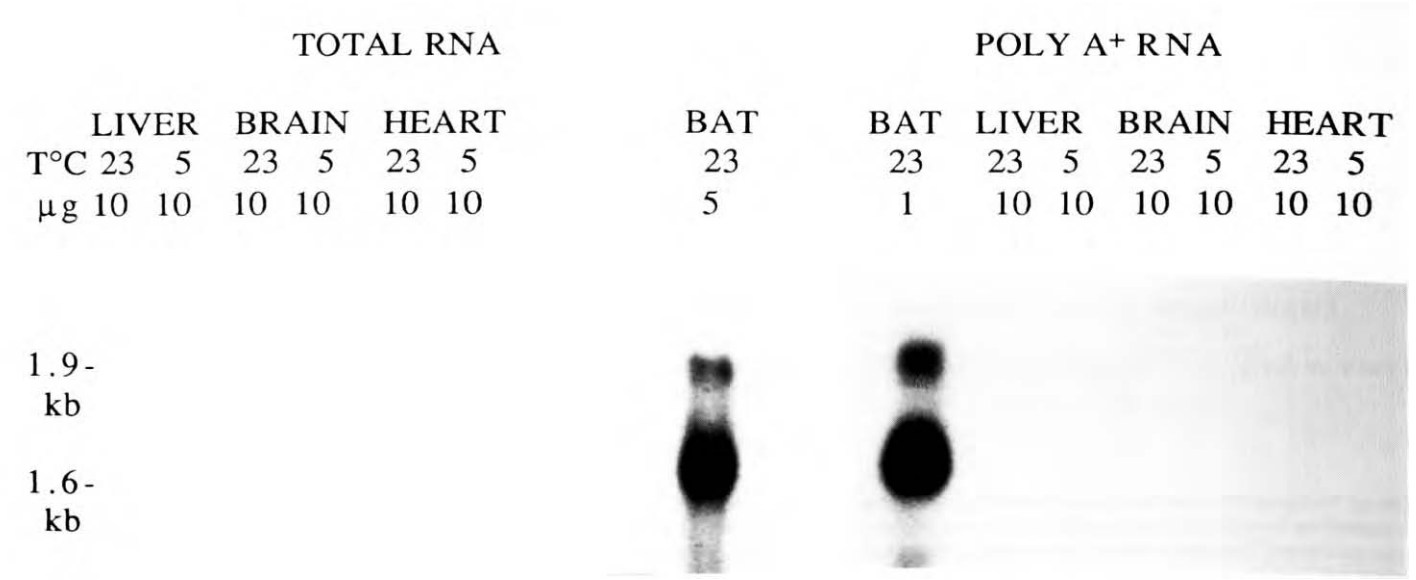

Fig. 1. Northern analysis of total or poly(A)$^+$ RNA of different tissues of control (kept at 23°C) or cold-exposed (kept at 5°C) adult rats. RNAs were hybridized with a ^{32}P-labeled rat UCP cDNA. The amount of analysed RNA is indicated (µg).

2.5. *Reverse transcription and polymerase chain reaction*

both cDNA synthesis and PCR reaction were performed in the same buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml nuclease-free BSA). The enzymes used were M-MLV (Gibco BRL) and Taq polymerase (Perkin Elmer/Cetus). All reactions were carried on in PCR apparatus (Hybaid). Total RNA (300 ng) was first denatured at 70°á for 10 min, then quickly cooled in ice; the first strand was polymerised at 42°C during 50 min in 20 µl volume containing 10 mM DTT, 100 ng oligo-dT, 1 mM deoxynucleotides, 30 U RNAsin, 100 U M-MLV. Reverse transcriptase was then inactivated by heat (95°, 6 min). DNA amplification (30 cycles) started by addition of PCR mix (80 µl) containing 150 pmol of each primer and 2.5 U Taq polymerase. Sense primer OL3 matches on UCP cDNA (EMBL data bank: RNUCPG) from position 589 to position 608 (GTGAAGGTCAGAATGCAAGC); antisense primer OL4 matches from position 785 to 766 (AGGGCCCCCTTCATGAGGTC). An aliquot of amplification pr oduct was analysed by electrophoresis in TBE buffer in 2% agarose gel containing ethidium bromide. The size of DNA fragments was checked by comparison with 1 kilobase ladder molecular weight standards (BRL).

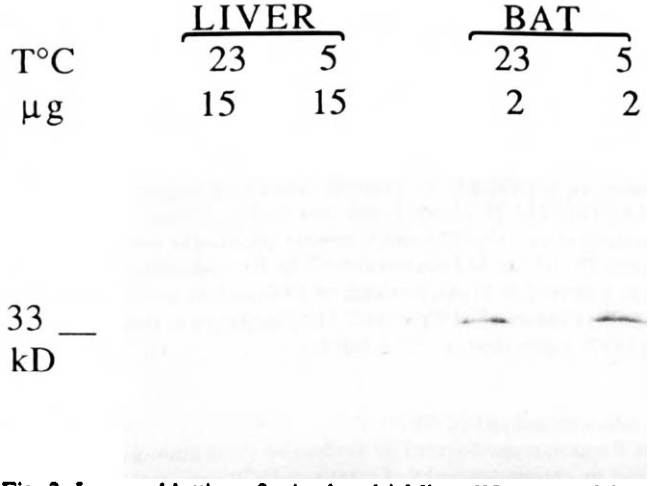

Fig. 2. Immunoblotting of mitochondrial liver (15 µg protein) and brown adipose tissue (2 µg) protein. The mitochondria were isolated from control (animals kept at 23°C) or cold-exposed (animals kept at 5°C) animals. The membrane was probed with purified anti-UCP antibodies.

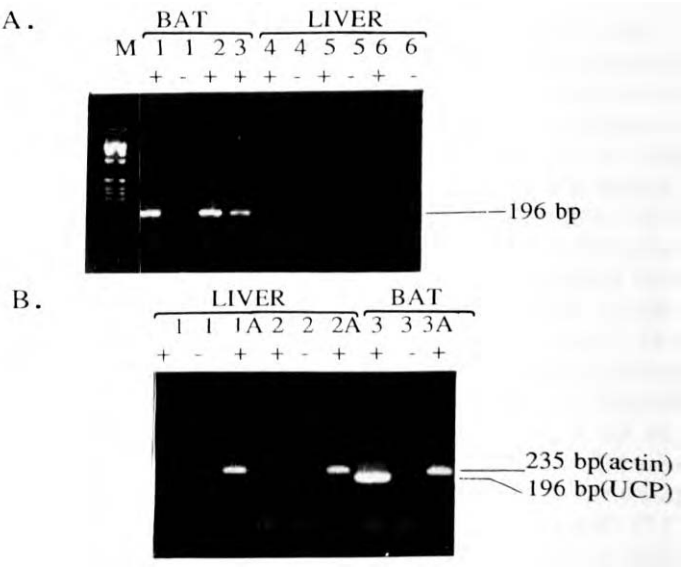

Fig. 3. Polymerase Chain Reaction analysis of UCP mRNA expression. PCR products were electrophoresed in agarose gel and stained with ethidium bromide. The experiments were carried out with (+) or without (−) reverse transcriptase. 2 different experiments (A and B) are shown. Experiment A: lanes 1, 2 and 3, PCR on 100, 50 and 10 ng adult rat BAT RNA, respectively; lanes 4 and 5, PCR on 300 ng liver RNA from end-term 21-day-old rat fetus and 6-h-old newborn rat, respectively; lane 6, PCR on 500 ng liver RNA from adult rat kept at 5°C for 8 days; M=molecular weight markers. Experiment B: lanes 1 and 2, PCR with 300 ng liver RNA of 1-day- (lane 1) and 3-day- (lane 2) -old rats; lane 3, experiment with 250 ng BAT RNA of adult rat. In this experiment, actin mRNA was also amplified from liver (lanes 1A and 2A) and BAT (lane 3A) RNA in order to check the method and the quality of RNAs.

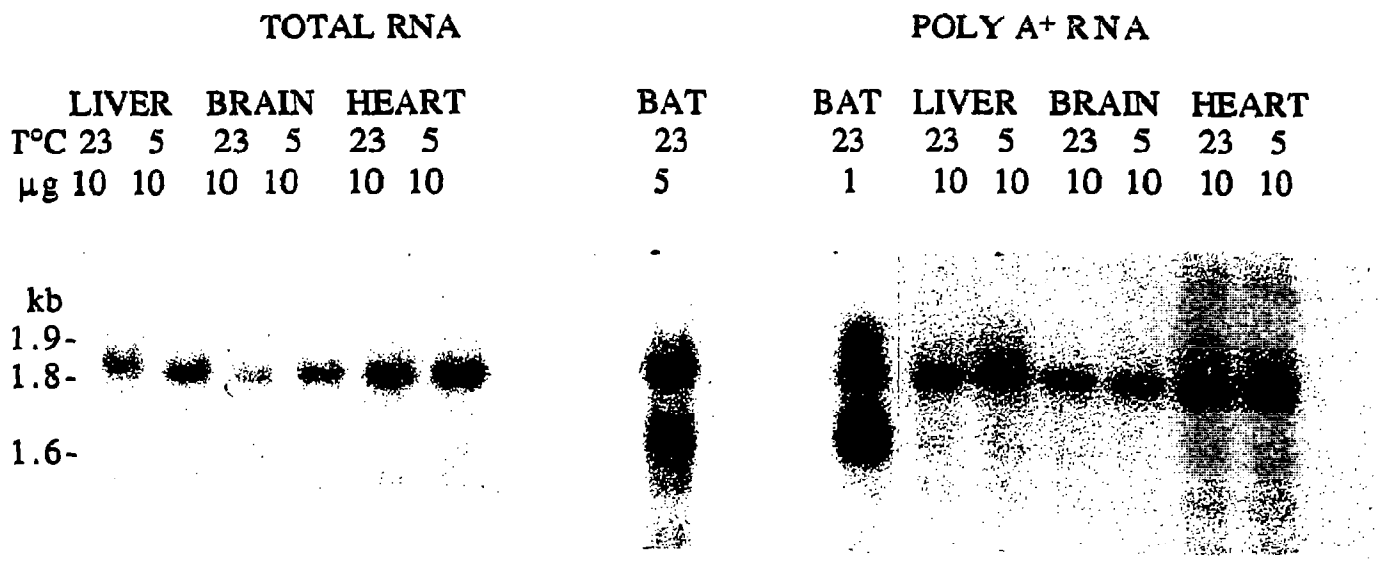

Fig. 4. Northern hybridization with the 3' extremity of the rat UCP cDNA probe. The blots shown in Fig. 1 were dehybridized and then probed with a *Bam*HI DNA fragment corresponding to C-terminal UCP aminoacids plus the following 112 nucleotides of the 3' non-coding domain and a 16 C stretch (see Materials and Methods). This picture was obtained after washing the blots in non-stringent conditions (2 SSC, 0.1% SDS at 65°C). After washing with 0.1 SSC, the 1.8 kb signal was strongly reduced but still visible.

3. RESULTS AND DISCUSSION

When electrophoresed rodent BAT RNA are hybridized with an UCP cDNA probe, 2 mRNAs are detected: an abundant mRNA of 1.6 kb and a minor 1.9 kb RNA [4,12–15,20]. This classical picture was also obtained by Shinohara et al. [17]. The examination of the hybridization signal they got in liver RNA indicates that this signal is obviously different from those present in BAT RNA: the size seems to be higher than 1.6 kb and only one band was visualized. Since their hybridization was obtained with only 5 µg total RNA of liver, it would have been easy for Shinohara et al. to repeat their experiment with poly(A)$^+$-enriched RNA. Such an experiment would have undoubtedly clarify their data. We have thus prepared total and poly(A)$^+$ RNA from BAT, liver, brain and heart of adult rats kept either at 23°C or at 5°C. These RNAs were electrophoresed in agarose gels in denaturing conditions, transferred to nylon membrane and hybridized with a rat radioactive UCP cDNA; the results of this experiment are given in Fig. 1. No signal was detected in total RNA (even after a low stringency wash), except for BAT RNA, for which a typical picture was observed. Whereas a strong signal was recorded with only 1 µg poly(A)$^+$ RNA of BAT, no hybridization was obtained with 10 µg poly(A)$^+$ RNA of the other tissues from control or cold-exposed animals. These data demonstrate that no UCP mRNA can be detected in rat liver using Northern blotting.

The possible presence of UCP in liver of control or cold-exposed rats was investigated using purified anti-UCP antibodies; these antibodies specifically react with UCP and particularly with the C-terminal extremity of the protein [19]. 2 µg BAT mitochondrial protein gave a signal with the antibodies, whereas no UCP was detected in 15 µg liver mitochondrial protein (Fig. 2). Thus, no UCP can be detected by antibodies in liver mitochondria of control or cold-exposed rats. This result agrees with previous studies made in many laboratories [4–11] and in particular with studies on liver mitochondria from cold-exposed rats [7,9].

An alternative method allowing the detection of low activity of genes is the PCR technique. The PCR analysis of UCP was set up and applied to liver RNA. Using this approach 10 ng total RNA of BAT of control adult rat was transcribed into DNA and amplified (Fig. 3). We also got UCP PCR product starting with only 1 ng total RNA of BAT (data non shown). With 300 ng RNA of end-term fetus and newborn rat liver, or with 500 ng RNA of liver of cold-exposed adult rat, no amplification of UCP was obtained (Fig. 3). These data allow us to conclude that no significant transcription of UCP gene occurs in liver of rats whatever they are newborn, adult or cold-exposed adult.

However it remains to explain the hybridization signal detected in liver RNA by Shinohara et al. [17]. Actually, using the entire rat UCP cDNA used by these authors, over the past years, we occasionally got a signal in Northern analysis of RNA prepared from many tissues of rat (generally when the specific radioactivity of the probe was very high). This signal was around 1.8 kb and in BAT RNA it was clearly located at a position intermediary between the 2 UCP mRNAs (1.6 and 1.9 kb). This signal was never observed in tissues of rodents other than rat. When RNA from different rat tissues (BAT, liver, brain, heart,...) was hybridized with a rat

UCP cDNA obtained by PCR (as done in Fig. 1), this extra 1.8 kb signal was never recorded. Since the main difference between the 2 cDNA probes lies in a shorter 3′ non-coding sequence in the PCR amplified probe (see Materials and Methods), we concluded that a non-specific hybridization could be due to the extremity of the 3′ non-coding do main of the full-length rat UCP cDNA. To check this hypothesis, a short probe containing the complete 3′ non-coding domain of the rat UCP cDNA was isolated and hybridized to the blots previously probed with the UCP cDNA (Fig. 1). In these conditions, this probe hybridized to a 1.8 kb band in RNA of all tissues (Fig. 4). With BAT RNA, as expected, the probe also hybridized to the 2 UCP mRNAs. The 1.8 kb signal detected in all tissues was clearly distinct from the 2 UCP mRNAs and was not induced in animals exposed to a cold environment. The reason for this hybridization is unclear. The similar intensity noticed in total and poly(A)$^+$ RNA could be explained by the hybridization of limiting amount of probe (20 ng) with the 18 S ribosomal RNA still present in poly(A)$^+$ RNA fraction. A simple explanation would be that the stretch of C present in the 3′ domain of our cDNA (see Materials and Methods) hybridized with the rat 18 S ribosomal RNA which is known to be very rich in poly(G/C) sequence [21].

4. CONCLUSION

As is generally admitted, the uncoupling protein is uniquely expressed in brown adipocytes. Using Northern analysis of poly(A)$^+$ RNA, immunodetection of UCP and detection of UCP mRNA using PCR method we were unable to identify UCP or its mRNA in other cells. We reject the conclusion drawn by Shinohara et al. [17] and consider that the expression of UCP in liver RNA has not been demonstrated. The signal observed by these authors is clearly distinct from UCP mRNA and probably results from an artefactual hybridization with the 3′ non-coding domain of the rat UCP cDNA or with poly(C) sequence present at its extremity. Shinohara et al. [17] observed no signal in RNA from control adult rat but only in liver of cold-exposed adult rat or in liver of newborn rat and they proposed that this was due to an appropriate physiological mechanism. We could not confirm this observation.

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