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Molecular approach to thermogenesis in brown adipose tissue: cDNA cloning of the mitochondrial uncoupling protein

(membrane/mitochondrial oxidation/adipocyte/noradrenergic control/pheochromocytoma)

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ABSTRACT **The uncoupling protein (UCP) of mammalian brown fat is a specialized and unique component responsible for energy dissipation as heat. Translation and immunoprecipitation from sucrose-fractionated mRNA indicated that the mRNA of UCP sedimented at 14–16 S. A recombinant cDNA library prepared from mRNA of thermoactive brown fat enriched for UCP mRNA has been constructed and cloned in *Escherichia coli*. Recombinant plasmids were screened by differential colony hybridization to a cDNA probe complementary to poly(A)$^+$ RNA isolated from thermogenic or from weakly thermogenic brown fat. Several differentially hybridizing plasmids were shown to contain UCP cDNA sequences by their ability to select a mRNA coding for an *in vitro* translation product that was immunoprecipitable with antibodies against UCP. Blot hybridization of brown fat mRNA to a ^{32}P-labeled UCP cDNA probe revealed two major species of mRNA (15S and 18S). As compared to non-thermogenic tissue, a strikingly increased hybridization to the probe was observed with brown fat mRNA from thermoactive tissue. Moreover, hybridization was observed with RNA of brown adipose tissue from rat, hamster, or mouse but not with RNA from rat or mouse liver.**

The mitochondrial uncoupling protein (UCP) of brown fat is responsible for energy dissipation as heat. This component is located in the inner membrane where it amounts to 10–15% of total mitochondrial proteins and functions as a proton short-circuit that uncouples substrate oxidation from ATP synthesis (1). The UCP of brown fat is thought to be specific and absent in mitochondria of other tissues (1–4). This protein is an inducible component that is highly increased when the thermogenic function of brown fat is activated during exposure of animals to cold (1–4). Different experimental approaches and clinical observations in animals and man suggest that the synthesis of UCP is controlled by local sympathetic nervous activity (3, 5) and probably by norepinephrine itself (3, 6–9). Cell-free synthesis and immunoprecipitation of translation products have indicated recently that UCP is not synthesized as a larger molecular weight precursor (10, 11) and that stimulated thermogenesis in the tissue is accompanied by an increased level of UCP mRNA (9, 10). The present work was undertaken because of the unique physiological function of the UCP and by the opportunity to contribute to DNA cloning of a mammalian mitochondrial component.

This paper reports the cloning of cDNA sequences complementary to UCP mRNA. Using these cloned cDNAs as hybridization probes, we could characterize UCP mRNA and document changes in the cellular concentration of this mRNA in different physiological situations. Such cDNA probes represent a powerful tool to the study of the molecular mechanisms involved in mitochondriogenesis and in the differentiation of brown fat cells.

MATERIAL AND METHODS

Materials. L-[^{35}S]Methionine, [α-^{32}P]dCTP, and [α-^{32}P]-dTTP were obtained from the Radiochemical Centre. Oligo(dT)-cellulose was obtained from Collaborative Research (Waltham, MA). Avian myeloblastosis virus (AMV) reverse transcriptase was purchased from Life Sciences (St. Petersburg, FL). *Escherichia coli* DNA polymerase I, S1 nuclease, calf thymus terminal deoxynucleotidyl transferase and restriction endonucleases were from Boehringer Mannheim and were used as recommended by the supplier.

Animals. Male Sprague–Dawley rats (200-g body weight) were either kept at room temperature (control; weakly thermoactive brown fat) or maintained at 5°C for 7 days (thermoactive brown fat).

Isolation and Fractionation of Interscapular Brown Fat mRNA. The methods for purification of total RNA and poly(A$^+$) RNA were as described (12, 13). Poly(A$^+$) RNA was fractionated by sucrose gradient centrifugation (*i*) in the presence of methylmercuric hydroxide and absence of NaCl (14) or (*ii*) in the presence of 0.1 M NaCl and absence of methylmercuric hydroxide (15).

Cell-Free Translation and Specific Immunoprecipitation. Cell-free translation of total or fractionated poly(A$^+$) RNA (16) and immunoprecipitation of synthesized UCP were carried out as described (9, 10).

cDNA Synthesis and Cloning. Poly(A$^+$) RNA from thermogenic brown fat was enriched for UCP mRNA by sucrose gradient centrifugation. The mRNA fraction sedimenting around 15S was used to synthesize double stranded (ds) cDNA according to standard procedures (17). Both first and second strands were prepared by using avian myeloblastosis virus reverse transcriptase. After hairpin loop cleavage with S1 nuclease and deproteinization with phenol and chloroform extractions, ds cDNA was size-fractionated by sedimentation on a sucrose gradient. Ten nanograms of a ds cDNA fraction (500 base pairs long) was tailed with calf thymus terminal deoxynucleotidyl transferase and dCTP (18) and was hybridized with 100 ng of dG-tailed, *Pst* I-linearized pBR327 (19, 20). Chimeric vectors were used to transform $CaCl_2$-treated *E. coli* C600, and transformant clones were selected on L medium agar plates containing 10 mg of tetracycline per liter. About 2000 recombinant clones were obtained and further characterized. A second library of recombinant clones bearing longer cDNA inserts were prepared from a

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Abbreviations: UCP, uncoupling protein; ds, double stranded; kb, kilobase.

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sucrose gradient fraction containing larger ds cDNA molecules.

Colony Screening. Recombinant cDNA clones were grown on duplicate sets of filters. After colony lysis and DNA adsorption (21), each set of filters was hybridized to a ^{32}P-labeled single-stranded cDNA probe (specific activity, 3.10^8 cpm/μg) synthesized from either thermoactive or control brown fat 15S mRNA fraction. Hybridized filters were washed, dried, and exposed 24 hr for autoradiography.

Hybrid-Selected Translation. Plasmid DNA was isolated by the alkaline lysis method of Ish-Horowicz and Burke (22), linearized by *Eco*RI, and bound onto nitrocellulose filters. The filters were hybridized to total poly(A$^+$) RNA from thermoactive brown fat essentially as described by Ricciardi *et al.* (23). Hybridized filters were washed, and selected RNA was eluted from each individual filter, extracted with phenol, and precipitated with ethanol in the presence of calf liver tRNA prior to cell-free translation analysis.

Blot Hybridizations. Total poly(A$^+$) RNA was denatured at 55°C in the presence of formamide and formaldehyde, electrophoresed on formaldehyde agarose gel, and transferred to nitrocellulose essentially as described (24, 25). Blots were hybridized to cDNA inserts excised from plasmids by *Pst* I digestion and labeled by nick-translation to a specific activity of 10^8 cpm/μg. Hybridization was carried out overnight at 42°C in 45% formamide/0.6 M NaCl/0.06 M Na citrate. After being washed in stringent (30 mM NaCl/3 mM Na citrate) or nonstringent (300 mM NaCl/30 mM Na citrate) conditions, blots were subjected to analysis by autoradiography.

Rat genomic DNA was digested by *Eco*RI, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose (26). Southern blots were probed with the cDNA inserts by using the conditions described by Wahl *et al.* (27). Hybridized blots were washed in nonstringent conditions and subjected to analysis by autoradiography.

Protein Electrophoresis and Autoradiography. Proteins were electrophoresed as in refs. 9 and 10. Dried NaDodSO$_4$ gels of proteins and hybridized nitrocellulose filters were exposed to Kodak X-Omat AR5 films at −70°C with a Dupont Cronex intensifying screen.

RESULTS

Fractionation of Brown Fat mRNA by Sucrose Gradient Centrifugation. Total poly(A$^+$) RNA of brown fat from control animals (weakly thermogenic tissue) or from cold-exposed rats (thermogenic tissue) was fractionated on sucrose gradients. An aliquot of each collected fraction corresponding to 1.2 μg of mRNA was used to direct cell-free translation. Fig. 1 shows the translation products of a 14S–16S fraction enriched in mRNA coding for a M_r 32,000 polypeptide (lanes 3 and 4) as compared with the total translation products (lanes 1 and 2). The translation products obtained from the 14S–16S-enriched fraction were then immunoprecipitated in the presence of antiserum against purified UCP and analyzed by NaDodSO$_4$ gel electrophoresis (Fig. 1, lanes 5 and 6). It was observed that the UCP was actually immunoprecipitated from the translation products of the 14S–16S mRNA fraction and that the level of UCP mRNA was markedly increased in cells from thermogenic tissue.

Construction and Identification of UCP cDNA Clones. A mRNA fraction from thermoactive brown adipose tissue enriched for UCP mRNA (15S fraction) was copied into ds cDNA by using reverse transcriptase. After sedimentation on a sucrose gradient, ds cDNA molecules from the peak fraction, around 500 base pairs in length, were inserted into the *Pst* I site of plasmid pBR327 by using the conventional dG-dC homopolymeric hybridizing procedure. About 2000 tetracycline-resistant clones were obtained after transformation of *E. coli* C600. These clones were screened *in situ* by differential hybridization to cDNA probes transcribed from 15S mRNA fractions originating from either control brown fat or similar thermoactive tissue. About 70 colonies gave a markedly increased hybridization signal with the probe from the thermoactive brown fat.

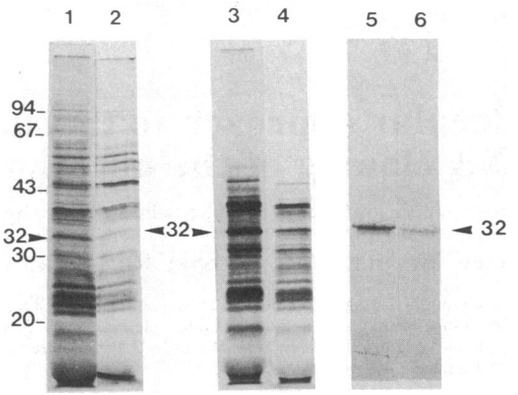

FIG. 1. NaDodSO$_4$/polyacrylamide gel autoradiography of translation products obtained with mRNA of brown fat from stimulated or control animals. Translation products were from unfractionated thermogenic brown fat mRNA (lane 1), unfractionated control brown fat mRNA (lane 2), the 15S fraction of sucrose gradient sedimentation of thermoactive brown fat mRNA (lane 3), and the 15S fraction of sucrose gradient sedimentation of control brown fat mRNA (lane 4). Immunoprecipitated translation products were from the 15S fraction of sucrose gradient sedimentation of thermoactive brown fat mRNA (lane 5) and the 15S fraction of sucrose gradient sedimentation of control brown fat mRNA (lane 6). Immunoprecipitation was carried out with anti-UCP antiserum by using the Sepharose-bound protein A procedure. Numbers indicate the molecular weight of marker polypeptides, shown × 10^{-3}. Arrowheads show the position of the M_r 32,000 protein.

cDNA inserts excised from a few clones were used as hybridization probes and matched against the 70 "differential" clones. These hybridization experiments revealed a major family of 16 clones containing sequences homologous to each other.

Plasmids extracted from several clones, including both "differential" and "nondifferential" bacterial colonies, were characterized by the positive mRNA hybridization-selection procedure. Poly(A$^+$) RNA selected by hybridization was translated in a reticulocyte lysate cell-free system. Translation products were identified by NaDodSO$_4$/polyacrylamide gel electrophoresis and immunoprecipitated with an anti-UCP antiserum as shown in Fig. 2. Plasmids pUCP383, pUCP455, and pUCP610, which all belong to the major family of "differential" clones, were able to select a mRNA encoding a M_r 32,000 protein, whereas other recombinant plasmids could not bind this mRNA species (Fig. 2). Moreover, by immunoprecipitation this M_r 32,000 polypeptide was not distinguishable from brown fat UCP (Fig. 2, lane 7). In addition, this immunoprecipitated M_r 32,000 peptide could be blocked by competition with cold, purified UCP (Fig. 2, lane 8).

Characterization of UCP mRNA. Fig. 3 shows an autoradiogram of a blot hybridization of poly(A$^+$) RNA from mouse liver and rat brown fat probed with the cDNA insert of clone pUCP455 [0.5-kilobase (kb) length]. This probe apparently detected two major species (15S and 18S) in brown fat mRNA (Fig. 3, lanes 2, 3, and 4) and did not hybridize to mouse or rat liver mRNA (Fig. 3, lanes 1 and 8). An important increase of both the 15S and 18S mRNA species could be observed after exposure to cold (Fig. 3, lane 3) as compared to control animals (Fig. 3, lane 2). These two mRNAs also were increased markedly in brown fat of animals bear-

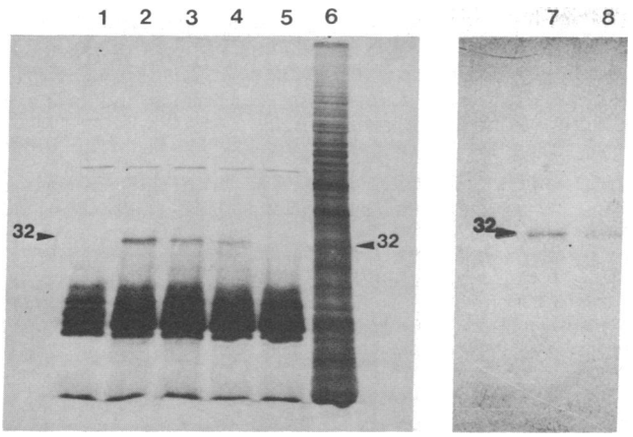

FIG. 2. Identification of clones containing brown fat UCP cDNA sequences by hybrid-selection translation. Poly(A$^+$) mRNA from thermoactive brown fat was hybridized to linearized immobilized recombinant plasmids. After being washed, the selected mRNA was eluted and translated. (*Left*) The translated products were electrophoresed and autoradiographed. Lanes: 1, no added selected mRNA (heavy background is due to methionyl-tRNA^{Met}); 2–4, mRNA respectively selected by pUCP383, pUCP455, and pUCP610 plasmids; 5, plasmid giving no stronger signal during differential screening of recombinant clones; 6, synthesis directed by total mRNA from brown fat. (*Right*) The product translated from mRNA selected by pUCP455 plasmid was immunoprecipitated with antibodies against purified UCP in the absence (lane 7) or presence (lane 8) of added unlabeled UCP excess in the immunoprecipitation mixture. The molecular weight of the UCP is shown $\times 10^{-3}$.

ing a (pheochromocytoma) tumor known to secrete excessive quantities of epinephrine and norepinephrine (Fig. 3, lane 4). Insert of clone pUCP455 was then used to screen a second cDNA library constructed with ds cDNA molecules

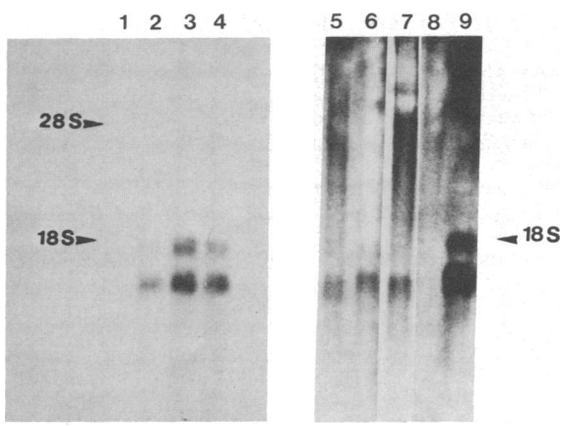

FIG. 3. Blot hybridization of UCP mRNA. (*Left*) Poly(A$^+$) RNA was purified from rat brown fat or mouse liver, electrophoresed on agarose, and transferred to nitrocellulose. Equivalent amounts of poly(A$^+$) RNA quantitated by absorbance at 260 nm were electrophoresed. The filter was probed with the ^{32}P-labeled cDNA insert of plasmid pUCP455. Lanes: 1, mRNA from mouse liver; 2, mRNA from brown fat of control animals; 3, mRNA from brown fat of cold-exposed rats (thermogenic tissue); 4, mRNA from brown fat of control animals bearing pheochromocytoma tumor (thermogenic tissue). (*Right*) Total cellular RNA or poly(A$^+$) RNA electrophoresed and transferred to nitrocellulose as previously. The filter was probed with a [^{32}P]cDNA insert of plasmid pUCP36. Lanes: 5, total cellular RNA from mouse brown fat; 6, total cellular RNA from hamster brown fat; 7, total cellular RNA from rat brown fat; 8, total cellular RNA from rat liver; 9, poly(A$^+$) RNA from rat brown fat. Rat 18S and 28S ribosomal RNAs were taken as size markers; 15 UCP mRNA is indicated by the lowest arrowhead.

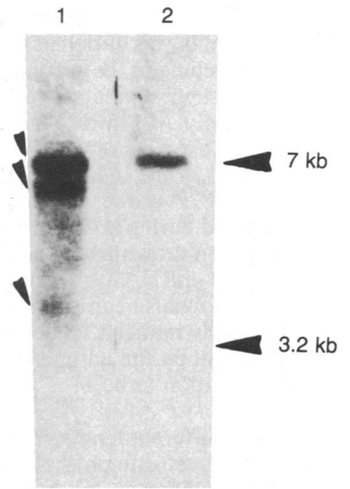

FIG. 4. Southern blot hybridization of *Eco*RI-digested rat liver DNA. The filter was probed with a [^{32}P]cDNA insert (1.9 kb) of plasmid pUCP36 (lane 1), and a ^{32}P-labeled insert (0.5 kb) of plasmid pUCP455. Arrowheads on the left indicate the positions of the hybridizing 7.0-, 6.5-, and 3.5-kb fragments.

longer than 0.5 kb; 69 positively hybridizing clones were obtained from 2250 recombinants. Plasmids of these positive clones were further analyzed and contained inserts ranging from 0.6 to 2.1 kb. The largest inserts (pUCP66 and pUCP36, 2.1 and 1.9 kb long, respectively) were used to probe mRNA blot hybridizations. Identical hybridization patterns were obtained with the longer probes as compared to pUCP455 (Fig. 3, lane 9). Interestingly, rat pUCP probes also detected a 15S RNA species in RNA preparations originating from brown fat from different rodents (Fig. 3, lanes 5–7).

Hybridization of UCP cDNA Probes to Rat Genomic DNA. In order to characterize a genomic DNA sequence of the brown fat UCP, Southern blots of *Eco*RI digests of rat liver DNA were probed with cDNA inserts of variable length. Probe pUCP455 detected a single band of 7 kb, whereas probe pUCP36 detected the same band of 7 kb and two lower additional bands of 6.5 kb and 3.5 kb (Fig. 4), although this latter probe did not contain any internal *Eco*RI site. Probe pUCP66 could not be used on rat genomic DNA blots because of the presence of a repeated sequence in this insert.

DISCUSSION

The aim of this work was the molecular cloning of DNA sequences complementary to the UCP mRNA of brown fat mitochondria. Immunoprecipitation and cell-free translation following sucrose gradient fractionation of brown fat mRNA confirmed previous reports of the molecular weight of the synthesized protein (9–11) and the induction of UCP mRNA in thermoactive brown fat (9, 10).

Sucrose gradient sedimentation led to a 5- to 10-fold enrichment of UCP mRNA as measured by the relative increase of the immunoprecipitable M_r 32,000 protein in the translation products. Since UCP mRNA is inducible, it was possible to synthesize two types of cDNA probes corresponding to both thermogenic (induced) and control (noninduced) mRNAs and, thus, to identify by differential hybridization a family of homologous cDNA sequences appearing during the exposure to cold. Immunoprecipitation of translation products of the mRNA selected by hybridization to several cloned cDNAs of this family demonstrated that this inducible sequence codes for UCP. At least 0.8% of the recombinant clones of the first library (16 of 2000) contained UCP cDNA sequences. Since only the clones displaying an unambiguous differential hybridization signal were screened fur-

ther, this value is probably an underestimate of the percentage of UCP mRNA present in this enriched fraction. A value of 3% obtained in the screening of the second library is obviously more accurate and suggests that the actual abundance of UCP mRNA in total poly(A^+) mRNA of induced brown fat is about 0.5%.

A significant proportion of the induced cDNA sequences did not hybridize to a UCP probe, suggesting that other mRNA species are induced during activation of the tissue. A similar conclusion has been drawn from cell-free translation experiments of brown fat mRNA (9) obtained from either cold-exposed animals or animals bearing a pheochromocytoma tumor. These differently induced sequences are valuable potential tools to further study the adrenergic control of the differentiation and activation processes of brown adipocytes.

UCP cDNA inserts of different lengths have been used as hybridization probes to investigate physiological and molecular aspects of UCP biosynthesis. No hybridization signal was detected on blot hybridizations probed with UCP cDNA sequences of liver mRNA, whereas a significant hybridization was observed with brown fat from several rodents. These data are in good agreement with the fact that UCP has never been found in non-brown fat tissues (1–4). However, it will be interesting to test for the hypothetical existence of UCP mRNA in tissue such as muscle and white fat. The hybridization signal was strongly enhanced with mRNA of thermoactive brown fat. This increase was greater with mRNA of cold-exposed rats than with mRNA of rats bearing pheochromocytoma. This result agrees with the well-documented estimation of UCP in thermoactive brown fat (1–4, 6), where this increase was also greater in cold-exposed rats than in animals with PC 12 pheochromocytoma tumor (28). These blot-hybridization experiments are consistent with the possibility that UCP biosynthesis is essentially controlled at the transcriptional level.

Several possibilities can account for the essentially dual hybridization signal corresponding to 15S and 18S mRNA. (*i*) The 18S signal could be ascribed to artifactual hybridization with contaminating ribosomal RNA. Since this signal was not enhanced in blot-hybridization experiments using total RNA (Fig. 3), such a hypothesis can be ruled out. (*ii*) Each band could correspond to the products of different transcription units. (*iii*) The two bands could be produced by the same gene and be the result of either differential splicing or incomplete processing. However, Southern blot-hybridization patterns of rat genomic DNA probed with the short UCP cDNA sequences (pUCP455) show a single band suggesting the existence of a single gene copy for UCP. Hybridization of the same blots with the longer probe pUCP36 reveals two additional bands and is a clue to the presence of introns in the UCP gene, since there is no *Eco*RI site in the cDNA sequence.

The existence of two mRNA species raises several additional questions. Are both species functional? Do they code for the same product? These questions and that of the genetic origin of the two species can be approached through the sequence determination of cDNAs of both mRNAs. It is probable that the two largest cloned sequences (pUCP36) both originate from the 18S mRNA species, whereas the 15S species should correspond to a nucleotide sequence of 1.5–1.6 kb.

In conclusion, these UCP cDNA probes can be used for the detection and the quantitation of UCP mRNA in various cells in different physiological, pharmacological, and pathological situations. In addition, this system could be an interesting model to study the expression of a gene implicated in thermogenesis and controlled by the noradrenergic nervous system. It can be pointed out that this is one of the first reports on cloning of cDNA encoding proteins of the mitochondrial membrane of mammals. cDNA sequencing also will provide useful information concerning the primary structure of this membrane protein.

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