# RABBIT BROWN ADIPOSE TISSUE

## UNCOUPLING PROTEIN CDNA

Dedicated to my aunt Elizabeth Garamvolgyi with all my love

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# SEQUENCING OF RABBIT BROWN ADIPOSE TISSUE

## UNCOUPLING PROTEIN CONA: CHARACTERIZATION OF RAT

AND RABBIT UNCOUPLING PROTEIN mRNAs

by

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#### ABSTRACT

A cDNA clone encoding the entire amino acid sequence of rabbit brown adipose tissue uncoupling protein has been isolated and sequenced. The coding region of this cDNA is 80.6% identical to that of the rat uncoupling protein cDNA.

In contrast to rat uncoupling protein for which there are two mRNAs of 1500 and 2000 nucleotides there is only one rabbit uncoupling protein mRNA of 2000 nucleotides. Whereas the rat cDNA hybridizes more strongly to the shorter rat uncoupling protein mRNA the rabbit cDNA hybridizes more strongly to the longer rat uncoupling protein mRNA. Primer extension and Northern blot analysis were performed to try to account for the difference of  $430 \pm 75$  nucleotides between the two rat uncoupling protein mRNAs. Northern blot analysis indicated the presence of 355 more nucleotides in the 3'-untranslated region of the 2000 nucleotide long rat uncoupling protein mRNA than in the 1500 nucleotide long rat uncoupling protein mRNA. The two rat uncoupling protein mRNAs could therefore arise by differential processing. Primer extension revealed that the two rat uncoupling protein mRNAs have a 5'untranslated region of approximately 186 nucleotides.

The deduced amino acid sequence of rabbit UCP is 86% identical with both the rat and hamster proteins. Several regions are conserved in all three uncoupling proteins. The two longest regions of conservation are residues 52 to 69 and 82 to 100 of the mature proteins and

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correspond to two of several basic regions of the protein that have been suggested as possible targeting sequences. These conserved regions fall within amino acids 52 to 104 of the mature rat protein, which has been shown by others to target a passenger protein to mitochondria. Helical wheel diagrams that correspond to residues 52 to 68 and residues 72 to 92 reveal possible amphiphilic  $\alpha$ -helical formations that may be involved in targeting. Regions corresponding to those conserved in the three UCPs are also conserved in three mammalian ADP/ATP carriers and may indicate a common role for these regions, perhaps including targeting.

There is almost complete conservation of lysine, arginine, and cysteine residues that are thought to be involved in nucleotide binding and proton transport in the three UCPs.

There is a threenine to alanine change at the carboxyl-terminus of the rabbit protein compared to the rat protein. This amino acid difference may explain the differential reactivities of rabbit and rat UCP with an antibody preparation against rat UCP.

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# LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
BAT	brown adipose tissue
bis-acrylamide	N,N'-methylene bis-acrylamide
qd	base pairs
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CDNA	complementary DNA
CDTA	trans 1,2-diaminocyclohexane N,N,N',N'-tetracetic acid
Da	daltons
datp	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dITP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
DIT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
FDM	formamide dye mix
GDP	guanosine diphosphate
GIP	guanosine triphosphate
HEPES	[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]

IPIG	isopropyl- $\beta$ -D-thiogalactopyranoside
kbp	kilobase pairs
kDa	kilodaltons
Klenow fragment	large fragment of DNA polymerase I
LB	Luria broth
MOPS	(3-[N-morpholino]propane sulfonic acid)
mRNA	messenger RNA
NBRF	National Biomedical Research Foundation
NTP	nucleoside triphosphate
<b>ОСТ</b> .	ornithine carbamoyltransferase
PAGE	polyacrylamide gel electrophoresis
PIPES	[1,4 piperazinebis(ethanesulfonic acid)]
PIR	Protein Identification Resource
RF	replicative form of M13
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	saline sodium citrate
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	Tris-EDTA-saline
Tris	Tris(hydroxymethyl)aminomethane
UCP	uncoupling protein
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

#### 1 INTRODUCTION

#### 1.1 PREFACE

Recent advances in molecular biology have enabled the cloning and sequencing of numerous genes and their cDNAs. The isolation of a cDNA allows a study of the expression of a gene at the mRNA level. In addition the nucleotide sequence of a cDNA allows the deduction of the amino acid sequence of the corresponding protein.

Knowledge of a protein's primary structure has many useful applications. The primary structure allows predictions of secondary structural features of the protein, its disposition if it is a membrane protein and the presence of antigenic determinants. A comparison of the amino acid sequence of the same protein from different sources, and different proteins that carry out similar functions, can reveal conserved elements that may represent common structural and/or functional domains.

The approach of cDNA cloning has been used in our laboratory to gain insight into the structure/function relationships of the uncoupling protein (UCP) of brown adipose tissue (BAT) mitochondria. This protein is being studied as a model system for mitochondrial biogenesis, particularly with respect to targeting. Using a monospecific antibody directed against rat UCP (Freeman <u>et al.</u>, 1983) together with molecular biological techniques allowed the isolation of the cDNA for rat UCP

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(Ridley <u>et al</u>., 1986a,b). This cDNA is being used to determine how the protein is targeted to mitochondria to achieve its final conformation and correct disposition in the inner mitochondrial membrane, and which regions of the protein are involved in carrying out its specific functions of nucleotide and free fatty acid binding, and of proton transport.

## 1.2 IMPORT OF PROTEINS INTO MITOCHONDRIA

The vast majority of mitochondrial proteins are nuclear encoded, synthesized on free polysomes in the cytosol and imported posttranslationally. Newly synthesized nuclear encoded mitochondrial proteins must be correctly targeted to mitochondria. They must also find their way into or across one and in most cases two mitochondrial membranes in order to achieve their final location in one of the four submitochondrial compartments: the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane or the matrix.

A number of excellent reviews are available on the import of proteins into mitochondria (Douglas <u>et al.</u>, 1986; Freeman <u>et al.</u>, 1988; Hay <u>et al.</u>, 1984; Nicholson and Neupert, 1988; Pfanner and Neupert, 1987a). The import of a mitochondrial matrix protein, such as <u>Neurospora</u>  $F_1$ -ATPase subunit  $\beta$  (shown in Fig. 1), illustrates the main features of this process. Protein import can be divided into five basic steps (Hay <u>et al.</u>, 1984; Nicholson and Neupert, 1988). The protein is first synthesized on free polysomes in the cytosol as a precursor with an amino-terminal extension of 19 residues (1). This extension acts as a targeting sequence and mediates the second step in import which is the



Fig. 1. Import of  $F_1$ -ATPase subunit  $\beta$ . The requirement for a membrane potential  $(\Delta \Psi)$  and NTPs ([+++] representing high requirement) are indicated. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; R, receptor; P, matrix protease. As illustrated in Pfanner <u>et al</u>. (1987a).

NTP-dependent binding of the protein to a receptor at the outer face of the outer mitochondrial membrane (2) (Pfanner et al., 1987a). The third step is the translocation of the protein across both mitochondrial membranes. The membrane potential-dependent translocation of the aminoterminal presequence into the matrix occurs at regions of apposition of the outer and inner mitochondrial membranes such that the protein spans both membranes simultaneously (3a) (Schleyer and Neupert, 1985). This process may involve hydrophilic channels (Pfanner et al., 1987b). This is followed by the NIP-dependent membrane potential-independent unfolding of the regions of the protein outside of the mitochondria and the translocation of these regions into the matrix (3b). The fourth step is the cleavage of the presequence by a matrix protease (4). Once the protein has been completely translocated it can adopt its final conformation and usually forms homo- or hetero-oligomeric protein complexes (5).

### 1.2.1 MITOCHONDRIAL PROTEIN PRECURSORS

Matrix, intermembrane space and inner mitochondrial membrane proteins are usually but not always synthesized as precursors with amino-terminal extensions whereas outer mitochondrial membrane proteins lack such extensions (Hay <u>et al</u>., 1984; Nicholson and Neupert, 1988). Exceptions to the above include the ADP/ATP carrier (Hay <u>et al</u>., 1984) and the uncoupling protein (Freeman <u>et al</u>., 1983; Ricquier <u>et al</u>., 1983; Ridley <u>et al</u>., 1986b) which are inner mitochondrial membrane proteins that are synthesized as precursors of mature length and presumably contain mitochondrial targeting sequences within the sequence of the mature protein.

## 1.2.2 CHARACTERISTICS OF MITOCHONDRIAL PRESEQUENCES

Mitochondrial presequences, as shown in Table 1, are found to be between 20 and 80 amino acids in length and have some common features (Nicholson and Neupert, 1988). They are rich in basic residues (arginine, lysine), hydroxylated residues (serine, threonine) and hydrophobic residues and almost always lack acidic residues (Douglas <u>et</u> <u>al</u>., 1986). Arginine, serine and leucine are found more frequently in the amino-terminal 40 residues of mitochondrial than cytosolic proteins, whereas aspartic acid, glutamic acid, valine and isoleucine are found less frequently (von Heijne, 1986). These presequences lack sequence homology and therefore charge distribution, secondary and/or tertiary structure may play an important role in their function (Douglas <u>et al</u>., 1986).

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Table 1. Amino-terminal sequences of proteins of the various submitochondrial compartments. Presequences (bold type) and mature portions (regular type) are indicated using the single letter amino acid code. Positively charged residues (+), hydroxylated residues ( $\cdot$ ), negatively charged residues (-) and cleavage sites ( $\uparrow$ ) are indicated. Matrix targeting domains are underlined and indicated only for those proteins for which they are known. Adapted from Nicholson and Neupert (1988).

1. yeast cytochrome c oxidase subunit IV-matrix MLSLROSIRFFKPATRT LCSSRYLL OOKPVVKTAQ + • + • + + •+• † ••+ † + +• 2. yeast alcohol dehydrogenase III-matrix MIRISSIFIRRVOPSIFSRNIIRLOST AAIPKIOKOV + + • • • + + • • + + • • + 3. yeast cytochrome  $\underline{c}_1$ -matrix MFSNLSKRWAORTISKSFYSTATGAASKSGKLIJOKLVTAGVAAAGTTASTILYADSLIJAFA + • •++ +• •+• •• • •+• + • + • . . . -· · - † 4. yeast 70 kDa protein-outer membrane MKSFTTRNKTAILATVAATGTAIGAYYYYNOLOOOORGKKNTINKDEKK ++• •+ +• • • • + ++ • +--++ 5. human ornithine carbamoyltransferase (OCT)-matrix MLFNLRILINNAAFRNGHNFMVRNFRCGOPLO NKVQLKGRDL + + + + + ↑ + + +<del>-</del> 6. rat malate dehydrogenase-matrix **MLSALARPVGAALRRSFSTSAONN** + • + ++ • • • • 7. <u>Neurospora</u> ATPase  $F_1\beta$ -matrix **MVLPRLYTATSRAAFKAAK** + + • • • + + 8. Neurospora Fe/S protein-intermembrane space MAPVSIVSRAAMRAAAAPARAVRA LITISTALO GSSSSIFESP + • •+ + + + + + •••• + •••• -• 9. yeast cytochrome  $\underline{b}_2$ -intermembrane space MLKYKPLLKISKNCEAAIIRASKIRINTIRAYGSTVPKSKSFDQDSRKRIQSWIALRVGAILAATSSVAYL-NWHNGOIDN - t

# 1.2.3 PRESEQUENCE TARGETING FUNCTION: ROLE OF POSITIVE CHARGE AND AMPHIPHILICITY

The targeting sequences of a number of yeast mitochondrial proteins have been localized (as seen in Table 1) using gene deletion and gene fusion studies. These procedures involve the fusion of the coding portion for a mitochondrial presequence to the gene of a passenger protein, such as dihydrofolate reductase or  $\beta$ -galactosidase. Import of the fusion protein is then assessed either <u>in vivo</u> by a determination of the extent of correct mitochondrial localization, or <u>in vitro</u> by the incubation of a transcription/translation system with isolated mitochondria.

The targeting sequences for a number of yeast proteins have been determined. As shown in Table 1, these include cytochrome <u>c</u> oxidase subunit IV (1) (Hurt <u>et al.</u>, 1985a), alcohol dehydrogenase III (2) (van Loon <u>et al.</u>, 1986), cytochrome <u>c</u><sub>1</sub> (3) (van Loon <u>et al.</u>, 1987) and the 70 kDa protein of the yeast outer mitochondrial membrane (4) (Hurt <u>et al.</u>, 1985b).

Yeast targeting sequences generally appear to be at the extreme amino-terminus of the presequence as illustrated by the following examples. The first 12 amino acids of the 25 amino acid presequence of cytochrome <u>c</u> oxidase subunit IV (Hurt <u>et al.</u>, 1985a), the 27 residue presequence of alcohol dehydrogenase III (van Loon <u>et al.</u>, 1986) and the amino-terminal 16 residues of the 61 residue presequence of cytochrome  $c_1$  (van Loon <u>et al.</u>, 1987) were sufficient to direct mouse dihydrofolate reductase into the matrix of isolated yeast mitochondria. The first 12 amino acids of the 70 kDa outer mitochondrial membrane protein are sufficient to direct the import of mouse dihydrofolate reductase into the matrix of isolated yeast mitochondria and cytochrome <u>c</u> oxidase subunit IV lacking its own presequence into the matrix of yeast mitochondria in vivo (Hurt et al., 1985b).

Studies on mammalian mitochondrial targeting sequences have not progressed as far as for yeast mitochondrial targeting sequences. However, the targeting sequence of human ornithine carbamoyltransferase (OCT) has been studied. As shown in Table 1, the targeting sequence of human pre-OCT has been localized to the central portion of the 32 amino acid presequence (5) (Horwich <u>et al.</u>, 1986; 1987). Amino acids 8 to 22 were necessary for the import of pre-OCT whereas the amino- and carboxyl-terminal regions of the presequence were not (Horwich <u>et al.</u>, 1986). This is in contrast to yeast targeting sequences which are at the extreme amino-terminus of the presequence. The midportion of the OCT presequence (the initiator methionine, residues 8 to 25 and glutamine 32) was found to be sufficient to direct the import of OCT (Horwich <u>et al.</u>, 1987).

Positive charge has been shown to be important in the targeting function of the presequences of OCT (Horwich <u>et al.</u>, 1985; 1986; 1987) and malate dehydrogenase (Table 1, (6)) (Chu <u>et al.</u>, 1987). Mutations of the presequences which acted to decrease their positive character resulted in less efficient import of the respective proteins. Multiple mutations that decreased positive charge at more than one site simultaneously impaired import even further. Positive charge rather than the specific amino acid is required as replacement of arginine by lysine did not affect import (Chu <u>et al.</u>, 1987; Horwich <u>et al.</u>, 1986). Analysis of 23 mitochondrial presequences, the majority from yeast, showed that most are expected to form amphiphilic (amphipathic)  $\alpha$ -helices with high hydrophobic moments (von Heijne, 1986). Peptides corresponding to portions of the presequences of yeast cytochrome <u>c</u> oxidase subunit IV (Roise <u>et al</u>., 1986) and rat OCT (Epand <u>et al</u>., 1986) probably form amphiphilic  $\alpha$ -helices and are lipophilic. This lipophilicity was demonstrated as these peptides inserted spontaneously into phospholipid monolayers, disrupted liposomes and collapsed the electrochemical potential across the inner membrane of isolated mitochondria.

Using synthetic oligonucleotides encoding artificial mitochondrial presequences fused to the gene for a passenger protein it has been demonstrated that positively charged amphiphilic structures are necessary for import whereas an a-helical structure is not (Allison and Schatz, 1986; Roise et al., 1988). In addition the targeting function is unlikely to be sequence dependent but rather the result of the balance of basic, hydroxylated and hydrophobic amino acids (Allison and Schatz, 1986). Artificial mitochondrial presequences containing only initiator methionine, arginine, serine and leucine in a ratio adjusted to match that of natural presequences were active in import, whereas those containing only initiator methionine, arginine, serine and glutamine were not (Allison and Schatz, 1986; Roise et al., 1988). The chemically synthesized presequence peptides that were active in import were shown to be amphiphilic whereas those that were inactive were nonamphiphilic (Roise et al., 1988). Thus amphiphilicity and positive charge are important for targeting.

# 1.2.4 MITOCHONDRIAL IMPORT RECEPTORS AND COMMON PATHWAY (S) FOR MITOCHONDRIAL PROTEIN IMPORT

In order to be imported mitochondrial proteins must be recognized by the import machinery of mitochondria (Hay <u>et al.</u>, 1984). Mitochondrial targeting sequences may react either with outer mitochondrial membrane receptor proteins directly or with phospholipids followed by diffusion to receptors (Skerjanc <u>et al.</u>, 1987).

Numerous studies have suggested the presence of proteinaceous receptors that are necessary for the import of mitochondrial proteins, however, none have been isolated as yet. The presence of these receptors have been demonstrated in a number of ways. Protease treatment of isolated <u>Neurospora</u> mitochondria has been shown to inhibit the binding of the ADP/ATP carrier, the outer mitochondrial membrane protein porin (Zwizinski <u>et al.</u>, 1984) and the precursor of  $F_0F_1$ -ATPase subunit 9 (Pfanner <u>et al.</u>, 1987c). This protease inhibition of binding suggests the presence of proteinaceous receptors in the outer membrane. In addition it has been found that the <u>Neurospora</u> ADP/ATP carrier can bind to deenergized mitochondria and upon reenergization of the mitochondria is imported from the bound sites (Zwizinski <u>et al.</u>, 1983).

It is believed that there are only a limited number of receptor types as a number of mitochondrial proteins utilize common pathway(s) for import. This has been shown in import competition experiments. A peptide corresponding to the first 27 amino acids of rat OCT presequence has been found to block the import into rat heart mitochondria of not only pre-OCT, but also the precursors of malate dehydrogenase and UCP (Gillespie <u>et al.</u>, 1985). Rat liver 3-ketoacyl-CoA thiolase, a matrix enzyme synthesized as a mature length precursor, inhibits the import of the precursors of OCT, medium chain acyl-COA dehydrogenase and acetoacetyl-COA thiolase into isolated rat liver mitochondria (Mori <u>et</u> <u>al</u>., 1985). Treatment of <u>Neurospora</u> mitochondria with elastase, sufficient to inhibit import of the ADP/ATP carrier and porin, did not affect import of  $F_0F_1$ -ATPase subunits 2 and 9 and these proteins may be imported via different pathways (Zwizinski <u>et al</u>., 1984). In contrast, apocytochrome <u>c</u> does not compete for binding with any other mitochondrial proteins tested so far. It has a distinct receptor that may be located in the intermembrane space (Nicholson and Neupert, 1988).

## 1.2.5 ENERGY REQUIREMENTS FOR IMPORT OF PROTEINS INTO MITOCHONDRIA

Import of matrix, inner mitochondrial membrane and intermembrane space proteins requires an electrochemical potential across the inner mitochondrial membrane whereas import of outer mitochondrial membrane proteins does not (Hay <u>et al.</u>, 1984). The electrochemical gradient ( $\Delta p$ ) is a function of the proton gradient ( $\Delta pH$ ) and the membrane potential ( $\Delta \Psi$ ) caused by charge separation across the inner mitochondrial membrane. Of these two components of the electrochemical gradient, the membrane potential is the one necessary for import. The membrane potential requirement was demonstrated by the fact that the import of the ADP/ATP carrier into isolated <u>Neurospora</u> mitochondria was driven by a valinomycin-induced potassium diffusion potential in the presence of CCCP, a protonophore, and was dependent on the size of the potential (Pfanner and Neupert, 1985). The membrane potential is required to initiate import of proteins but not to complete it.

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NTPs are required for the translocation of proteins across the inner mitochondrial membrane (Chen and Douglas, 1987a; Chen and Douglas, 1987b; Eilers <u>et al</u>., 1987; Eilers <u>et al</u>., 1988; Pfanner and Neupert, 1986; Pfanner <u>et al</u>., 1987a; Verner and Schatz, 1987). This requirement for NTPs has been demonstrated in a number of experiments. The depletion of NTPs in an import system inhibits the import of various proteins (Pfanner and Neupert, 1986; Eilers <u>et al</u>., 1987). The addition of NTPs but not non-cleavable ATP analogs was found to restore import. Phosphodiester bond hydrolysis is required outside the inner mitochondrial membrane (Chen and Douglas, 1987a; Eilers <u>et al</u>., 1987). Import therefore requires both an electrochemical potential and NTPs.

Experiments in which fusion proteins were prevented from unfolding by the binding of specific ligands (Eilers <u>et al.</u>, 1987; Chen and Douglas, 1987b) and studies on the import of incompletely folded precursor proteins (Verner and Schatz, 1987) suggested that NTPs are required for the unfolding of the precursor proteins. Point mutations that resulted in the destabilization of the tertiary structure of a mitochondrial precursor protein resulted in increased import relative to the wild-type precursor (Vestweber and Schatz, 1988). Import of a ureadenatured precursor occurred much faster, more efficiently and at lower temperatures than the native protein (Eilers <u>et al.</u>, 1988). The latter two studies indicate that unfolded proteins are imported more efficiently than their folded counterparts. Precursors having identical presequences but different passenger proteins required different levels of NTPs for translocation and precursors were more sensitive to proteases in the presence of NTPs (Pfanner <u>et al.</u>, 1987a). From these types of results it has been suggested that an unfoldase that controls the unfolding of precursors such that they are maintained in an importcompetent conformation may utilize the energy of NTPs.

# 1.2.6 TRANSLOCATION INTERMEDIATES SPAN BOTH MITOCHONDRIAL MEMBRANES SIMULTANEOUSLY

By examining the import of <u>Neurospora</u>  $F_1$ -ATPase subunit  $\beta$ , cytochrome c1 (Schleyer and Neupert, 1985), ADP/ATP carrier (Pfanner and Neupert, 1987b; Pfanner et al., 1987a,d) and the Fe/S protein (Hartl et al., 1986) at low temperatures or after binding to antibodies it was found that precursors simultaneously spanned both the outer and inner mitochondrial membranes during import. That these translocation intermediates spanned both membranes was based on two observations. The first observation was that precursors were partially translocated aminoterminus first as indicated by matrix protease cleavage of those precursors having presequences. The second observation was that a major portion of the proteins was found at the outer face of the outer mitochondrial membrane as determined by sensitivity to proteases. Translocation therefore occurs at sites where the outer and inner mitochondrial membranes are in close proximity. As mitochondrial membranes are 5 nm thick translocation could occur at sites ranging from 10 nm (membranes in contact) to 20 nm thick (maximal allowable distance accounting for observations) (Nicholson and Neupert, 1988).

Translocation appears to occur at sites of contact between the outer and inner mitochondrial membranes (Schwaiger <u>et al.</u>, 1987). These translocation contact sites as they are called have been characterized

in at least two ways. Firstly, it was found that mitoplasts, which are mitochondria lacking most of their outer membrane due to treatment with digitonin, retain the ability to import proteins. Secondly, it was demonstrated that translocation intermediates, isolated by import at low temperature, were located in areas of close proximity between the inner and residual outer mitochondrial membranes. The translocation contact sites appeared to be stable in the absence of precursors. Contact sites are not stabilized by the membrane potential as demonstrated by the fact that import into mitoplasts occured after the isolation of mitochondria maintained in the absence of a membrane potential.

Import through translocation contact sites may involve hydrophilic, possibly proteinaceous, channels (Pfanner <u>et al.</u>, 1987b). This was demonstrated by the extractability of translocation intermediates of  $F_1$ -ATPase subunit  $\beta$ , the ADP/ATP carrier, and cytochrome <u>b</u><sub>2</sub> by reagents such as urea and sodium bicarbonate. These agents are known to remove peripheral but not integral membrane proteins and indicate that the intermediates are in a hydrophilic environment.

#### 1.2.7 INTRACELIULAR TARGETING AND INTRAMITOCHONDRIAL SORTING

The above findings have allowed the formulation of models for the import of mitochondrial proteins. According to Hurt and van Loon (1986) intracellular targeting and intramitochondrial sorting are proposed to involve matrix targeting domains, stop transfer domains and cleavage sequences. The presence of a matrix targeting domain results in the transport of a protein across both mitochondrial membranes unless it is followed by a stop transfer domain. The latter domain interrupts transfer across the mitochondrial membranes. Possibly depending upon the distance between the matrix targeting domain and stop transfer domain the protein will be targeted to the outer or inner mitochondrial membrane, or as described below, to the intermembrane space. This model holds well for the import of matrix and outer mitochondrial membrane proteins but not for all intermembrane space and inner mitochondrial membrane proteins.

Yeast cytochrome  $\underline{c}_1$ , an intermembrane space protein, is synthesized as a precursor with a 61 amino acid presequence that contains a matrix targeting sequence (van Loon <u>et al.</u>, 1987) and a proposed stop transfer domain consisting of 19 consecutive hydrophobic residues (van Loon and Schatz, 1987). The existence of a stop transfer domain was demonstrated through the use of a fusion protein consisting of the cytochrome  $\underline{c}_1$  presequence and cytochrome  $\underline{c}$  oxidase subunit IV. This fusion protein was imported into the intermembrane space of isolated mitochondria without passing through a matrix intermediate (van Loon and Schatz, 1987). As seen in Fig. 2, the hydrophobic stop transfer domain which spans the inner membrane is cleaved off by a second protease at the outer face of the inner mitochondrial membrane releasing the protein into the intermembrane space.



Fig. 2. Import of yeast cytochrome  $c_1$  (according to van Loon and Schatz (1987)). OM, outer membrane; IM, inner membrane; R, receptor; P, matrix protease; P', intermembrane space protease; H, heme; p, precursor; i, intermediate; m, mature. The matrix targeting domain is indicated by +++. The hydrophobic stop transfer sequence is indicated by the hatched box. As illustrated in Nicholson and Neupert (1988).

#### 1.2.8 EVOLUTIONARY MODEL OF MITOCHONDRIAL PROTEIN IMPORT

The import of yeast cytochrome  $\underline{b}_2$ , <u>Neurospora</u> cytochrome  $\underline{c}_1$  (70 residue presequence cf. 61 in yeast) (Hartl <u>et al.</u>, 1987) and the <u>Neurospora</u> Fe/S protein (shown in Fig. 3) (Hartl <u>et al.</u>, 1986) involve matrix intermediates. All three precursors are first completely imported into the matrix where a protease removes the matrix targeting domain of the presequence (Hartl <u>et al.</u>, 1986; 1987). The intermediates are then redirected back across the inner mitochondrial membrane. The differences in the import pathways of yeast and <u>Neurospora</u> cytochrome  $\underline{c}_1$  could be due to the differences between the yeast and <u>Neurospora</u> proteins or to the fact that Schatz's group did not use cytochrome  $\underline{c}_1$  but rather a fusion protein between the cytochrome  $\underline{c}_1$  presequence and

cytochrome <u>c</u> oxidase subunit IV. The hydrophobic stretches of the presequences may act as signals for export of the intermediates from the matrix (Hartl et al., 1987) and not as stop transfer domain as suggested by the results of van Loon and Schatz (1987). The results of the experiments of Hartl et al. (1986; 1987) led to the suggestion of an evolutionary model for the import of intermembrane space proteins. This model would imply that during evolution there was transfer of genes for proteins of the bacterial cell from endosymbiotic membrane protomitochondria to the nucleus (Baker and Schatz, 1987). In addition to the maintenance of the export targeting signals from inside the mitochondrial matrix this would require the acquisition of mitochondrial matrix targeting sequences at the amino-terminus of these proteins. Thus the final stages in the import of these intermembrane space proteins resembles protein export from bacteria.



Fig. 3. Import of the <u>Neurospora</u> Fe/S protein. The legend is as in Fig. 2. As illustrated in Nicholson and Neupert (1988).

The import of the ADP/ATP carrier differs from the models for import illustrated above. Among the differences are the lack of a presequence and its multitopic disposition across the inner mitochondrial membrane. Its import will be discussed after an examination of the uncoupling protein and the relationship between these two proteins.

## 1.3 BROWN ADIPOSE TISSUE AND THE UNCOUPLING PROTEIN

Brown adipose tissue (BAT) functions in heat production in newborn mammals, small mammals acclimated to the cold and in arousal of hibernators (Nicholls and Locke, 1984). The process, known as nonshivering thermogenesis, is dependent on the action of a unique 32 kDa protein of the BAT mitochondrial inner membrane known as the uncoupling protein (UCP). During oxidative phosphorylation an electrochemical gradient is formed as protons are expelled across the inner mitochondrial membrane by the action of the electron transport chain. The energy of this gradient is used for the generation of ATP as protons flow through ATP synthase. The action of UCP as a membrane potentialdriven proton translocator (Klingenberg and Winkler, 1985) uncouples oxidative phosphorylation as ATP synthase is bypassed resulting in the dissipation of the energy as heat.

#### 1.3.1 UNCOUPLING OF BAT MITOCHONDRIA BY UCP

A number of lines of evidence summarized by Nicholls and Locke (1984) led to the conclusion that the uncoupling of BAT mitochondria was due to the presence of a 32 kDa protein now commonly referred to as UCP

or thermogenin. Regulation of the uncoupling process is mediated by the purine nucleotide and free fatty acid binding of UCP. Purine nucleotides bind to UCP at the outer face of the inner mitochondrial membrane inhibiting proton transport (Heaton et al., 1978). There is a direct correlation between the affinity of purine nucleotides for the binding site and their inhibition of proton conductance (Heaton et al., 1978; Lin and Klingenberg, 1982). The binding affinities of purine nucleotides decreases in the order GTP > GDP > ATP > ADP (Lin and Klingenberg, 1982). The affinity of purine nucleoside monophosphates for UCP is about 100-fold lower than that of the di- and triphosphates. Although GTP has the greatest affinity for UCP ATP is probably the regulator in vivo by virtue of higher concentrations (Klingenberg, 1984; Nicholls and Locke, 1984). Purified UCP, isolated by detergent solubilization of the inner mitochondrial membrane followed by hydroxylapatite column chromatography, retains the ability to bind purine nucleotides (Lin and Klingenberg, 1982). The binding capacity of the purified protein for GDP (16 µmol/g UCP) together with cross-linking analysis revealed that UCP acts as a dimer (Lin and Klingenberg, 1982).

Free fatty acids act to increase proton transport by UCP. This fatty acid induced uncoupling of BAT mitochondria (Locke <u>et al.</u>, 1982) results from the lowering of the binding affinity of purine nucleotides for UCP (Rial <u>et al.</u>, 1983). The free fatty acids probably act not by displacing the nucleotides but by causing a conformational change in UCP (Rial <u>et al.</u>, 1983). The proton permeability induced by free fatty acids is decreased by increasing nucleotide concentrations.

Studies on BAT mitochondria do not demonstrate that the

uncoupling of these mitochondria is due to UCP and does not involve any other unrecognized protein(s) and/or mechanism(s). To demonstrate that uncoupling is caused by the action of UCP required that UCP be isolated and reconstituted into liposomes. Reconsitution of UCP has been accomplished by Strielman et al. (1985) and Klingenberg and Winkler (1985) who have studied UCP with respect to proton transport, nucleotide binding and the effect of free fatty acids. The latter group studied the effect of UCP reconstituted into K<sup>+</sup>-loaded liposomes. When valinomycin was added to these liposomes it was found that the pH outside the liposomes decreased indicating a proton influx into the liposomes. Since UCP was necessary for this proton influx it was concluded that UCP was a membrane potential-dependent proton translocator. Furthermore it was found that GDP inhibited this influx. These two results strongly suggested that UCP is the uncoupler of BAT mitochondria. Strielman et al. (1985) have further shown that fatty acids increase the proton permeability of liposomes containing UCP. This result suggested that free fatty acids are the activators of the uncoupling process in BAT mitochondria and act via UCP. These conclusions must be regarded with caution however as the UCP reconstituted into the liposomes contained 20% (Klingenberg and Winkler, 1985) or 10% (Strielman et al., 1985) or less of other proteins.

#### 1.3.2 CHLORIDE TRANSPORT BY BAT MITOCHONDRIA

In addition to a high proton permeability BAT mitochondria have a high chloride permeability that is also inhibited by purine nucleotides (Nicholls and Locke, 1984). There appear to be two independent pathways for the transport of  $H^+$  and  $Cl^-$  by BAT mitochondria (Kopecky <u>et al.</u>, 1984). This was demonstrated as a valinomycin-induced  $K^+$  potential resulted in passive uptake of  $Cl^-$  and active extrusion of  $H^+$  in BAT mitochondria (Kopecky <u>et al.</u>, 1984). There was no competition between  $H^+$  and  $Cl^-$  transport and it was found that  $Cl^-$  transport was more sensitive to inhibition by GDP than was  $H^+$  transport. "The  $H^+$  and  $Cl^-$  conducting pathways of BAT mitochondria are formed by two independent transport entities which could be represented by a two state transition of one channel or by two distinct types of channels" (Kopecky <u>et al.</u>, 1984, p190). It is still not resolved whether  $Cl^-$  transport is due to UCP as  $Cl^-$  transport has not been demonstrated with purified UCP reconstituted in liposomes (Klingenberg and Winkler, 1985).

#### 1.3.3 SIRUCTURE/FUNCTION RELATIONSHIPS OF UCP

Positively charged residues are important in the binding of purine nucleotides to UCP. Modification of mitochondria and isolated UCP with a lysine-specific reagent and isolated UCP with argininebinding reagents decreased nucleotide binding (Lin and Klingenberg, 1982). The binding of purine nucleotides is pH dependent with binding affinities increasing at pH values less than 6.4 (Lin and Klingenberg, 1982). Since the affinity of nucleotides for the binding site increases with decreasing pH Klingenberg (1988) it was proposed that lysines and
arginines involved in nucleotide binding form a salt bridge with aspartic and glutamic acid residues. As the pH decreases these acidic groups are protonated freeing the lysine, arginine and histidine groups of the nucleotide binding site to interact with the nucleoside di- or triphosphates.

Sulfhydryl groups are also involved in the nucleotide binding of UCP as modification of mitochondria with sulfhydryl reagents decreased nucleotide binding and nucleotide inhibition of Cl<sup>-</sup> transport (Rial and Nicholls, 1987). A single cysteine residue (287) seems to be implicated in nucleotide binding as there is loss of high affinity GDP binding when it is modified. In contrast to the decreased nucleotide inhibition of Cl<sup>-</sup> transport, modification of mitochondria with sulfhydryl reagents did not significantly decrease nucleotide inhibition of H<sup>+</sup> transport (Rial and Nicholls, 1987).

Sulfhydryl groups may also be involved in  $H^+$  translocation by UCP (Jezek, 1987). Hydrophilic sulfhydryl reagents were found to inhibit  $H^+$  translocation but did not affect Cl<sup>-</sup> transport suggesting that a water accessible sulfhydryl group on the outer face of the inner mitochondrial membrane is involved in proton translocation. Cysteine 304 was suggested as a possibility as it is on the outer face of the inner membrane in the membrane disposition model of hamster UCP proposed by Aquila <u>et al</u>. (1985) (Jezek, 1987).

# 1.3.4 STRUCTURAL RELATIONSHIP OF THE UNCOUPLING PROTEIN, THE ADP/ATP CARRIER AND THE PHOSPHATE CARRIER

The primary structure of hamster UCP has been determined by direct sequencing of the protein (Aquila <u>et al.</u>, 1985). The protein consists of 306 amino acids with a molecular mass of 33,215 Da (Aquila <u>et al.</u>, 1985). There is micro-heterogeneity with leucine and phenylalanine at position 128. The 28 positively charged residues and 19 negatively charged residues give UCP a net positive charge of 9 at a pH at which all residues are charged. UCP contains 40% hydrophobic residues (30% alicyclic amino acids, 10% aromatic amino acids) and has a polar amino acid composition of 42%.

After the direct sequencing of hamster UCP had been completed a full length cDNA clone for rat UCP was isolated in our laboratory (Ridley <u>et al.</u>, 1986b). The predicted protein sequence of rat UCP (Ridley <u>et al.</u>, 1986b; Bouillaud <u>et al.</u>, 1986) was found to be 91.5% homologous with that of hamster UCP (Aquila <u>et al.</u>, 1985). The mature protein consists of 306 amino acids and is 33,084 Da in size. The amino-terminal 30 amino acids of UCP were determined by protein sequencing. The deduced amino-terminal methicnine which is removed from the precursor protein (Ridley <u>et al.</u>, 1985). These results confirm previous results that UCP is synthesized as a mature length precursor (Freeman <u>et al.</u>, 1983; Ricquier <u>et al.</u>, 1983; Freeman and Patel, 1984; Freeman <u>et al.</u>, 1985).

Knowledge of the primary sequences of hamster and rat UCPs allowed the prediction of a possible secondary structure and membrane disposition of the protein. Standard hydropathy calculations according to the method of Kyte and Doolittle (1982) suggested the existence of six membrane spanning domains in UCP, as illustrated in Fig. 4 (Runswick et al., 1987). Calculations of hydropathy that take into account sided (amphiphilic)  $\alpha$ -helices and  $\beta$ -strands were applied to UCP and suggested the existence of 6  $\alpha$ -helices and an amphiphilic  $\beta$ -strand (Aquila et al., 1985; 1987). A model of the membrane disposition of hamster UCP (Aquila et al., 1985) is shown in Fig. 5a. The membrane disposition model of UCP (Fig. 5a) differs from the hydropathy plot (Fig. 4) for the protein in that at least seven transmembrane segments are predicted former while only six are predicted in the latter, the in the difference being a  $\beta$ -strand between  $\alpha$ -helices A and B. This difference would lead to an opposite position of the amino-terminus with respect to the carboxyl-terminus in the two models.

The primary structure of the UCPs revealed several features that were very similar to that of the bovine heart ADP/ATP carrier whose primary structure had been previously determined (Aquila <u>et al.</u>, 1982). The ADP/ATP carrier was found to consist of 297 residues and has a molecular mass of 32,906 Da. The membrane disposition of the protein was studied by modification of mitochondria, submitochondrial particles and solubilized protein with the lysine-specific labelling reagent pyridoxal 5-phospate. The results of these modifications were projected into a transmembrane folding model consisting of six membrane spanning  $\alpha$ -helices and a  $\beta$ -sheet as shown in Fig. 5c (Bogner <u>et al.</u>, 1986). There appears to be a transmembrane segment that crosses back across the membrane towards the carboxyl-terminus of the protein as seen in Fig. 5c



Fig. 4. Hydropathy profiles of the three mitochondrial transport proteins. There is a window of 11 amino acids used in the calculations. The horizontal line is the average hydrophobicity of sequenced proteins according to Kyte and Doolittle (1982). Transmembrane helices are represented by Roman numerals and hydrophilic stretches by letters. As illustrated in Runswick <u>et al</u>. (1987).



Suggested membrane disposition model of hamster UCP (Aquila  $\underline{et}$   $\underline{al}$ ., 1985). The six membrane spanning  $\alpha$ -helices are labelled A-F. A possible  $\beta$ -strand is shown between helix A and helix B. Stretches of unknown structure are indicated by shaded boxes. The single letter amino acid code is used. Areas with residues more hydrophobic than glycine are shaded gray.

b)

c y 10 s 0 l



Suggested membrane disposition model of the phosphate carrier (Aquila <u>et al.</u>, 1987). The six membrane spanning  $\alpha$ -helices are labelled A-F. A possible  $\beta$ -strand is between helices A and B. Intervening hydrophilic segments are indicated by hatched boxes as secondary structure is unclear. The single letter amino acid code is used.



Suggested membrane disposition model of the ADP/ATP carrier (Bogner et al., 1986). Membrane spanning  $\alpha$ -helices and  $\beta$ -strands were derived from hydrophobicity calculations for sided  $\alpha$ -helices and  $\beta$ -strands. The direction of the segments toward the cytosolic or matrix was based on lysine incorporation into mitochondria and submitochondrial particles. The position of lysines in the phospholipid headgroup region is based on comparisons of incorporation into solublized protein, mitochondria and sub-mitochondrial particles. Inaccessible lysines are marked by a Lysines accessible in a carboxyatractylate-protein dashed circle. complex are indicated by a full circle. Lysines accessible in a bongkrekate-protein complex are indicated by a rectangle. Possible assignment of lysines to the translocation path is indicated by omission of background pattern.

Fig. 5. Membrane disposition models of the three mitochondrial transport proteins. a, hamster UCP as illustrated in Aquila <u>et al</u>. (1985) b, bovine phosphate carrier as illustrated in Aquila <u>et al</u>. (1987) c, bovine heart ADP/ATP carrier as illustrated in Bogner <u>et al</u>. (1986).

The membrane disposition model of UCP does not have this transmembrane segment as seen in Fig. 5a. As similar studies have not yet been performed on UCP it is not known whether UCP also contains this transmembrane segment. This discrepancy between the membrane disposition models of UCP and the ADP/ATP carrier, together with the result that the carboxyl-terminus of UCP is on the cytosolic face of the inner mitochondrial membrane (Eckerskorn and Klingenberg, 1987) indicates that either the membrane disposition model of UCP (Aquila <u>et al.</u>, 1985) is incorrect or that it is in fact correct and the two proteins have different dispositions in the inner membrane.

The primary sequences of three mitochondrial carriers, UCP (Aquila et al., 1985), the ADP/ATP carrier (Aquila et al., 1982) and the phosphate carrier (Runswick et al., 1987) have been compared and are very similar (Aquila et al., 1985; 1987; Runswick et al., 1987). **A**11 three proteins are found to exhibit a tripartite structure of three 100 residue repeats and all nine repeats in the three proteins are related to each other (Aquila et al., 1987; Runswick et al., 1987). There is a distant homology amongst the full sequences as well as between individual repeats. There is a high percentage of polar residues, with more basic than acidic amino acids, resulting in a large net positive There is a distinct conservation of critical residues, charge. glycine, proline and of charged and aromatic residues in all three repeats of each of the three proteins (Aquila et al., 1987).

The similarity in the primary sequences and membrane disposition models of these three mitochondrial transport proteins, UCP, the ADP/ATP carrier and the phosphate carrier has led to the suggestion that they evolved by gene triplication followed by diversification (Aquila <u>et al.</u>, 1987). It has also been proposed that UCP belongs to the subgroup of  $H^+/anion$  cotransporters and that UCP evolved by deletion of the anion binding site (Aquila <u>et al.</u>, 1985). UCP could therefore be considered as a degenerated  $H^+/anion$  cotransporter.

Despite the great deal of similarity amongst the three mitochondrial transport proteins a major difference exists in the structure of their precursors. The bovine phosphate carrier is synthesized with a 49 amino acid presequence (Runswick <u>et al.</u>, 1987). This is in contrast to the precursors of the ADP/ATP carrier and UCP which are both synthesized as mature length precursors. The targeting and import of the phosphate carrier may therefore differ from that of UCP and the ADP/ATP carrier.

## 1.3.5 IMPORT OF THE ADP/ATP CARRIER: A MODEL FOR UCP IMPORT

The structural similarity between UCP and the ADP/ATP carrier suggests that both of these inner mitochondrial membrane proteins would be imported via a similar mechanism. In fact both of these proteins are synthesized as precursors of mature length and presumably contain mitochondrial targeting sequences within the sequence of the mature protein. As discussed previously targeting sequences tend to be rich in positively charged residues. Several regions of high positive charge exist within UCP that represent ideal candidates for targeting sequences (Ridley <u>et al.</u>, 1986 b,c). Current knowledge of the targeting of the ADP/ATP carrier will therefore serve as a starting point for considering the targeting sequence(s) and import pathway of UCP.

A targeting sequence of the yeast ADP/ATP carrier has been localized to the first 115 amino acids as a fusion protein of these residues with  $\beta$ -galactosidase was imported into isolated yeast mitochondria (Adrian et al., 1986). It has been recently suggested that the region between residues 72 and 111 of the yeast ADP/ATP carrier is involved in targeting (Smagula and Douglas, 1988a,b). This is based on the import of a protein containing the first 111 amino acids of the ADP/ATP carrier fused to dihydrofolate reductase and the lack of import of a fusion protein containing only the first 72 residues of the ADP/ATP carrier. It remains to be demonstrated however that this region by itself is sufficient for targeting. In contrast to these studies a precursor of the Neurospora ADP/ATP carrier lacking the first 103 amino acids was still imported into isolated mitochondria (Pfanner et al., 1987d). However this result is not suprising since this protein exhibits a tripartite structure of three 100 residue repeats. It may be that each of the tripartite segments or at least more than one has a targeting sequence.

There are at least two targeting sequences in rat UCP (Liu <u>et</u> <u>al</u>., 1988) paralleling the results obtained with the ADP/ATP carrier. The first signal, located between residues 12 and 104 of the mature protein, is necessary for targeting and membrane insertion. This was based on the import into isolated rat heart mitochondria of a protein consisting of residues 12 to 104 of UCP fused to the carboxyl-terminal 209 amino acids of OCT and its resistance to alkaline extraction. The second signal, located downstream of residue 100, functions only to target UCP to mitochondria. This was demonstrated as the carboxylterminal 206 residues of UCP were imported into isolated rat heart mitochondria but were not resistant to alkaline extraction, indicating lack of integration into the inner membrane.

A model for the import of the ADP/ATP carrier has been proposed (Pfanner <u>et al.</u>, 1987d; Pfanner and Neupert, 1987b), and is illustrated in Fig. 6. The ADP/ATP carrier is synthesized as a mature length precursor on free ribosomes in the cytosol (1). The next step in the import pathway is the NTP-dependent, membrane potential-independent binding of the protein to a receptor in the outer membrane (2). The protein is then translocated into saturable protease protected sites within the outer membrane in an NTP-dependent process (3). The protein is extractable from these sites at alkaline pH indicating that the protein is in a hydrophilic environment. Translocation from the outer membrane to the inner membrane requires a membrane potential and occurs

NTP: dependent



Fig. 6. Import of the ADP/ATP carrier. The requirement for a membrane potential  $(\Delta \Psi)$  and NTPs ([+++] representing high requirement and [-] representing no requirement) are indicated. The legend is as in Fig. 3. As illustrated in Pfanner <u>et al</u>. (1987a).

at translocation contact sites (4). If import occurs at low temperature it is found that there is an intermediate on the import pathway that spans both mitochondrial membranes simultaneously (Pfanner <u>et al</u>., 1987c). The completion of translocation into the inner membrane is a membrane potential independent process (5).

The import of the phosphate carrier may differ from those of UCP and the ADP/ATP carrier as the phosphate carrier possesses a presequence (Runswick <u>et al.</u>, 1987). It is known that the phosphate carrier is present in prokaryotes whereas the ADP/ATP carrier is found in mitochondria, while UCP is found only in mammalian mitochondria (Aquila <u>et al.</u>, 1987). If the phosphate carrier evolved into the ADP/ATP carrier which subsequently evolved into UCP it could mean an alteration in the import pathway has occured during evolution. This could be the result of the loss of a mitochondrial presequence by the ADP/ATP carrier.

# 1.4 ANTIGEN-ANTIBODY INTERACTIONS: AN ANTIGENIC DETERMINANT OF UCP

A number of criteria can be used to predict the location of antigenic determinants (Berzofsky, 1985). An antigenic determinant should be surface accessible so that it can interact with antibodies (Berzofsky, 1985). Hydrophilicity has been suggested to be an important factor (Hopp and Woods, 1981) and is generally a reflection of surface exposure. Hopp and Wood's algorithm, however, is only 56% successfull as hydrophilic regions that are predicted to be antigenic determinants can interact with one another and therefore be buried and nonantigenic (Berzofsky, 1985). Mobility and flexibility may also contribute to the antigenicity of a particular region of the protein (Berzofsky, 1985; van

## Regenmortel, 1986).

Antigenicity may also be determined by local stereochemistry (Geyson <u>et al.</u>, 1987) and is correlated with high mobility, low packing density, surface exposure and convex surface shape. Further initial binding to solvent exposed residues may lead to a conformational change in the protein, allowing interactions with previously buried hydrophobic residues (Getzoff <u>et al.</u>, 1987).

Antigenic determinants tend to be about 6 to 8 residues in length (Hopp and Woods, 1981; Novotony <u>et al.</u>, 1986). They may be classified as continuous, being composed of a linear sequence of amino acids, or discontinous, being composed of residues far apart in the primary sequence but brought close together by the folding of the protein (Barlow <u>et al.</u>, 1986). Most determinants are likely to be discontinous and the most continous regions of the protein are located in surface loops (Barlow <u>et al.</u>, 1986). Amino- and carboxyl-terminal regions tend to be surface oriented and therefore antigenic (van Regenmortel, 1986). This explains why hydrophilicity (Hopp and Woods, 1981), surface accessibility (Novotony <u>et al.</u>, 1986), mobility and protrusion are reasonably accurate predicters of antigenicity (Barlow <u>et al.</u>, 1986).

Antigenicity of protein regions can be assessed by the ability of peptides corresponding to those regions to inhibit the reaction of a protein with a specific antibody (Lando and Reichlin, 1982). The standard procedure is to perform a radioimmune assay and determine the displacement of radioactive protein from the antibody by the peptide (Lando and Reichlin, 1982). Problems with peptides arise, however, as the conformation of the peptide in solution may differ from its conformation in the native protein.

How do these ideas relate to the antigenicity of UCP? There is an antigenic determinant within the carboxyl-terminal 11 amino acids of UCP (Ridley et al., 1986a). This was demonstrated by the immunological screening of a rat BAT cDNA library in Escherichia coli with a rabbit antibody preparation against rat UCP (Freeman et al., 1983) which revealed five possible clones (Ridley et al., 1986a). Two of these clones encoded the carboxyl-terminal 54 amino acids of UCP and three others encoded the carboxyl-terminal 11 amino acids of UCP. This suggested the presence of an antigenic determinant within the carboxylterminal 11 amino acids. The carboxyl-terminal 11 residues possess several antigenic characteristics (Berzofsky, 1985), namely hydrophilicity and a possible  $\beta$ -turn conformation.

The immunological relationships of UCP from rat, mouse, hamster and rabbit have also been investigated (Afong <u>et al.</u>, 1985) using a rabbit antibody preparation against rat UCP (Freeman <u>et al.</u>, 1983). There was complete cross reactivity of the antibody preparation with rat, mouse and hamster UCP but only 25% cross reactivity with rabbit UCP (Afong <u>et al.</u>, 1985). The structure of the antigenic determinant(s) for this antibody preparation is therefore highly conserved in closely related species such as rat, mouse and hamster. If the carboxylterminal antigenic determinant is the dominant determinant in the antibody preparation the complete identity of the carboxyl-terminal 11 amino acids in the rat and hamster proteins would explain the complete cross reactivity of the two proteins. This antigenic determinant may be less highly conserved among more distantly related mammalian species such as rabbit the lesser reactivity of the rabbit protein may be due to amino acid difference(s) in the carboxyl-terminal 11 residues of rat and rabbit UCP.

Subsequent to this it was demonstrated that the carboxylterminal 10 amino acids of UCP are found to be on the cytosolic side of the inner mitochondrial membrane (Eckerskorn and Klingenberg, 1987). Proteolytic digestion of UCP, UCP within a detergent micelle and UCP within intact BAT mitochondria resulted in cleavage of the protein releasing a 2 kDa fragment. The presence of this carboxyl-terminal portion on the outer face of a protein detergent micelle correlates well with the antibody detection of a cDNA clone encoding the carboxylterminal 11 amino acids of rat UCP as a rat UCP-detergent micelle was used to immunize rabbits (Ridley et al., 1986a).

#### 1.5 SCOPE AND GOALS OF PROJECT

The aim of this project was to isolate and sequence the cDNA for rabbit UCP. The sequence of this UCP was to be compared to the sequences of rat and hamster UCP to determine which regions are conserved in all three proteins and which could therefore represent functional domains. On the other hand the lack of conservation in particular regions proposed to be involved in UCP's function could provide evidence against their involvement in a particular function. A comparison of the protein sequence with that of the structurally related ADP/ATP carriers (Aquila <u>et al</u>., 1985; 1987) could indicate whether there is conservation of regions possibly involved in targeting and nucleotide binding.

A second aim of this project was to determine whether there were two rabbit UCP mRNAs as had been previously found in both rat (Bouillaud <u>et al.</u>, 1985; Ridley <u>et al.</u>, 1986a) and mouse (Jacobson <u>et al.</u>, 1985). In addition it had been previously suggested that the two rat UCP mRNAs could be due to differential processing (Bouillaud <u>et al.</u>, 1985). Whether this is the case could be determined by examining the two rat UCP mRNAs using cDNA probes.

A third aim of this project was to try to determine the basis of the differential cross-reactivity of rabbit and rat UCP with an antibody preparation against rat UCP (Afong <u>et al.</u>, 1985). Sequence differences at the carboxyl-terminus of the rabbit protein compared to the rat protein could explain this if the carboxyl-terminal antigenic determinant was the dominant one in the antibody preparation used in the above study.

#### 2 MATERIALS

#### 2.1 <u>Plasmid Preparation Solutions</u>

Tetracycline: Sigma T3383 - 10 mg/ml in 50% ethanol (v/v)

Ampicillin: Sigma A9518 - 25 mg/ml

Chloramphenicol: Sigma 0378 - 40 mg/ml in absolute ethanol

LB medium: 10 g bactotryptone, 5 g bactoyeast extract, 10 g NaCl; to one liter after pH adjusted to 7.5 with NaOH. Autoclaved for 30 min. 2X YT medium: 16 g bactopeptone, 10 g bactoyeast extract, 10 g NaCl; pH to 7.5 with HCl, to one liter. Autoclaved for 30 min. Bactotryptone, bactoyeast extract, bactopeptone, bactoagar: Difco Lysozyme solution: 50 mM glucose, 10 mM CDTA, 25 mM Tris-HCl, pH 8.0.

Autoclaved for 15 min.

Lysozyme: Sigma L6876

Alkaline-SDS solution: 0.2 N NaOH autoclaved for 30 min; SDS added to 1% and the solution filter sterilized.

High salt solution: 3 M potassium acetate, 1.8 M formic acid: 60 ml 5 M potassium acetate (autoclaved 30 min), 35 ml sterile water and 5 ml 98% formic acid.

Acetate CDTA-MOPS solution: 0.1 M sodium acetate, 1 mM CDTA, 0.05 M MOPS, pH 8.0 with NaOH; made in sterile manner and filter sterilized. CDTA-Tris solution (1X): 1 mM CDTA, 10 mM Tris-HCl, pH 7.5. Autoclaved for 30 min.

LiCl solution: 5 M LiCl, 1 mM CDTA, 0.05 M MOPS, pH 8.0; made in a sterile manner and filter sterilized.

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## CSC1: BRL ultrapure

#### 2.2 DNA Dot Blots

Tris-EDTA (TE) Buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 7.6. Autoclaved for 30 min.

Nitrocellulose: Bio-Rad or Mandel Scientific

20X SSC (saline sodium citrate): 3 M NaCl, 0.3 M trisodium citrate, pH to 7.0 with HCl. Autoclaved for 30 min.

Hybridization solution (40 ml): 10.0 ml 20X SSC, 18.8 ml deionized formamide, 4.0 ml 50X Denhardt's solution, 4.0 ml 10X orthophosphate (0.5 M sodium dihydrogen orthophosphate/0.5 M disodium hydrogen orthophosphate (685:315, v/v)), 2.0 ml 20X pyrophosphate (100 mM  $Na_4P_2O_7$ ), 1.0 ml denatured salmon sperm DNA (10 mg/ml), 0.2 ml 20% SDS 50X Denhardt's solution: 5 g ficoll, 5 g polyvinylpyrrolidone, 5 g BSA (pentax fraction V) to 500 ml

BSA, DNase/RNase free: Pharmacia - 10 mg/ml

#### 2.3 Random Primer Labelling of CDNA

IS (labelling solution): 1 M HEPES, pH 6.6/DS/OS (25:25:7, V/V/V)

DS (deoxyribonucleotide solution): 100  $\mu M$  each of dATP, dGTP and dTTP in TMS

**TMS (Tris-magnesium solution):** 25 mM MgCl<sub>2</sub>, 50 mM  $\beta$ -mercaptoethanol, 250 mM Tris-HCl, pH 8.0

OS (oligodeoxyribonucleotide solution): 1 mM EDTA, 1 mM Tris-HCl, pH 7.5 with 90  $A_{260}$  units of oligodeoxyribonucleotides/ml

a-[<sup>32</sup>P]dCTP: Amersham - 410 Ci/mmol

Klenow fragment: Pharmacia - (5-10 U/µl) Calf liver tRNA: Boehringer Mannheim - 2 mg/ml

## 2.4 <u>Reverse Transcription of mRNA</u>

DE81 filter paper: Whatman

AMV reverse transcriptase: Pharmacia - (10-20 U/µl)

**Phenol/chloroform/isoamyl alcohol:** (25:24:1, v/v/v) - saturated with TE buffer, pH 7.6

#### 2.5 Competent Cell Preparation/Transformation/Plating

Cells: JM107: BRL; MV1193: Dr. A.B. Futcher; <u>E. coli</u> 294: Dr. C.B. Harley

Plasmids: M13mp18 and M13mp19 RF forms: BRL or Pharmacia; pUC118: Dr. A.B. Futcher

Bacteriophage: M13K07: Dr. A.B. Futcher

IB bottom agar: 12 g bactoagar/l IB medium

LB top agar: 7 g bactoagar/l LB medium

B medium: 10 g bactopeptone, 8 g NaCl, pH to 7.5, to one liter. Autoclaved for 30 min.

B bottom agar: 12 g bactoagar/l B medium

B top agar: 6 g bactoagar/1 B medium

X-gal: IBI or Research Organics - 2% in dimethylformamide

IPIG: BRL - 100 mM

#### 2.6 Agarose Gels

#### Seaken LE agarose: Mandel Scientific

10X Tris-borate EDTA (TBE): 109 g Tris, 55 g boric acid, 9.3 g EDTA, pH to 8.3 with Tris or boric acid, to one liter. Autoclaved for 30 min. FDM (formamide dye mix): 9 ml deionized formamide, 1 ml 0.25 M EDTA, 1% xylene cyanol, 1% bromophenol blue

# 2.7 <u>Restriction Enzyme Digests</u>

Bann HI: ERL - 10 U/µl or Pharmacia - 18 U/µl; Bgl I: Pharmacia-12.5 U/µl; Bgl II: IBI - 20 U/µl; Eco RI: ERL -10 U/µl or Pharmacia-15 U/µl; Hinc II: Pharmacia - 8 U/µl; Hind III: ERL -10 U/µl; Kon I: ERL - 5 U/µl or Pharmacia - 15 U/µl; Pst I: ERL or Pharmacia - 10 U/µl; Rsa I: Boehringer Mannheim - 20 U/µl; Sal I: ERL - 9 U/µl; Sma I: Pharmacia - 10 U/µl

10X restriction buffers used were those supplied by the manufacturer or as specified by Maniatis et al. (1982), as follows.

Low salt buffer: (10X) 100 mM MgCl<sub>2</sub>, 10 mM DTT, 100 mM Tris-HCl, pH 7.5 Medium salt buffer: (10X) 500 mM NaCl, 100 mM MgCl<sub>2</sub>, 10 mM DTT, 100 mM Tris-HCl, pH 7.5

High salt buffer: (10X) 1 M NaCl, 100 mM MgCl<sub>2</sub>, 10 mM DIT, 500 mM Tris-HCl, pH 7.5

RNase A: Sigma R5125 (1 mg/ml)

#### 2.8 Northern Blot Analysis

10X gel running buffer: 0.2 M boric acid, 2 mM EDTA, pH 8.3. Autoclaved for 30 min.

#### 3 MM paper: Whatman

The hybridization solution for Northern blot analysis was the same as that for DNA dot blots with the following exceptions: 20.0 ml deionized formamide instead of 18.8 ml; omit 2.0 ml 20X pyrophosphate; add 0.8 ml water.

## 2.9 Ligations

10X ligase buffer: IBI - 100 mM MgCl<sub>2</sub>, 10 mM DIT, 4 mM ATP, 250 mM Tris-HCl, pH 7.8 T<sub>4</sub> DNA ligase: IBI or BRL - (1-1.5 U/μl)

Gene clean kit: BIO101

# 2.10 Single Stranded DNA Preparation

TES (Tris-EDTA-saline) solution: 10 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5. Autoclaved for 30 min.

## 2.11 Sequencing Reactions

The Sequenase kit and modified  $T_7$  DNA polymerase (Sequenase) (Tabor and Richardson, 1987) were purchased from United States Biochemical.  $\alpha-[^{32}P]dATP: NEN - 3000 Ci/mmol$ 

# 2.12 Sequencing Primers

Additional primers were purchased from the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University and were synthesized using an Applied Biosystems 381A Automated DNA Synthesizer (Applied Biosystems Inc., Foster City, CA). These were diluted to 0.5  $pmol/\mu l$  in sterile water.

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AB322: 5'-GCCTGGCGGACGTGATC-3'; AB332: 5'-CAACTGGAGGCGTGGCG-3';
AB341: 5'-CTCCAGTGGATGTGGTG-3'; AB350: 5'-GCACCCGGGTCATCAAG-3'.
These correspond to nucleotides 71-87, 359-375, 686-702 and 961-977
respectively of the noncoding strand of rabbit UCP cDNA.
AB362: 5'-GTCTTCATTTGCCTTTG-3'; AB363: 5'-GCATATTGTCCCGGTGG-3';
AB364: 5'-CGCCACGCCTCCAGTTG-3'; AB365: 5'-CGTCCGTGGTCGTGGTG-3'.
These correspond to nucleotides 1069-1053, 740-724, 375-359 and 22-6
respectively of the noncoding strand of rabbit UCP cDNA.
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#### 2.13 Sequencing Gels

8% instagel solution: 38 g acrylamide, 2 g bis-acrylamide, 210 g urea, 50 ml 10X TBE, to 500 ml and filtered twice through Whatman 3 MM paper.

#### 2.14 5' End-Labelling of Oligonucleotides

 $\tau$ -[<sup>32</sup>P]ATP: NEN - 3000 Ci/mmol T<sub>4</sub> polynucleotide kinase: Pharmacia - 7.5 U/µl

#### 2.15 Primer Extension

Reaction Mix (90  $\mu$ l): 5.0  $\mu$ l 1 M Tris-HCl, pH 8.2, 5.0  $\mu$ l 0.2 M DTT, 5.0  $\mu$ l 0.12 M MgCl<sub>2</sub>, 2.5  $\mu$ l 100 mg actinomycin D/ml, 2.0  $\mu$ l 25 mM dATP, 2.0  $\mu$ l 25 mM dCTP, 2.0  $\mu$ l 25 mM dGTP, 5.0  $\mu$ l 10 mM dTTP, 60.5  $\mu$ l of sterile water and 1.0  $\mu$ l (10 U) AMV reverse transcriptase.

The primer used for primer extension of rabbit BAT  $poly(A)^+$  RNA was AB365 (see sequencing primers). A primer (Ridley <u>et al.</u>, 1986b) complementary to nucleotides 174-158 of the coding strand of rat UCP cDNA was used for primer extension of rat BAT  $poly(A)^+$  RNA.

# 2.16 Computer Programs

Wordperfect: Version 4.2 for wordprocessing

Microgenie: Beckman Instruments Inc., Palo Alta, CA (Queen and Korn, 1984) for sequence analysis.

Grapher: for Figs. 25 and 26

#### 3 METHODS

## 3.1 Previously Prepared Materials

At the start of this project several materials were available. The rabbit UCP cDNA clone was selected from a 31 day fetal rabbit BAT cDNA library prepared in <u>E. coli</u> by Dr. R.G. Ridley from sucrose-density gradient fractionated RNA enriched for UCP mRNA following a previously described method (Ridley <u>et al.</u>, 1986a). Labelled first strand cDNA prepared by H. Patel from 31 day fetal rabbit BAT mRNA and rabbit liver mRNA according to the method of Okayama and Berg (1982) was used for probing of dot blots. Total RNA prepared by H. Patel based on a modified phenol/chloroform extraction procedure (Lomedico and Saunders, 1976) was used for Northern blot analyses. Poly(A)<sup>+</sup> RNA obtained from total RNA by passage over an oligo(dT)<sub>18</sub> column was used for primer extension reactions.

# 3.2 Small Scale Plasmid Preparation

Plasmid DNA was prepared according to a modified version of the alkaline lysis procedure of Birnboim and Doly (1979). A single bacterial colony was inoculated into 3 ml of LB medium containing 10  $\mu$ g tetracycline/ml. The culture was incubated overnight at 37°C with vigorous shaking and 1.5 ml was recovered by centrifugation for 5 min in an Eppendorf microcentrifuge. The supernatant was discarded and the pellet was suspended by vortex mixing in 100  $\mu$ l of lysozyme solution containing 1 mg lysozyme/ml and left on ice for 5 min. Alkaline-SDS

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solution (200  $\mu$ l) was added and the sample mixed vigorously on a vortex mixer until it became viscous and clear. After 5 min on ice 150  $\mu$ l of high salt solution was added, the sample mixed on a vortex mixer and placed on ice for 15 min.

The sample was centrifuged for 5 min and 350  $\mu$ l of the supernatant transferred to another Eppendorf tube. Ice cold 95% ethanol (900  $\mu$ l) was added and the sample left on ice for 10 min. The sample was centrifuged for 5 min and the supernatant discarded. The pellet was dissolved in 100  $\mu$ l of acetate-CDTA-MOPS solution, 200  $\mu$ l of ice cold 95% ethanol added and the nucleic acid precipitate recovered by centrifugation after 10 min on ice. The precipitation step was repeated once more. The final pellet was dried in a vacufuge for 5 min and dissolved in 40  $\mu$ l 0.1X CDTA-Tris solution.

## 3.3 Large Scale Plasmid Preparation

Large scale preparations of plasmid DNA were performed by following a modified version of the procedure of Birnboim and Doly (1979) with the following modifications. A loop of cells from a single bacterial colony was inoculated into 3 ml of LB medium (for pER322derived plasmids) or 1X YT medium (for pUC118-derived plasmids) containing the appropriate antibiotic (10  $\mu$ g tetracycline/ml for Y11.28 or 150  $\mu$ g ampicillin/ml for pUC118 recombinant) and grown overnight at 37°C. These were used to inoculate 500 ml of LB or YT medium and grown at 37°C to an A<sub>600</sub> of 0.8 to 1.0 for pER322-derived plasmids or overnight for the pUC118-derived plasmid. For pER322-derived plasmids chloramphenicol was added to a final concentration of 170  $\mu$ g/ml and the culture incubated at 37°C overnight.

DNA was isolated and the pellet obtained was purified by CsCl density gradient centrifugation as described by Maniatis <u>et al</u>. (1982). The DNA pellet was dissolved in 10.2 ml of 1X CDTA-Tris solution and 10.6 g of CsCl and 160  $\mu$ l of 10 mg ethidium bromide/ml added. The solution was transferred to a Beckman quick seal tube, sealed and centrifuged at 40,000 rpm in a Ti80 rotor for 72 hr in a Beckman ultracentrifuge.

After the centrifugation was complete the top of the quick seal tube was cut off and scotch tape was placed over the tube where the injection was to be made. The lower visible band of plasmid DNA was recovered with a 3 ml syringe and 19 guage  $1\frac{1}{2}$  inch needle and 2 to 3 ml of water-saturated butanol added to remove the ethidium bromide. Five more butanol washes were performed until all traces of ethidium bromide were removed. Two volumes of sterile H<sub>2</sub>O were added, the volume measured and two volumes of ice cold 95% ethanol added. The DNA was precipitated at 0°C for 30 min, the pellet collected by centrifugation at 7,000 rpm for 30 min at 4°C in an SS34 rotor and dried under vacum. The pellet was dissolved in 200 µl of 1X CDTA-Tris solution and 10 µl of this sample was diluted in 1.5 ml of water to measure the A<sub>260</sub> and A<sub>280</sub> and the absorbance from 220 rm to 320 rm.

# 3.4 DNA Dot Blots

The procedure for DNA dot blotting was adapted from Maniatis <u>et</u> <u>al</u>. (1982). The DNA from a small scale plasmid preparation (~250 ng DNA) was denatured by adding 5  $\mu$ l of 1 N NaOH and the sample left at room temperature for 20 min. Fifteen microliters of a solution containing 1 M NaCl, 50 mM EDTA, and 50 mM Tris-HCl, pH 8.0, and 25  $\mu$ l of 1 M Tris-HCl, pH 6.8 were added to each sample which was then mixed on a vortex mixer and the volume brought to 100  $\mu$ l with sterile water. A portion of the sample (10  $\mu$ l at 2.5 ng DNA/ $\mu$ l) was diluted 1 in 5 with TE buffer, pH 7.6 and an aliqout of 50  $\mu$ l of this solution was spotted under slight vacuum to each of three replicate nitrocelluose filters on a dot blot apparatus.

The filters were air dried for 10 min on Whatman 3 MM paper followed by baking in a vacuum oven at 80°C for 2 hr. The filters were wetted for 5 min in 5X SSC and then placed in separate seal-a-meal bags with 10 ml of hybridization solution. The filters were prehybridized for 4 hr at 42°C in a water bath. Radioactive probes  $(2-5 \times 10^6 \text{ cpm})$ were denatured by boiling for 10 min and then quickly placed on ice. One milliliter of hybridization solution was removed from the appropriate seal-a-meal bag, added to the denatured probe and then added back to the appropriate bag. Hybridization to labelled reversetranscribed mRNAs was overnight at 42°C whereas that for random-primer labelled pUCP15 insert (rat UCP cDNA) was 72 hr at 42°C.

Following hybridization the non-hybridized radioactive probe was removed from the filters as follows. All three filters were first washed four times at room temperature for 15 min in 2X SSC containing 0.1% SDS. The random primer-labelled pUCP15 insert filter was then washed four times for 15 min in 0.1X SSC containing 0.1% SDS at 30°C, whereas the reverse-transcribed mRNA labelled filters were washed four times for 15 min in 0.1X SSC containing 0.1% SDS at 50°C. The filters were dried on Whatman 3 MM paper for 15 min and then exposed to Kodak X-Omat AR5 film with Dupont lightning-plus intensifying screens for appropriate lengths of time as required at -78 °C.

#### 3.5 Random Primer Labelling of CDNA

Random primer labelling was performed according to the procedure of Feinberg and Vogelstein (1983). Insert (2.5 µl (50-200 ng) isolated by the gene clean procedure) was mixed with 5  $\mu$ l sterile H<sub>2</sub>O in a sterile Eppendorf tube and heated at 100°C for 2 min. The sample was cooled on ice, 11.4 µl labelling solution, 1.0 µl BSA solution (10 mg/ml), 4.0  $\mu$ l  $\alpha$ -[<sup>32</sup>P]dCIP (410 Ci/mmol) and 1.0  $\mu$ l (7 U) Klenow fragment added and the sample left at room temperature overnight. Serile  $H_2O$  (50 µl) was added and 1 µl of the mixture spotted on Whatman DE81 filter paper. The sample was extracted once with 20 µl phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) equilibrated in TE buffer. The aqueous phase was recovered and  $2 \ \mu$ l of calf liver tRNA (2 mg/ml) and 10 µl of 10 M ammonium acetate added. Two volumes of ice cold 95% ethanol were added and the sample left at -70°C for 30 min and the precipitate recovered by centrifugation for 30 min at 4°C. The supernatant was discarded and 50 µl of 2.5 M ammonium acetate added gently to the pellet down the side of the Eppendorf tube. The sample was heated at 37°C for 5 min, 100 µl of ice cold 95% ethanol added and the sample then placed on ice for 30 min. The pellet was recovered by centrifugation, dried in a vacufuge for 5 min and then resuspended in 100 µl TE buffer. One microliter was spotted on each of 2 DE81 filter papers.

One of the final two filters was counted directly and the other two filters were washed as follows: 6 times in  $0.5 \text{ M Na}_2\text{HPO}_4$  for 5 min, 2 times in  $H_2O$  for 2 min, and twice in 95% ethanol for 1 min. The filters were dried and then placed in scintillation vials with 3 ml toluene containing 4 g omnifluor/1 and counted.

## 3.6 Competent Cell Preparation

Competent cell preparation was according to the method of Mandel and Higa (1970) as described by Maniatis <u>et al</u>. (1982). A single colony of JM107 cells (for M13mp RF forms) or MV1193 cells (for pUC118 derived plasmids) was inoculated into 3 ml of 1X YT medium. A single colony of <u>E. coli</u> 294 cells (for pER322-derived plasmids) was inoculated into 3 ml of LB medium. These cultures were grown overnight at  $37^{\circ}$ C with vigorous shaking. The next day 100 ml of the appropriate medium was inoculated with 1.2 ml of the overnight culture for competent cell preparation and a second 100 ml of 1X YT medium was inoculated with 0.5 ml of overnight JM107 culture for lawn cell preparation. The cultures were grown at  $37^{\circ}$ C with vigorous shaking.

The cultures to be used for competent cell preparation were grown to an  $A_{500}$  of 0.4 to 0.5 and immediately placed on ice for 20 min. Twenty milliliters of the cells were then transferred to each of two chilled 30 ml Sorvall tubes and centrifuged at 2,500xg for 10 min at 4°C in an SS34 rotor. The supernatants were discarded and a half of the original volume of a chilled solution containing 50 mM CaCl<sub>2</sub> and 10 mM Tris-HCl, pH 8.0 solution added to the cell pellets which were resuspended by gentle swirling. The cells were left on ice for 30 min

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and then centrifuged at 2,500xg for 15 min at 4°C. The supernatants were removed, the cell pellets gently resuspended in 1/10 of the original volume of a chilled solution containing 50 mM CaCl<sub>2</sub> and 10 mM Tris-HCl, pH 8.0 solution and the cells left at 4°C for 1 to 24 hr before use.

# 3.7 <u>Cell Transformation</u>

The procedure followed for cell transformation was as described by Maniatis <u>et al</u>. (1982) and is based on the method of Mandel and Higa (1970). The appropriate competent cells (100-300  $\mu$ l) were placed in chilled sterile glass tubes. An appropriate amount of DNA (1-40 ng) was added directly to the cells which were then gently shaken and left on ice for 20-40 min.

# 3.8 Plating

The procedure followed for plating transformed cells was that of Mandel and Higa (1970) as described by Maniatis <u>et al</u>. (1982). 3.8.1 For JM107 (with M13 RF) or MV1193 (with pUC118)

B top agar (M13 RF) or LB top agar (pUC118) was melted and maintained at 48°C. For each 3 ml of top agar required, 50  $\mu$ l of 2% X-gal and 10  $\mu$ l 100 mM IPTG were added and in the case of MV1193 (with pUC118) 150  $\mu$ g ampicillin/ml. The transformed cells were heat shocked at 42°C for 2 min. Top agar solution (3ml) was added to the transformed cells and mixed well. In the case of M13 RF 200  $\mu$ l of the lawn cell preparation was also added and mixed well. The mixture was then poured over B bottom agar (M13) or LB bottom agar (MV1193) and the plate allowed to solidify for 30 min at room temperature. The plates were then placed in a 37°C incubator overnight.

3.8.2 For E. coli 294 with pBR322 derived plasmids

One milliter of LB medium was added to heat shocked (42°C for 2 min) transformed cells and grown at 37°C for 45 min. One hundred microliter, 200  $\mu$ l and 300  $\mu$ l aliquots were plated onto LB plates containing 10  $\mu$ g tetracycline/ml.

## 3.9 Agarose Gel Preparation

The procedure followed for 1% agarose gel preparation was as described by Maniatis <u>et al.</u> (1982). The gel was equilibrated in 1X TBE. DNA (0.1-1  $\mu$ g) was applied to the gel by adding 2  $\mu$ l of FDM to the DNA sample and bringing the volume up to 20  $\mu$ l with sterile H<sub>2</sub>O. Gels were mobilized at between 20 V (for overnight gels) to 200 V (for 1 hr gels). The gels were stained by placing them in 200 ml of water containing 20  $\mu$ g ethidium bromide/ml. Gels were visualized over ultraviolet light and results recorded by photography.

# 3.10 <u>Restriction Enzyme Digests</u>

Restriction enzyme digests were carried out according to the specifications of the manufacturer or supplier.

## 3.10.1 Small scale plasmid preparations

Digests contained 7.5  $\mu$ l (300 ng) of a small scale plasmid preparation, 1.0  $\mu$ l sterile H<sub>2</sub>O, 1.0  $\mu$ l of the appropriate 10X restriction enzyme buffer and 0.5  $\mu$ l of the restriction enzyme. The mixture was incubated at 37°C for 1 hr. One microliter of RNase A (1 mg/ml) was added (after heat inactivating any contaminating DNase by treatment for 15 min at  $100^{\circ}$ C) and the sample incubated at  $37^{\circ}$ C for 10 min.

### 3.10.2 Large scale plasmid preparations

Digests contained DNA (<1  $\mu$ g), 2.0  $\mu$ l of the appropriate 10X reaction buffer and 1.0  $\mu$ l of the restriction enzyme in a final volume of 20  $\mu$ l with sterile H<sub>2</sub>O. The mixture was incubated at 37°C for 1 to 2 hr.

#### 3.11 Northern Blot Analysis

The procedure followed for Northern blot analysis was adapted from the method of Derman <u>et al</u>. (1981). A denaturing 1% agarose gel was prepared as follows: 1.5 g of Seakem LE agarose was mixed with 15 ml of 10X gel running buffer and 108 ml sterile  $H_2O$  and the mixture was heated until the agarose dissolved. After the solution cooled to  $60^{\circ}C$ , 26.8 ml of 37% formaldehyde was added and the mixture poured as described by Maniatis <u>et al</u>. (1982). The gel was equilibrated in 1X gel running buffer.

The sample was made up of 10  $\mu$ g of RNA in 4.5  $\mu$ l sterile H<sub>2</sub>O, 2.0  $\mu$ l of 10X gel running buffer, 3.5  $\mu$ l of formaldehyde and 10.0  $\mu$ l of deionized formamide. The RNA was denatured at 55°C for 15 min, 2  $\mu$ l of FDM added and 20.0  $\mu$ l of the sample applied to the gel. The RNA was mobilized at 100 V for 3 to 4 hr. Liver total RNA was run as a control and the migration of 18S and 28S rRNAs visualized by staining in ethidium bromide.

After the electrophoresis was complete the gel was placed in a

tray of 10X SSC. The gel was set on 3 MM paper on a sponge, both of which had been soaked in 10X SSC, in a Tupperware box. Saran wrap was carefully placed around all four borders of the gel to isolate subsequent layers from the 10X SSC. Nitrocellulose cut to fit the gel was first wetted on water for 5 min, then soaked in 10X SSC for 5 min and placed over the gel. Two sheets of 10X SSC-soaked 3 MM paper were placed over the nitrocellulose followed by 5 to 10 cm of paper towels. It was important to ensure that no air bubbles were trapped. An appropriate amount of 10X SSC (200 to 300 ml) was poured into the Tupperware box and the lid closed so as to exert pressure to aid the transfer process.

After an overnight transfer of the RNA the paper towels and top two 3 MM papers were removed and the well positions marked on the nitrocellulose with a ballpoint pen. The filter was dried on 3 MM paper and then baked in a vacuum oven at 80°C for 2 hr.

The filters were prehybridized by first wetting in 5X SSC and then placed in a seal-a-meal bag with 10 ml of Northern blot hybridization solution. Prehybridization was at 42°C for 4 hr in a water bath. The probes  $(2-5 \times 10^6 \text{ cpm})$  were denatured by boiling for 10 min and then quickly placed on ice. One milliliter of hybridization solution was removed from the appropriate seal-a-meal bag, added to the probe and added back to the appropriate bag. Hybridization was overnight at 42°C. After hybridization was complete the filters were washed four times for 15 min at room temperature in a solution of 2X SSC containing 0.1% SDS and four times for 15 min at 40°C in a solution of 0.1X SSC containing 0.1% SDS. The filters were then dried on 3 MM paper and exposed to Kodak X-Omat AR5 film with Dupont lightning-plus intensifying screens for an appropriate length of time.

## 3.12 <u>Subcloning</u>

#### 3.12.1 Y11 insert into pUC118

Five micrograms (5  $\mu$ l) of a large scale plasmid preparation of Y11.28 was mixed with 2  $\mu$ l 10X high salt buffer, 12  $\mu$ l H<sub>2</sub>O and 1  $\mu$ l Pst I and the sample incubated at 37°C for 1 hr. A sample was analyzed by 1% agarose gel electrophoresis to ensure that digestion was complete. Two micrograms (2  $\mu$ l) of pUC118 was mixed with 2  $\mu$ l 10X high salt buffer, 15  $\mu$ l H<sub>2</sub>O and 1  $\mu$ l Pst I and the mixture incubated at 37°C for 1 hr. The Pst I digests were heated at 65°C for 10 min to inactivate the enzyme. The ligation of pUC118 and Y11.28 was carried out as follows: 500 ng (2  $\mu$ l) of the pUC118 Pst I digestion was mixed with 500 ng (5  $\mu$ l) of the Y11.28 Pst I digestion to give a molar ratio of insert to vector of 2:1. Two microliters of 10X ligase buffer, 10  $\mu$ l H<sub>2</sub>O and 1  $\mu$ l (0.025 U) T<sub>4</sub> DNA ligase were added and the ligation was at 4°C overnight. The ligations were stopped by adding 2  $\mu$ l of 0.2 M EDTA, pH 7.0 and one-half run on an agarose gel for analysis.

## 3.12.2 pUC118 Recombinant Insert Into M13mp18/M13mp19

The pUC118 recombinant insert was isolated via the gene clean procedure after a Pst I digestion of the recombinant was separated on a 1% agarose gel. The insert was ligated into M13mp18 or M13mp19 cut with Pst I such that the molar ratio of insert to vector was 2:1. Two microliters of 10X ligase buffer, 10  $\mu$ l H<sub>2</sub>O and 1  $\mu$ l (0.025 U) T<sub>4</sub> DNA ligase were added and the ligations were at 4°C overnight. Ligations were stopped by the addition of  $2 \ \mu$ l of 0.2 M EDTA, pH 7.0.

#### 3.13 Gene Clean Procedure

The procedure followed was as outlined in the manual supplied by the manufacturer. A Pst I digest of a large scale plasmid preparation of pUC118 recombinant was run on a 1% agarose gel for 1 hr at 200 V so that there would be complete separation of insert from vector. The gel was stained in ethidium bromide and examined over a UV light box. The agarose piece containing the insert was excised from the gel and cut into very small pieces and placed into a tared Eppendorf tube. The weight of agarose (~0.4 g) was measured by difference. Two to three volumes of NaI stock solution (1000  $\mu$ l) was added and the sample mixed on a vortex mixer. The sample was placed in a 50°C water bath for 2 min, mixed on a vortex mixer and then returned to the water bath for a further 3 min. Glassmilk suspension  $(10 \ \mu l)$  was added, the sample mixed vigorously for 1 min and then placed on ice for 5 min. The sample was centrifuged for 15 sec and the supernatant discarded. The pellet was resuspended in 1 ml of NEW solution by vigorous mixing, centrifuged for 15 sec and the supernatant discarded. This was repeated twice and then the final pellet was resuspended in 15  $\mu$ l of water or TE buffer and the sample placed in a 50°C water bath for 3 min. The sample was centrifuged for 30 sec and the supernatant was removed into a fresh tube. A portion of the isolate was run on a 1% agarose gel for analysis. The insert could then be used for random primer labelling or for further subcloning.

## 3.14 Single-Stranded DNA Preparation

Single-stranded DNA was isolated by the procedure outlined in the BRL M13 Cloning/Dideoxy Sequencing Instruction Manual.

## 3.15 Sanger Dideoxy Sequencing

Dideoxy sequencing (Sanger <u>et al.</u>, 1977) was performed as described in sequencing manual provided with the USB Sequenase kit and was based on the use of a modified  $T_7$  DNA polymerase (Tabor and Richardson, 1987). This modified enzyme has high processivity, is very fast, does not discriminate against nucleotide analogs and has no 5'-3' exonuclease activity (Tabor and Richardson, 1987). dITP was used in all sequencing reactions in order to sequence through the poly dG-dC tail, to eliminate compression and to minimize the effects of secondary structure.

# 3.16 Sequencing Gels

Sequencing gels were made according to the procedure of Davis <u>et</u> <u>al</u>. (1986). Seventy-five millilters of 8% instagel was deaerated for 5 min, 450  $\mu$ l of fresh 10% ammonium persulfate and 20  $\mu$ l TEMED were added and the solution mixed and poured immediately.

# 3.17 Radioactive Markers for Primer Extension

The procedure for the preparation of radioactive pBR322 Eco RI/ Hinf I fragment markers was as described by Davis <u>et al</u>. (1986). pBR322 (1 µg) was added to 2 µl of 5X buffer (600 mM NaCl, 60 mM MgCl<sub>2</sub>, 60 mM  $\beta$ -mercaptoethanol, 60 mM Tris-HCl, pH 7.4), 4 µl of  $\alpha$ -[<sup>32</sup>P]ATP (3000 Ci/mmol), 0.5 U of Eco RI, 0.5 U of Hinf I, and 12  $\mu$ l sterile water and the mixture was incubated at 37°C for 30 min. To this reaction was added 0.5  $\mu$ l (3 U) of Klenow fragment and incubation was continued at 37°C for a further 15 min followed by heat inactivation at 65°C for 10 min. Free nucleotides were removed by passing the sample through a spun column (Maniatis <u>et al.</u>, 1982).

# 3.18 <u>5' End-Labelling of Oligonucleotides</u>

End-labelling was performed as described by Geliebter (1987). Oligonucleotide (100 ng) was added to 100  $\mu$ Ci  $\tau$ -[<sup>32</sup>P]ATP (3000 Ci/mmol), 7.5 U T<sub>4</sub> polynucleotide kinase and 11  $\mu$ l of reaction buffer (10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM spermidine, 50 mM Tris-HCl, pH 7.4). The mixture was incubated at 37°C for 30 min followed by heat inactivation of the enzyme by incubation at 65°C for 5 min. Sterile water (7  $\mu$ l) was added to the sample to give a final concentration of labelled primer of 5 ng/ $\mu$ l.

## 3.19 Primer Extension

The procedure followed was as described by Hames and Higgins (1985). 5'-end labelled primer (14 pg) was added to 300 ng of  $poly(A)^+$  RNA, 2 µl of 5X annealing buffer (2M NaCl, 50 mM PIPES, pH 6.4) and sterile water to a final volume of 10 µl. The reaction was sealed in a 50 µl glass capillary tube or a 500 µl Eppendorf tube and annealing was for 6 hr at 53°C. The contents of the capillary or Eppendorf tube were added to 90 µl of reaction mixture and the primer extension carried out for 1 hr at 50°C. To the sample was added 10 µl of 3 M sodium acetate
and 250  $\mu$ l of ice cold 95% ethanol. The sample was placed in liquid nitrogen until frozen and then centrifuged for 5 min. The supernatant was discarded and the pellet rinsed with 200  $\mu$ l of ice cold 95% ethanol. The pellet was dried for 5 min in a vacufuge and resuspended in 10  $\mu$ l of TE buffer and 10  $\mu$ l FDM solution. The sample was denatured at 90°C for 2 min prior to loading onto an 8% sequencing gel.

#### 4 RESULTS

## 4.1 ISOLATION OF A CDNA CLONE FOR RABBIT UCP

The approach used to identify a clone for rabbit UCP cDNA was differential hybridization. A clone was expected to give positive signals with both random primer-labelled rat UCP cDNA and labelled reverse-transcribed rabbit BAT mRNA and a negative signal with labelled reverse-transcribed rabbit liver mRNA. Small scale plasmid preparations of approximately 250 colonies from the 31 day fetal rabbit BAT cDNA library were used to prepare DNA dot blots in triplicate. Each one of these dot blots was screened with one of the three radioactive probes. One clone having a high probability of containing rabbit UCP cDNA (Y11) together with a few possible positive clones were found to fit the criteria (see Fig. 7).

These possible positive clones were retested by performing a second set of DNA dot blots and again screening with the three radioactive probes. On the basis of the results of this experiment only one clone (Y11) was found to have a high probability of containing rabbit UCP cDNA as all others gave signals with the rabbit liver cDNA (see Fig. 8) and were, therefore, not specific to BAT.

<u>E.</u> <u>coli</u> 294 cells were transformed with the small scale plasmid preparation of Y11 in order to amplify the clone. Small scale plasmid preparations of 29 transformants revealed a plasmid corresponding to 6.0 kbp in 25 cases. Pst I digests of these preparations revealed

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Fig. 7. Autoradiogram of DNA dot blots of small scale plasmid preparations from 31 day fetal rabbit BAT cDNA library. DNA isolated by small scale plasmid preparations was denatured and spotted on one of three nitrocellulose filters as described in the methods. Each spot repesents approximately 25 ng of DNA. The nitrocellulose filters were prehybridized and then hybridized to one of three radioactive probes as desribed in the methods. a, Probed with random primer-labelled rat UCP cDNA; b, probed with labelled reverse-transcribed rabbit BAT mRNA; c, probed with labelled reverse-transcribed rabbit liver mRNA. -, pBR322; +, pUCP 15;  $\rightarrow$ , Y11.





Fig. 8. Autoradiogram of DNA dot blots showing retesting of possible positive clones. The legend is as in Fig. 7. In b and c rows 3 and 4 are spread out relative to rows 3 and 4 in a.

an insert of approximately 1.6 kbp. DNA dot blots of 9 of these preparations gave positive signals with random primer-labelled rat UCP cDNA and reverse-transcribed rabbit BAT mRNA and negative signals with reverse-transcribed rabbit liver mRNA as expected (see Fig. 9). Thus it appeared that a cDNA clone for rabbit BAT UCP had been detected and isolated. A plasmid containing a portion of the cDNA for rabbit cytochrome g oxidase subunit I was included as a control. This clone was originally isolated as a clone for rabbit UCP cDNA. This cDNA hybridized strongly with both random primer-labelled rat UCP cDNA and reversetranscribed rabbit BAT mRNA, as seen in Figs. 9a and 9b respectively. However, unlike the rabbit UCP cDNA, the cytochrome g oxidase subunit I cDNA gave a strong signal with reverse-transcribed rabbit liver mRNA as seen in Fig. 9c. The large signal of cytochrome g oxidase I cDNA in BAT (Fig. 9b) compared with that in liver (Fig. 9c) is due to the increased numbers of mitochondria in the latter tissue.

## 4.2 RESTRICTION ENZYME ANALYSIS OF THE RABBIT UCP CDNA

Restriction enzyme digests were performed on the rabbit UCP cDNA in order to characterize the cDNA and to determine possible sites for subcloning. Partial restriction analyses were performed on large scale plasmid preparations of two of the above colonies, Y11.16 and Y11.28. The insert in both cases was found to lack sites for Bam HI, Eco RI, Hind III, and Sal I. Pst I digestion released a 1550 bp insert. The insert was found to have one Hinc II site, dividing Y11 into fragments of 4.36 kbp, 1.1 kbp and 0.5 kbp, and two Bgl II sites which released a 300 bp fragment.



Fig. 9. Autoradiogram showing DNA dot blots of small scale plasmid preparations of clone Y11. The legend is as in Fig. 7. \*, 500 bp fragment of cDNA for rabbit cytochrome  $\underline{c}$  oxidase subunit I (the sequenced region encoded amino acids 71-149).

To further characterize the insert double restriction enzyme digests were performed. The Pst I/Hinc II digestion revealed that the Hinc II site divided the insert into fragments of approximately 1300 bp and 250 bp. The Bgl II/Pst I digestion revealed that the two Bgl II sites divided the insert into fragments of approximately 1000 bp, 300 bp and 250 bp. A Rsa I/Pst I double digestion revealed the presence of at least 3 Rsa I sites in the insert.

### 4.3 SUBCLONING OF THE RABBIT UCP CDNA INSERT

The rabbit UCP cDNA insert from a large scale plasmid preparation of Y11 was subcloned into pUC118, M13mp18 and M13mp19 to allow for isolation of single-stranded DNA that would enable the sequencing of the insert. A Pst I digestion of the large scale plasmid preparation of Y11.28 was ligated into Pst I-cut pUC118 in a molar ratio of insert to vector of 2:1. Competent MV1193 cells were transformed with this ligation and plated onto LB bottom agar in LB top agar containing X-gal, IPTG and 150 µg ampicillin/ml to select for transformants. Two colonies, pUC118.3 and pUC118.12, contained pUC118 recombinants and a 1.6 kbp insert was released upon Pst I digestion. However, there was extensive smearing suggesting the presence of a second plasmid from a mixed colony. These recombinants were replated, with pUC118.3 revealing 29 white and 18 blue colonies, while pUC118.12 gave 20 white and 2 blue colonies, thereby demonstrating the presence of mixed colonies. A small scale plasmid preparation of a pUC118 recombinant containing a 1.6 kbp insert, pUC118.3.7, was used for a large scale plasmid preparation of the pUC118 recombinant.

A Pst I digestion of the large scale plasmid preparation of the pUC118 recombinant was used for isolation of the rabbit UCP cDNA insert using the gene clean procedure after agarose gel eletrophoresis. This insert was random primer-labelled and used to probe Northern blots or for further subcloning.

The rabbit UCP cDNA insert was subcloned into Pst I-cut M13mp18 and M13mp19 to allow easier isolation of single-stranded DNA for sequencing. Competent JM107 cells were transformed with these ligations and plated onto LB bottom agar in LB top agar containing X-gal and IPIG. A number of presumptive white colonies were picked and the M13 RF forms Thirty-eight preparations of M13mp18 recombinants were isolated. revealed 7 colonies that were of the right size. Thirty-eight preparations of M13mp19 recombinants revealed 3 colonies of the right size. These 10 preparations were digested with Pst I and all revealed a 1.6 kbp insert. Clones M13mp18.15 and M13mp18.18 were not used for sequencing, however, as digestion with Pst I revealed the presence of an extra band. Single-stranded DNA was made from the remaining eight recombinants. As the three sequencing templates used, M13mp18.3, M13mp19.34 and M13mp19.36, gave the same 5' sequencing information (see Fig. 10) an attempt was made to subclone the insert again so as to get sequencing information of the opposite strand. The rabbit UCP cDNA insert was therefore subcloned into Pst I-cut M13mp19. Competent JM107 cells were transformed and plated onto LB bottom agar in LB top agar containing X-gal and IPTG. Single-stranded DNA preparations were made from 12 transformants. Four of these transformants were used in sequencing. Two of these, MP13mp19.6 and M13mp19.7 gave inserts with a



Fig. 10. pUCL18 and ML3 recombinants showing orientation of rabbit UCP cDNA insert relative to the universal priming site. These were determined by digestion of small scale plasmid preparations of recombinants with Bgl II and Hinc II. M, multiple cloning site; U, universal primer site; P, Pst I; B, Bam HI; K, Kpn I; E, E $\infty$  RI. Numbering corresponds to position of restriction sites within the insert in addition to Bgl II sites outside of the insert. Number preceding 1 refers to the size of the recombinant. AAA indicates the poly(A) tail and the 3'- end of the cDNA.

poly(A) tail upon sequencing. Two others M13mp19.1 and M13mp19.5 did not have poly(A) tails upon sequencing and did not correspond to the 5' end of the gene. They were probably dimerized vectors.

#### 4.4 SEQUENCING OF THE pUC118 RECOMBINANT

MV1193 cells containing the pUC118 recombinant were superinfected with M13K07 phage in order to produce single-stranded DNA template for sequencing. Dideoxy sequencing (Sanger <u>et al.</u>, 1977) was performed using USB's Sequenase kit which utilizes a modified  $T_7$  DNA polymerase (Tabor and Richardson, 1987). Sequencing of the pUC118 recombinant revealed a G tail of 26 bases followed by the 5' end of rabbit UCP cDNA.

### 4.5 SEQUENCING OF ML3 RECOMBINANTS

Sequencing of clones M13mp18.3, M13mp19.34 and M13mp19.36 revealed the 5' end of rabbit UCP cDNA and cloning into the Pst I site. Sequencing of clones M13mp18.2, M13mp18.4, M13mp18.9 and M13mp18.32 revealed sequences not related to rabbit UCP and analysis of the sequences of two of these, M13mp18.2 and M13mp18.9, revealed M13mp18 sequences indicating dimerization of the vector.

Sequencing of M13mp18.3 revealed a Bgl II site approximately 170 nucleotides into the sequence. Sequencing of this clone, however, revealed a G tail of only 4 bases, in contrast to the 26 base tail in the pUC118 recombinant. This was puzzling at first but it has recently been reported that M13 can delete portions of dG-dC homopolymer tails during replication (Martin, 1987).

It was decided that sequencing of one clone would be optimal and that the sequencing would be performed by walking along the gene using oligonucleotide primers determined from previous sequencing. M13mp18.3 was sequenced once over its entire length and again over the 5' 80% of the sequence using this method, as seen in Fig. 11. M13mp19.6 and M13mp19.7 were sequenced using the universal primer and four oligonucleotide primers complementary to M13mp18.3. M13mp19.6 was sequenced twice over virtually its entire length and M13mp19.7 was sequenced only using the universal primer, as shown in Fig. 11.

### 4.6 THE SEQUENCE OF RABBIT UCP CDNA

The complete nucleotide sequence of the rabbit UCP cDNA is presented in Fig. 12. It was found to be 1511 nucleotides long including a poly(A) tail of 17 nucleotides but not including a 5'-tail of 26 Gs and a 3'-tail of 18 Cs giving a total length of 1555 nucleotides for the insert. The cDNA excluding all tails has an A/T content of 51.9%. The 5'-untranslated region is 122 nucleotides long and the 3'-untranslated region is 451 nucleotides long. The poly(A) tail of 17 nucleotides was found to be 17 nucleotides downstream of the consensus polyadenylation signal of AATAAA (Proudfoot and Brownlee, 1976) at nucleotides 1347-1352. This is within the 11-30 nucleotide range found in mammalian mRNAs (Proudfoot and Brownlee, 1976). There is a second consensus polyadenylation signal at nucleotides 979-984.

There is one open reading frame with the ATG initiating codon starting at nucleotide -3 as determined by alignment with rat UCP cDNA. The open reading frame is 921 nucleotides long and encodes 306 amino

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Strategy for sequencing. Single-stranded DNA was isolated and Fig. 11. sequenced as described in the methods. M13 recombinants were sequenced using 17 nucleotide long synthetic primers determined from previous The upper scaled line represents the length of the sequencing. nucleotide sequence. The lower scaled line represents the structure of the cDNA with the thick bold line representing the coding region and the narrower lines representing untranslated regions. The extent of each sequencing reaction is indicated by the arrows with each set of sequencing reactions grouped together. Open cirles represent the use of the universal primer. Closed circles represent the use of synthetic oligonucleotide primers. The synthetic primer used for sequencing is indicated between each grouping of sequencing reactions for a particular recombinant.

nc 666 -121 -125 -120 GTG GGA GGG CG3 TTC CCG AGG TCA GAG AGA GCC CAG TGA CCA GGC AGG AAA GGG AAC TTA +61 -60 CAT CTT CGG AGA TTG CAG CCC TTA TCC TCT TGC ACC TGC CTC TTG CTC AGA GTG AAG ATG -1 -ĩ l GTG GGC ACC ACG ACC ACG GAC GTG CCC CCA ACC ATG GGG GTC AAG ATC TTC TCA GCT GGA  $_1$  val Gly Thr Thr Thr Thr Asp Val Pro Pro Thr Met Gly Val Lys Ile Phe Ser Ala Gly 60 20 120 61 GTG GCA GCC TGC CTG GCG GAC GTG ATC ACC TTT CCG CTG GAC ACC GCC AAA GTC CGG CAA 40 21 Val Ala Ala Cys Leu Ala Asp Val Ile Thr Phe Pro Leu Asp Thr Ala Lys Val Arg Gin 180 121 CAG ATE CAA GGE GAG TTE CEG ATE ACE AGE GGE ATE AGG TAE AAA GGT GTE ETG GGG ACA 60 4] Gin Ile Gin Gly Glu Phe Pro Ile Thr Ser Gly Ile Arg Tyr Lys Gly Val Leu Gly Thr 240 181 ATC ACC ACC CTG GCA AAA ACG GAA GGG CCC CTG AAA CTC TAC AGC GGG TTG CCC GCC GGC 80 of lie Thr Thr Leu Ala Lys Thr Glu Gly Pro Leu Lys Leu Tyr Ser Gly Leu Pro Ala Gly 241 CTC CAG AGA CAA ATC AGC TTC GCC TCG CTC AGG ATC GGC CTC TAC GAC ACG GTG CAG GAG 81 Leu Gin Arg Gin Ile Ser Phe Ala Ser Leu Arg Ile Giy Leu Tyr Asp Thr Val Gin Giu 300 100 301 TTC TTC ACC TCG GGG GAA GAA ACA CCG AGT TTA GGA AGC AAG ATC TCG GCC GGC CTA ACA 101 Phe Phe Thr Ser Gly Glu Glu Thr Pro Ser Leu Gly Ser Lys Ile Ser Ala Gly Leu Thr 360 120 361 ACT GGA GGC GTG GCG GTG TTC ATC GGG CAG CCC ACA GAG GTC GTG AAA GTC AGG CTG CAA 121 Thr Gly Gly Val Ala Val Phe Ile Gly Gln Pro Thr Glu Val Val Lys Val Arg Leu Gln 420 140 421 GCG CAG AGC CAC CTG CAC GGT CTC AAG CCT CGC TAC ACG GGG ACG TAC AAT GCC TAC AGG 141 Ala Gin Ser His Leu His Gly Leu Lys Pro Arg Tyr Thr Gly Thr Tyr Asn Ala Tyr Arg 480 160 481 ATT ATA GCT ACA ACT GAG AGC TTG ACC AGT CTG TGG AAA GGG ACA ACT CCT AAT CTG TTA 161 Ile Ile Ala Thr Thr Glu Ser Leu Thr Ser Leu Trp Lys Gly Thr Thr Pro Asn Leu Leu 540 1.80 600 541 AGG AAT GTC ATT ATT AAC TGT ACA GAG CTC GTA ACC TAC GAC CTA ATG AAG GGG GCC CTT 181 Arg Asn Val Ile Ile Asn Cys Thr Glu Leu Val Thr Tyr Asp Leu Met Lys Gly Ala Leu 200 601 GTG AGA AAC GAA ATA CTA GCA GAT. GAT GTT CCC TGC CAC TTA CTG TCA GCT CTT ATC GCT 660 201 Val Arg Asn Glu Ile Leu Ala Asp Asp Val Pro Cys His Leu Leu Ser Ala Leu Ile Ala 220 661 GGA TIT TGC ACA ACG CTT CTG TCC TCT CCA GTG GAT GTG GTG AAA ACC AGA TIT ATT AAC 720 221 Gly Phe Cys Thr Thr Leu Leu Ser Ser Pro Val Asp Val Val Lys Thr Arg Phe Ile Asn 240 780 721 TCT CCA CCG GGA CAA TAT GCG AGT GTG CCC AAC TGT GCA ATG ACA ATG TTC ACT AAG GAA 241 Ser Pro Pro Gly Gin Tyr Ala Ser Val Pro Asn Cys Ala Met Thr Met Phe Thr Lys Glu 260 840 781 GGA CCA ACG GCT TTT TTC AAA GGA TTT GTA CCT TCC TTC CTG CGA CTC GGA TCA TGG AAC 261 Giv Pro Thr Ala Phe Phe Lys Gly Phe Val Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn 280 841 GTC ATC ATG TTC GTG TGC TTT GAA AAG CTG AAA GGA GAA CTC ATG AGG TCA AGG CAG ACT 900 281 Val Ile Met Phe Val Cys Phe Glu Lys Leu Lys Gly Glu Leu Met Arg Ser Arg Gin Thr 300 960 901 GTG GAC TGT GCC ACA TAA TCA GCT TCA AGA AAA GGA CAC CAC ATC CCA GTG GGA ACC CCT 301 Val Asp Cys Ala Thr 305 961 GCA CCC GGG TCA TCA AGA AAT AAA ACC TTG TTC ACT TTA TTT TAC CCT AAA AAC TAA AGA 1020 1021 AAT CCC AGT AGG GAG TTT TGG ACT TTT TTT TTC AAA GGC AAA TGA AGA CCT ATT TTG TTT 1080 1081 AAG TTT TAT CCT CAG IGC CTT AGG AGB AGA AAG CCA AAC ATA CAT CTG GCA AAT GTA ACG 1140 1141 CCC AAA TAA GGA ACA GCA CTT GGT TGA CCA TTT TGG AGG TGC AAT GGT ATA ATT GAA TAT 1200 1201 GAA GAA CCT TTA TAT ATT TTA ATA TTT GAA ACT GAT GGT AGA GGA AAA CTB AGT GAA ATG 1260 1261 CAT TTT ATG AAT ACT TTA AAA TGA AGT TGT CAA AGA AAA TAT TAG TTT CTT TTT ATT 1320 1321 TAA CCA CAC TEC CAB CTA ATA TAT TCA ATA AAG TAT TCT AAT ACC CTT TAA AAA AAA AAA 1380 1386 1381 666 666

Fig. 12. Complete nucleotide sequence of rabbit UCP cDNA. Singlestranded DNA was isolated and sequenced as described in the methods. Initiation and termination codons are underlined at positions -3 to -1 and 916-918 respectively. The consensus polyadenylation signals are located at nucleotides 979-984 and 1347-1352 and are underlined. The deduced amino acid sequence is presented below the nucleotide sequence. This was determined by applying the translation function of the Microgenie program and by comparison of the nucleotide sequences of the rat and rabbit UCP cDNAs. The numbering of this diagram differs from that of Fig. 11 due to inclusion of the initiating codon in the translated region of the former. acids. The mature protein has a deduced Mr of 32,935. There are 28 basic amino acids and 21 acidic amino acids for a net positive charge of 7.

The restriction map of rabbit UCP cDNA is given in Fig. 13 and reveals the presence or absence of all previously examined restriction sites (see section 4.2). The two Bgl II sites divide the cDNA into fragment sizes of 1063, 297 and 195 bp compared with fragment sizes of approximately 1000, 300 and 250 bp from the restriction digests. The single Hinc II site divides the sequence into fragment sizes of 1314 and 241 bp compared with fragment sizes of approximately 1300 and 250 bp from the digests. There are four Rsa I sites dividing the sequence into fragments of 550, 300, 300, 250 and 100 bp.

## 4.7 NORTHERN ANALYSIS

Northern analysis was performed on total RNA from rabbit liver, 31 day fetal rabbit BAT and cold acclimated rat BAT. The blots were probed with either random primer-labelled rat UCP cDNA or random primerlabelled rabbit UCP cDNA. There was no hybridization to rabbit liver total RNA with either of the two probes, as seen in lanes 2 and 8 of Fig. 14, thus showing the specificity of UCP mRNA for BAT. Both the rat and rabbit cDNAs hybridized to a rabbit BAT mRNA of about 2050 nucleotides as seen in lanes 3 and 9. The rat cDNA hybridized rather weakly to this mRNA (lane 3) whereas the rabbit cDNA hybridized very strongly to this mRNA (lane 9).

Both the rat and rabbit cDNAs hybridized to two rat BAT mRNAs of approximately 1500 and 2000 nucleotides (lanes 5 and 11). From a



Fig. 13. Location of restriction endonuclease sites within the nucleotide sequence of rabbit UCP cDNA. The location of restriction sites was determined by using the Microgenie program on the nucleotide sequence. The presence of restriction sites as determined by digests of large scale plasmid preparations was confirmed as indicated by \*.



Fig. 14. Autoradiogram of Northern analysis of total RNA from rabbit liver, 31 day fetal rabbit BAT and cold acclimated rat BAT. Total RNA was isolated, run on a denaturing agarose gel and transferred to nitrocellulose as described in the methods. Prehybridization and hybridization to either of two radioactive probes was performed as outlined in the methods. Lanes 2 and 8, 10  $\mu$ g of rabbit liver total RNA; lanes 3 and 9, 10  $\mu$ g of 31 day fetal rabbit BAT total RNA; lanes 5 and 11, 10  $\mu$ g of cold acclimated rat BAT total RNA. Lanes 1, 6, 7 and 12 contain no sample. Lanes 1 to 6 were probed with random primerlabelled rat UCP cDNA (Ridley <u>et al</u>., 1986b). Lanes 7 to 12 were probed with random primer-labelled rabbit UCP cDNA. The migration of 18 S rRNA is indicated. Lanes 1 to 4 were exposed for three times as long as lanes 4 to 12. number of Northern blots the difference in the sizes of the two rat UCP mRNAs was determined to be 430  $\pm$  (s.d.) 75 nucleotides. The presence of two UCP mRNAs has been previously reported both in rat (Bouillaud <u>et al.</u>, 1985; Ridley <u>et al.</u>, 1986a) and mouse (Jacobsson <u>et al.</u>, 1985). It has been previously suggested that the two rat BAT UCP mRNAs may result from differential splicing or processing (Bouillaud <u>et al.</u>, 1985). In the case of rat UCP cDNA there is greater hybridization to the smaller rat BAT mRNA of 1500 nucleotides than to the longer mRNA of 2000 nucleotides (lane 5). The opposite was true with rabbit UCP cDNA (lane 11) which hybridized more strongly to the 2000 nucleotide rat BAT mRNA. This suggests that the longer 3'- sequence of rabbit UCP cDNA is hybridizing to a sequence in an mRNA of 2000 nucleotides to which the rat UCP cDNA does not hybridize or to which it hybridizes to a lower extent.

In order to test this possibility rabbit UCP cDNA was digested with Hinc II which cuts the 3'-untranslated region in half, producing fragments of 1314 and 241 nucleotides. The fragments of the digest were separated on a 1% agarose gel, isolated via the gene clean procedure, random primer-labelled and used to probe a second Northern blot of total RNA from 31 day old fetal rabbit BAT and cold acclimated rat BAT. The blots were hybridized to either of three probes: the full length rabbit UCP cDNA, the 1314 nucleotide long Pst I/Hinc II fragment of rabbit UCP cDNA (containing the 5'-untranslated region, the translated region, and the 5'- half of the 3'-untranslated region) and the 241 nucleotide long Hinc II/Pst I fragment of rabbit UCP cDNA (containing the terminal half of the 3'-untranslated region).

All three probes hybridized to a 2050 nucleotide long rabbit BAT mRNA (lanes 2, 7 and 12 of Fig. 15). The results in the case of the two rat BAT mRNAs with the three different probes provided insight into the origin of the hybridization seen with the full length rabbit CDNA (Fig. 14). As in the previous Northern blot (Fig. 14) there was hybridization to rat BAT mRNAs of 1500 and 2000 nucleotides with the complete insert (Fig. 15, lane 4) with greater hybridization to the 2000 In the case of the 1314 nucleotide long insert nucleotide long mRNA. fragment there was again hybridization to the two rat BAT mRNAs as would be expected (lane 9). Hybridization however was now greater with the 1500 nucleotide long mRNA, paralleling the results obtained with the rat cDNA which has a short 3'-untranslated region (lane 5, Fig. 14). The results with the 241 nucleotide insert fragment were not unexpected. Hybridization occurred only to the 2000 nucleotide long mRNA (Fig. 15, lane 14). It should be noted that the hybridization of the 2000 . nucleotide mRNA with the 3'- region of the rabbit UCP cDNA (lane 14) was greater than with the coding region (lane 9). This proves that the greater signal with the 2000 nucleotide long region of BAT mRNA compared to the 1500 nucleotide region is due to sequences at the 3'- end of the rabbit UCP cDNA. The nature of this mRNA species will be considered in the discussion.



Fig. 15. Autoradiogram of Northern analysis of total RNA from 31 day fetal rabbit BAT and cold acclimated rat BAT. Probes were made by random primer-labelling inserts isolated by the gene clean procedure as oulined in the methods. Lanes 2, 7 and 12, 10  $\mu$ g of 31 day fetal rabbit BAT total RNA; lanes 4, 9 and 14, 10  $\mu$ g of cold acclimated rat BAT total RNA. Lanes 1, 3, 5, 6, 8, 10, 11, 13 and 15 contain no sample. Lanes 1 to 5 were probed with random primer-labelled full length rabbit UCP cDNA insert; lanes 6 to 10 were probed with random primer-labelled large insert fragment of rabbit UCP cDNA; lanes 11 to 15 were probed with random primer-labelled small insert fragment of rabbit UCP cDNA. The migration of 18 S and 28 S rRNAs are indicated.

## 4.8 PRIMER EXTENSION

## 4.8.1 Rabbit BAT Poly(A)<sup>+</sup> RNA

Primer extension was performed on rabbit BAT  $poly(A)^+$  RNA in order to determine whether the rabbit UCP cDNA clone obtained was full length. This was carried out using an oligonucleotide corresponding to nucleotides 22-6 of the noncoding strand of rabbit UCP cDNA. This oligonunucleotide starts 147 nucleotides from the end of the rabbit UCP cDNA. A major primer extension product of approximately 235 nucleotides and a minor product of about 150 nucleotides were obtained, as seen in Fig. 16, lanes 1 and 3. The longer primer extension product indicates that the rabbit UCP mRNA extends to a point approximately 88 nucleotides 5'- to the end of the rabbit UCP cDNA reported here, giving a 5'-untranslated region of at least 210 nucleotides. The smaller primer extension product of approximately 150 nucleotides corresponds to the end of the rabbit cDNA reported here.

This indicates that our cDNA is about 90 nucleotides short of being full length. The inability to obtain a full length clone may be because the AMV reverse transcriptase fell off the template in the first strand synthesis of the cDNA due to secondary structure effects of the UCP mRNA. This is supported by at least two observations. The first is that when the primer extension reaction was performed at  $42^{\circ}$ C (not shown) primer extension products did not extend to the end of the cDNA. The second line of evidence comes from the fact that even when primer extension was performed at  $53^{\circ}$ C, which presumably reduced secondary structure over that present at  $42^{\circ}$ C, a small portion of the primer extension reaction gives a product that terminates at a point equivalent



Fig. 16. Autoradiogram of primer extension of rabbit BAT  $poly(A)^+$  RNA. Poly(A)<sup>+</sup> RNA was isolated as described in the methods. pBR322 Eco RI/Hinf I markers prepared as described in the methods are indicated. Lanes 1 and 3 represent the primer extension reaction carried out on 300 ng rabbit BAT  $poly(A)^+$  RNA using 14 pg of primer as described in the methods. The primer extension products are indicated by arrows. to the end of the cDNA. An attempt to determine the sequence of these extra 5'- 88 nucleotides using sequencing of the RNA (Geliebter, 1987) was unsuccessfull.

## 4.8.2 Rat BAT Poly(A) + RNA

Primer extension was also performed on rat BAT  $poly(A)^+$  RNA in order to determine if some of the 430  $\pm$  75 (s.d.) nucleotide difference between the two rat UCP mRNAs was at the 5'-end. Primer extension products obtained using an oligonucleotide corresponding to nucleotides 49-33 of the noncoding strand of rat UCP cDNA is seen in Fig. 17, lanes 2, 3 and 5. This oligonucleotide starts 174 nucleotides from the end of rat UCP cDNA. A major product is present at about 270 nucleotides and a minor product is present at about 420 nucleotides (see Fig. 17). The major product of 270 nucleotides indicates that the rat UCP mRNA(s) extend to a point 96 nucleotides beyond the end of the rat UCP cDNA. Thus together with the 91 nucleotides already determined at the 5'-end of rat UCP cDNA gives a 5'-untranslated region of 187 nucleotides for the rat UCP mRNA(s). This primer extension on rat BAT  $poly(A)^+$  RNA does not however enable a distinction to be made between the two rat UCP mRNAs. The origins of the 420 nucleotide long product will be considered in the discussion.

To determine the exact length of the 5'-untranslated region of each of the two rat UCP mRNAs  $poly(A)^+$  RNA was separated on a denaturing agarose gel as for Northern blotting. Bands corresponding to approximately the 1500 and 2000 nucleotide long  $poly(A)^+$  RNAs were separately excised from the gel, electroeluted as described by Maniatis



Fig. 17. Autoradiogram of primer extension of rat BAT  $poly(A)^+$  RNA· Lane 1 represents pBR322 Eco RI/Hinf I markers prepared as described in the methods. Lanes 2,3 and 5 represent the primer extension reaction carried out on 560 ng rabbit BAT  $poly(A)^+$  RNA using 32 pg of primer as described in the methods. Major primer extension products are indicated by the arrows. et al. (1982) and precipitated. A small portion of each sample was used for primer extension and sequencing (Geliebter, 1987). These proved to be unsuccessfull.

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### 5 DISCUSSION

#### 5.1 IDENITTY BETWEEN RABBIT AND RAT UCP CONAS

An alignment of the sequence of rabbit UCP cDNA with that of rat UCP cDNA (Ridley <u>et al</u>., 1986b) revealed 80.6% identity between the coding regions of the two sequences, as seen in Fig. 18. This compares with only 52.4% identity in the 5'-untranslated region and 50.4% identity in the overlapping portions of the 3'-untranslated regions, not including the rat poly(A) tail.

The deduced amino acid sequence of rabbit UCP is 306 amino acids long compared with 307 in the case of rat UCP. This one less amino acid in rabbit UCP is in addition to 41 amino acid differences between the two proteins. The amino acid differences can be accounted for by examining changes at the nucleotide level between their respective cDNAs. Eighteen differences are the result of one nucleotide change/codon, twenty are the result of two nucleotide changes/codon and three are the result of three nucleotide changes/codon. There are silent substitutions in 105 codons, 101 of which are the result of a single nucleotide change/codon and four of which are the result of two nucleotide changes/codon in conserved leucine residues.

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:	CC6 66615656366C56715CC56A961C43646A6A6C5C56A56A6C6466C466A4686 11 1 1 11 11 11 11 11 11 11 11 11 11 11	877 847	ACTANGGANGGACCAACDGCTTTTTCAAAGGATT9GTACCTDCTTCCTGCGACGTOBBA II 1111111111 I 11 1111111111 IIII IIII IIIIII
60 47	AACTIACATCTTCGOAGATTGCAGCECTTATCCTCTGCACCTGCETCTTGCTCAGAGTG	957 929	TOATGGAACSTCATCATGTTCGTGCTTTTGAAAASCTGAAASGABAACTCATGAGGTCA
120 199	AGOTOG TGGGCACCACGACCACGGAC07GCCCCCAACCATGGGGGTCAAGATCTTCTCA 111111111111111111111111111111111111	1017	ABCABACTBTBBACTGTBCCACATABTCABCTTCAABAAABBACACCACATCCCABTG INTIT INTIT I
180	CT GGAGTGGCAGCCT GCCTGGCGGACG TGATCACCTTTCCGCTGGACACCGCCAAGTC	1077	
240 209	CEGGAACABATECAAGECBAGTTCECEATCACCAECBGCATCAGETACAAAGETBTCCTE	1134 1103	MACTAAASAAATCCCASTASSSASTITTSSACTITITTTCAAASGCAAATSAASACC
300		1176	TATTTETTTANDITTATCCTCASTSCCTTASSASBAGAAGCCAAACATACATCTBBC
356	SCC69CCTCCASAGACAAATCAGCTTCSCCTC8CCTA8GATC86CCTCTACBACAC9676	1254	AMATETAACECCCAAATAAEBAACABCACTTERTTEACCATTTTEBAGGTECAATERTAT
420 389	CAGGAGTTCTTCACCTCB5G5GAAGAAACACCCG AGTTTA5GAAGCAAGATCTDBCCC 11 1111 1111 1111111111111111111111	1316	AATTBAATATBAABAACCTTTAATATTTTAATATTTBAAACTBATQBTAGABBAAAACT
477 449	SECTIONAL SECTION SECT	1376	GASTEMANTECATTITATEMATACTITAMAATEMASTTETCAAABAAAATATTASTTTCT
537 509		1434	TTTTATTTATTAACDACACTEDCASCYAATATATTCAATAAASGTATTCTAATACDCTTTA
547 569	CCTACAGGATTATAGCTACAACTGAGAGCTTGACCAGTCTBTGGAAAGGGACAACTGCT : :::::::::::::::::::::::::::::::::::	1496	*****
637 629	AATCTSTTAASGAATSTCATTATTAACTSTACAGASCTCSTAACTACSACCTAATSAAS	Hatches - Langth -	- 865 - Nignatchup - 233 - Unastchud - 378 1516 - Ratchup/Langth - 57-1 percent
717 689	BGGGCCCTTGTGAGAAACGAAATACTAGCAGATGATGATGTTCCCCTGCCACTTACTGTCAGCT 		
777 749			
837 809			

Fig. 18. Alignment of the sequence of rabbit UCP cDNA with that of rat UCP cDNA. Rabbit UCP cDNA is the upper and rat UCP cDNA (Ridley <u>et al.</u>, 1986b) is the lower sequence in each pair of lines. Identities are indicated by a colon. The coding regions are bounded by []. The initiating and terminating codons and consensus polyadenylation signals are underlined. The alignment was obtained by the alignment function of Microgenie.

### 5.2 NORTHERN ANALYSIS

## 5.2.1 There is a Single Rabbit UCP mRNA

An alignment of the rabbit and rat UCP cDNAs (see Fig. 18) indicates the presence of the consensus polyadenylation signal, AATAAA (Proudfoot and Brownlee, 1976) in approximately the same position in the two cDNAs. This corresponds to the first of two such signals in the rabbit UCP cDNA at positions 979-984, as shown in Figs. 12 and 18. In the case of the rat the use of this polyadenylation signal results in the 1500 nucleotide long mRNA (Figs. 14 and 15), whereas in the rabbit this signal is not used, as evidenced by the fact that there is only one rabbit BAT UCP mRNA of 2050 nucleotides (Figs. 14 and 15). This indicates that there must be sequences in addition to the polyadenylation signal consensus sequence for polyadenylation to occur. Sequences downstream of the AATAAA hexanucleotide have been found to be necessary for polyadenylation (Conway and Wickens, 1985; Gil and Proudfoot, 1984; McDevitt et al., 1984; Sadofsky and Alwine, 1984; Woychick et al., 1984). Among the possible downstream sequences that have been proposed are the following: YGTGTTYY (McLauchlan et al., 1985; Birnstiel et al., 1985; Gil and Proudfoot, 1984) and CATT (Gil and Proudfoot, 1984). An analysis of the rabbit UCP cDNA sequence (see Figs. 12 and 18) downstream of the first consensus polyadenylation signal does not reveal the presence of sequences similar to either of the above sequences. The absence of these additional sequences may therefore be the reason why the first rabbit polyadenylation signal is not used. It is not possible from this data to determine whether there

was loss/gain of the consensus polyadenylation signal or downstream sequences by rabbit/rat UCP mRNA.

Primer extension of rabbit BAT  $poly(A)^+$  RNA indicated that the rabbit UCP mRNA has a 5'-untranslated region of at least 210 nucleotides. This 5'-untranslated region of 210 nucleotides, together with a translated region of 921 nucleotides and a 3'-untranslated region of 451 nucleotides represents 1582 nucleotides of the rabbit UCP mRNA which by Northern blot analysis appears to be 2050 nucleotides long. This value of 2050 nucleotides together with the calculated length of the poly(A) tail of 468 nucleotides are both very approximate and should not be taken as absolute. This will be discussed in section 5.2.4.

### 5.2.2 The Two Rat UCP mRNAs May Be the Result of Differential Processing

A number of lines of evidence have indicated the presence of two UCP mRNAs of about 1500 and 2000 nucleotides in both rat and mouse. Several groups have demonstrated the hybridization of two BAT mRNAs with a rat UCP cDNA probe (Bouillaud <u>et al.</u>, 1985; Ridley <u>et al.</u>, 1986a) and with a mouse UCP cDNA probe (Jacobsson <u>et al.</u>, 1985). The two mRNAs are absent in white adipose tissue from ambient or cold-adapted mice, liver (see also Fig. 14), skeletal muscle, intestine, pancreas, spleen, testis, cerebellum and cerebral cortex (Jacobsson <u>et al.</u>, 1985). These two mRNAs have been found to increase upon exposure of rats (Ricquier <u>et al.</u>, 1983; Bouillaud <u>et al.</u>, 1984; Ricquier <u>et al.</u>, 1984; Bouillaud <u>et</u> <u>al.</u>, 1985; Ridley <u>et al.</u>, 1986a; Reichling, 1987) and mice (Jacobsson <u>et</u> <u>al.</u>, 1985) to cold. There has been no direct demonstration that both mRNAs encode UCP as they have not been separated and individually translated.

UCP is not the first protein for which multiple mRNAs have been found to exist. A number of genes have been found to contain multiple polyadenylation signals. Among these genes are the following: mouse  $\alpha$ amylase (Tosi <u>et al.</u>, 1981), mouse dihydrofolate reductase (Setzer <u>et al.</u>, 1982), yeast ADH I (Bennetzen <u>et al.</u>, 1982), chicken ovomucoid (Gerlinger <u>et al.</u>, 1982), mouse  $\beta_2$  microglobulin (Parnes <u>et al.</u>, 1983), human pro  $\alpha_2(I)$  collagen (Myers <u>et al.</u>, 1983), chicken pro  $\alpha_2$  (I) collagen (Aho <u>et al.</u>, 1983) and cytochrome <u>c</u> (Scarpulla, 1984).

It has been suggested that the two rat UCP mRNAs arise by differential processing or splicing (Bouillaud et al., 1985). If this were the case one would expect the presence of two polyadenylation signals in the rat UCP gene transcript. In addition one would expect the 3'- end of rabbit UCP cDNA to be similar to the 3'- end of the 2000 nucleotide rat UCP mRNA. While the 3'- end rabbit UCP hybridizes to a sequence within a 2000 nucleotide-long rat BAT mRNA (lane 14, Fig. 15) it is not known whether this is to the 3'- end of the 2000 nucleotide rat UCP mRNA or to a sequence within some other 2000 nucleotide rat BAT mRNA. The latter is a strong possibility because, unexpectedly, the hybridization of the 3'- end of rabbit UCP cDNA to the 2000 nucleotide long region is greater than that obtained by hybridization of the coding region. The latter hybridizes in roughly the same ratio to the 1500 and 2000 nucleotide long regions as rat UCP cDNA (Ridley et al., 1986a; Reichling et al., 1987). Since the identity of the rabbit and rat cDNAs is 52.4% and 50.4% in the comparable 5'- and 3'-untranslated regions respectively and 80.6% in the translated region, it must be concluded that if the 3'- end of rabbit UCP cDNA (nucleotides 1315-1555) is hybridizing to the 3'- end of a longer rat UCP mRNA then the identity must be greater than that in the coding region. In general it is found that the 3'-untranslated regions of genes from different species are less conserved than the translated regions (Yaffe <u>et al.</u>, 1985). There are, however, a few examples of genes in which there is high sequence identity in the 3'-untranslated regions amongst species. Among these examples are the following  $\alpha$ -crystallin (Dodemont <u>et al.</u>, 1985), brain B creatine kinase (Billadello <u>et al.</u>, 1986), procollagen  $\alpha 1(IV)$  (Myers <u>et al.</u>, 1986) and actins (Yaffe <u>et al.</u>, 1985). Yaffe <u>et al.</u> (1985) have suggested that these conserved sequences may be involved in the regulation of tissue specific expression of these mRNAs.

After the work for this thesis was completed the gene for rat UCP was isolated and sequenced (M.F. Bouillaud, personal communication). This sequence contained two polyadenylation signals which supports the theory that the two rat UCP mRNAs arise by differential processing (Bouillaud <u>et al.</u>, 1985). S<sub>1</sub> nuclease mapping of rat BAT mRNA using the 3'- end of the rat gene revealed two products that differed in length by 370 nucleotides (M. F. Bouillaud, personal communication). This value corresponds approximately to the difference of 355 nucleotides between the 3'-untranslated regions of the rat and rabbit UCP cDNAs, 96 and 451 nucleotides long respectively. This 355 nucleotide difference represents the lower limit of the 430  $\pm$  75 (s.d.) nucleotide difference between the two rat BAT mRNAs. The agreement between Bouillaud's value of 370 nucleotides and the value of 355 nucleotides determined from the

rabbit UCP cDNA suggests that the 2000 nucleotide long rat BAT mRNA is for UCP and that it has a 3'-untranslated region 355-370 nucleotides longer than that of the 1500 nucleotide long rat UCP mRNA. This of course does not prove that the 3'- end of the rabbit UCP cDNA is hybridizing to the longer rat UCP mRNA but a comparison of the 3'sequences of rabbit UCP cDNA and the rat UCP gene to see if there is high sequence identity would help to clarify this.

# 5.2.3 The 5'- Ends Of The Two Rat UCP mRNAs Appear to Be Identical

Primer extension of rat BAT poly(A)<sup>+</sup> RNA revealed a major primer extension product of 270 nucleotides indicating that an mRNA(s) extended to a point 96 nucleotides beyond the end of rat UCP cDNA giving a 5'-untranslated region of 187 nucleotides. An additional minor product at 420 nucleotides, differing by 150 nucleotides from the major product, suggested that up to 150 nucleotides of the 430  $\pm$  75 (s.d.) nucleotide difference between the two rat UCP mRNAs was at the 5'- end of the longer mRNA. While this is a possibility  $S_1$  nuclease mapping of the 5'- end of rat BAT mRNAs using the rat UCP gene has revealed a single product corresponding to a 5'-untranslated region of 183 nucleotides (M.F. Bouillaud, personal communication). This value of 183 nucleotides is approximately the same as the 187 nucleotides determined here by primer extension. Thus the 5'- ends of the two rat UCP mRNAs appear to be identical. This also suggests that the 420 nucleotide primer extension product does not reflect the true end of a rat UCP mRNA. This product could therefore be due to snapping back during primer extension or to hybridization of the primer to some other mRNA.

#### 5.2.4 One Rabbit and Two Rat UCP mRNAs

A schematic diagram of the rabbit UCP mRNA together with the two rat UCP mRNAS, as determined by sequencing, Northern blot analysis (Figs. 14 and 15) and primer extension (Figs. 16 and 17), is shown in Fig. 19. The lengths of the mRNAs as determined by Northern blot analysis are approximate and should not be considered as absolute. The



Fig. 19. A comparison of the lengths of rabbit UCP mRNA and the two rat UCP mRNAs. The sequencing of rat UCP cDNA (Ridley et al., 1986b) and rabbit UCP provide most of the information for this diagram. The additional 5'-untranslated regions were determined from primer extension of rabbit BAT poly(A)<sup>+</sup> RNA (Fig. 16) and rat BAT poly(A)<sup>+</sup> RNA (Fig. 17) and are indicated by \*. The extra 3'-untranslated region of the longer nucleotide long rat UCP mRNA was determined from Northern blot analysis (Fig. 15) and is indicated by <sup>+</sup>. The length of the mRNAs as determined by Northern analysis are approximate and the probable values as determined by the experimental results are indicated in brackets at the extreme right. Rabbit UCP mRNA is the upper, the 1850 nucleotide long rat UCP mRNA the middle, and the 1500 nucleotide long rat UCP mRNA the lower scaled line. Figures in brackets for the 5'-untranslated regions represent totals. TR, translated region; UNT, untranslated region. The lengths of the poly(A) tails were determined by the difference between mRNA sizes given above and length of determined sequences. These values are given in brackets as they are not absolute.

length of the poly(A) tails in this figure represent the difference between the length of the mRNAs as determined by Northern blot analysis and the length of sequences determined by sequencing, primer extension

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and Northern blot analysis and they should also not be taken as absolute. The average poly(A) tail for mammalian mRNAs is 260-300 nucleotides long (Brawerman, 1981). If the values for the length of the mRNAs determined from Northern blotting are taken as approximate the only difference between the two rat UCP mRNAs could be the difference of 355-370 nucleotides in the 3'-untranslated region. This would represent the lower limit of the difference between the lengths of the two mRNAs and the larger mRNA would therefore be about 1850 nucleotides long. Thus the true size of the rabbit UCP mRNA would be closer to 1900 nucleotides long giving it a poly(A) tail of 318 nucleotides that is approximately the same length as for the two rat UCP mRNAs and close to the range given above.

## 5.3 PROPERTIES OF THE THREE UCPS

A comparson of the properties and amino acid composition of the three UCPs is presented in Table 2. As previously stated rabbit UCP has one less amino acid than rat and hamster UCP. In addition the relative molecular mass of rabbit UCP is the smallest of the three proteins. Rabbit UCP also has a smaller net positive charge than either rat or hamster UCP. On the basis of these properties rabbit UCP would be expected to migrate faster than rat UCP on SDS-PAGE. Rabbit UCP, however, migrates more slowly than rat UCP on SDS-PAGE (Freeman <u>et al</u>., 1985). This is may due to rabbit UCP binding either a different amount of SDS or the rabbit UCP-SDS complex having a different conformation than the rat UCP-SDS complex. On the basis of its smaller net positive,

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i.e. greater negative charge, rabbit UCP might be expected to bind less SDS.

Table 2. A comparison of the properties and amino acid composition of the three UCPs. P, precursor; M, mature; Mr, relative molecular mass of protein (for Mr. P initiator methionine removed); acidic, number of glutamic and aspartic acid residues in mature protein; basic, number of lysine and arginine residues in mature protein; net positive, overall charge obtained by subtracting number of acidic residues from number of basic residues in mature protein; hydrophobic, number of basic residues in mature protein; hydrophobic, number of aromatic, isoleucine, leucine, methionine and valine residues in mature protein; \*, microheterogeneity; \*, based on observation that when the penultimate amino acid is valine the initiator methionine is removed (Tsunasawa et al., 1985).

PROPERTY	HAMSTER UCP	RAT UCP	RABBIT UCP
RESIDUES.P	307	307	306
RESIDUES.M	306	306	305+
Mr.P	33347	33215	33067
Mr.M	33215	33084	32935
ACIDIC	19	19	21
BASIC	28	28	28
NET POSITIVE	9	9	7
AROMATIC	26/27*	25	26
HYDROPHOBIC	106	106	106

There is conservation in the number of hydrophobic, aromatic and basic residues amongst all three UCPs. Rabbit UCP has two less acidic residues than rat and hamster UCPs with the result that it has a smaller net positive charge. The changes that account for the difference in net positive charge are presented in Table 3. Table 3. Amino acid differences that account for differences in net positive charge amongst the three UCPs. The residue refers to the position in the mature hamster and rat proteins. Lysine and argininine are counted as +1, glutamic and aspartic acid as -1 and other amino acids as neutral. Histidine is considered neutral following Aquila <u>et al</u>. (1985).

RESIDUE	HAMSTER UCP	RAT UCP	RABBIT UCP
106	lysine	arginine	glutamic acid
115	arginine	lysine	lysine
203	asparagine	asparagine	arginine
205	glutamine	histidine	glutamic acid
290	glutamine	glutamine	lysine
293	lysine	lysine	glycine
297	lysine	lysine	arginine

## 5.4 HOMOLOGY AMONGST HAMSTER, RAT AND RABBIT UCPS

An alignment of the deduced amino acid sequence of rat UCP (Ridley <u>et al.</u>, 1986b) with the amino acid sequence of hamster UCP (Aquila <u>et al.</u>, 1985) revealed 91.5 % identity between the two sequences, as shown in Fig. 20. An alignment of the deduced amino acid sequence of rabbit UCP with the hamster UCP sequence (Aquila <u>et al.</u>, 1985) revealed 85.3% identity between the two proteins as seen in Fig. 21. An alignment of the deduced amino acid sequences of rabbit and rat UCP (Ridley <u>et al.</u>, 1986b) revealed 86.3% identity between the two proteins as shown in Fig. 22. The one less amino acid within rabbit UCP corresponds to residue 110 of the mature rat protein as determined by a comparison of the cDNA sequences (Fig. 18).
RATUCP 306 residues rat uncoupling protein - rat HAMUCP 306 residues hamster uncoupling protein - hamster 70 1 10 20 30 40 50 60 VSSTISEVOPIMEVKIFSAGVSACLADITTFPLDTAKVRLOIQGEGQASSTIRYKGVLGTITTLAKTEGL VNPTTSEVHPIMGVKIFSAGVAACLADIITFPLDTAKVRLQIQGECQISSTIRYKGVLGTITTLAKTEGL 71 80 90 100 110 120 130 140 . E. . 1 . 1 ŧ PKLYSGLPAGIOROISFASLRIGLYDIVOEYFSSCRETPASLGSKISAGLMIGGVAVFIGOPTEVVKVRM PKLYSGLPAGIQRQISFASIRIGLYDIVQEYFSSGKETPPTLGNRISAGIMIGGVAVLICQPTEVVKVRL 141 190 200 210 150 160 170 180 **QAQSHLHGIKPRYTGIYNAYRVIATTESLSTLWKGITPNLMRNVIINCTELVTYDIMKGALVNHHILADD QAQSHLHGIKPRYTGTYNAYRIIATTESFSTIWKGTTPNLLRNVIINCVELVTYDLMKGALVNNQILADD** 211 220 230 240 250 260 270 280 t . . 1 . . VPCHLISALVAGFCTTLIASPVDVVKTRFINSLPGOYPSVPSCAMIMYTKEGPAAFFKGFAPSFLRLGSW VPCHLLSAFVAGFCTTFLASPADVVKTRFINSLPGQYPSVPSCAMIMLITKEGPTAFFKGFVPSFLRLASW

281 290 300 NVIMFVCFEQLKKEIMKSRQIVDCTT

Fig. 20. Alignment of the sequence of rat UCP with that of hamster UCP. Rat UCP (Ridley <u>et al</u>., 1986b) is the upper and hamster UCP (Aquila <u>et al</u>., 1985) the lower sequence in each pair of lines. Identities are indicated by a colon, similar amino acids are indicated by + and conservative changes according to Lipman and Pearson (1985) are indicated by a period. Similar changes are also conservative. Numbering corresponds to residues of the mature hamster protein. The alignment was obtained by the alignment function of PIR.

rabbit HAMUCP hamste	uncouplir 306 re r uncoupli	ng protein esidues ing proteir	- rabbit n - hamster				
1	10	20	30	40	50	60	70
VGTT	TIDVPPIMG	KIFSAGVAAC	LADVITFPLD	TAKVRQQIQC	EFPITSGIR	(KGVLGTTTTI	
VNPI	:++:.:::: ISEVHPIMG	/KIFSAGVAAC	LADIITFPLD	JIAKVRLQIQO	EGQISSTIR	KGVLGTITTI	LAKTEQL
71 _	80 1	90 '	100	110	120	130	140 '
IKLY PKLY	SGLPAGLQR ::::::+::: SGLPAGIQR	QISFASIRIGI QISFASIRIGI	YDIVQEFFTS	GEETP-SLGS :.::: +::. GKETPPTLGN	SKISAGLITIGO +::::: ::: IRISAGIMIGO	VAVFIGQPI TOTOTOTICS TOTOTOTICS TOTOTOTICS TOTOTOTICS TOTOTOTICS	EVVKVRL
141	150 '	160 '	170	180 '	190	200	210
QAQSI :::: QAQSI	HLHGLKPRYI ::::+:::: HLHGIKPRYI	IGTYNAYRII IIIIII IGTYNAYRII	ATTESLITSLWK 	GTTPNLLRNV GTTPNLLRNV	/IINCTELVTY	'DLMKGALVRN ''''''''''''''''''''''''''''''''''''	VEILADD
211	220	230	240	250 '	260	270	280
VPCH :::: VPCH	LISALIAGFO ::::.+:::: LISAFVAGFO	CTTLLSSPVDA :::::+:::: CTTFLASPADA	VKIRFINSPP :::::::: VKIRFINSLP	GQYASVPNCA CQYPSVPSCA	MIMFTKEGPI MIMLITKEGPI	'AFTKGFVPSI INFTKGFVPSI	FIRLGSW FIRLASW
201	200	200					

281 290 300

RABUCP

305 residues

Fig. 21. Alignment of the sequence of rabbit UCP with that of hamster UCP. Rabbit UCP is the upper and hamster UCP (Aquila <u>et al.</u>, 1985) the lower sequence in each pair of lines. Numbering corresponds to residues of mature hamster protein. The legend is as for Fig. 20. Inversions are boxed.

RABUCP 305 residues rabbit uncoupling protein - rabbit RATUCP 306 residues rat uncoupling protein - rat 70 60 1 10 20 30 40 50 VGITTTDVPPIMGVKIFSAGVAACIADVITFPLDIAKVRQQIQGEFPITSGIRYKGVLGITTTIAKTEGP VSSTISEVOPIMGVKIFSAGVSACLADIITFPLDTAKVRLOIOGEGOASSTIRYKGVLGTITTTAKTEGL 71 80 90 100 110 130 140 120 1 ٠ ×. ÷. . T. 1 . LKLYSGLPAGLOROISFASIRIGLYDIVOEFFTSGEETP-SLGSKISAGLITGGVAVFIGOPTEVVKVRL PKLYSGLPAGIQRQISFASIRIGLYDTVQEYFSSCRETPASIGSKISAGIMICGVAVFICQPTEVVKVRM 141 150 160 170 180 190 200 210 OAOSHIHGI KPRYTGIYNAYRI I ATTESIIISI WKGITPNLIRNVI INCTELVIYDI MKGALVRNEI LADD QAQSHLHGIKPRYTGIYNAYRVIATTESISTIWKGTTPNIMRNVIINCTEIVTYDIMKGALVNHHIIADD 211 220 240 270 280 230 250 260 . . ŧ . . VPCHLISALIAGFCITLLSSPVDVVKTRFINSPPGQYASVPNCAMIMFTKEGPTAFFKGFVPSFLRLGSW VPCHILISALVAGFCITTLLASPVDVVKTRFINSLPGQYPSVPSCAMIMYTKEGPAAFFKGFAPSFLRLGSW 281 290 300

NVIMFVCFEKLKGELMRSRQIVDCAT

Fig. 22. Alignment of the sequence of rabbit UCP with that of rat UCP. Rabbit UCP is the upper and rat UCP the lower sequence in each pair of lines. Numbers correspond to residues of mature rat protein. The legend is as in Fig. 21. A summary of the degree of homology among the three UCPs is presented below in Table 4. Of the amino acid changes between the three proteins 30-51% represent similar amino acids i.e those having common characteristics such as charge and hydrophobicity. Greater than 80% of the changes are conservative representing changes that have occured frequently during evolution (Lipman and Pearson, 1985).

Table 4. Homology amongst hamster, rat and rabbit UCPs.\*, Calculation includes amino acid differences in addition to one less amino acid in rabbit UCP. Similar refers to amino acid residues having common characteristics such as charge and hydrophobicity. Conservative refers to amino acid changes that have occured frequently during evolution (Lipman and Pearson, 1985). Conservative includes similar amino acids. Figures in brackets repesent percentage of total differences.

Comparison	Amino Acid Differences	% Identity	Similar	Conservative
Rat/ Hamster	26	91.5	8 (30)	23 (88)
Rabbit/ Hamster	44	85.3*	16 (36)	36 (82)
Rabbit/ Rat	41	86.3*	21 (51)	33 (80)

An alignment of all three UCP sequences is presented in Fig. 23. Over the length of the entire protein there are 53 positions, 17.3% of the sequence, at which amino acid substitutions occur. If the protein is divided into membranous and nonmembranous regions according to the membrane spanning model of Aquila <u>et al</u>. (1985), 149 amino acids are found outside the membrane and 157 amino acid residues are found within the membrane. Of the 53 substitutions 28 occur outside the membrane, representing a 18.8% change in these regions, and 25 occur within the membrane, representing a 15.9% change in these regions. Ten (40%) of the

C 1 Val Asn Pro Thr Thr Ser Glu Val Kis Pro Thr Met Gly Val Lys Ile Phe Ser Ala Gly Ser Ser Gly Thr Thr Asp Pro 21 Val Ala Ala Cys Leu Ala Asp Ile Ile Thr Phe Pro Leu Asp Thr Ala Lys Val Arg Leu Ser Val Cin 41 GIN ILE GIN GIY GIN GIN GIN FILE SET FOR THE ILE AND THE GIN GIY VAL LEU GIY THE ALA Phe Pro The Gly 61 Ile Thr Thr Leu Ala Lys Thr Glu Cly Leu Pro Lys Leu Tyr Ser Gly Leu Pro Ala Gly Pro Leu 81 Ile Gin Arg Gin Ile Ser Fhe Ala Ser Lau Arg Ile Gly Lau Tyr Asp Thr Val Gin Giu (C) 101 Tyr Phe Ser Ser Gly Lys Glu Thr Pro Thr Leu Gly Asn Arg Ile Ser Ala Gly Leu Arg Rhe Thr Glu — Ser Ser Lys 121 Mer Thr Gly Gly Val Ala Val Leu Ile Gly Gln Proton For Glu Val Val Lys Val Art Leu Phe Thr Phe Phe 141 Gin Als Gin Ser His Leu His Gly Ile Lys Pro Arg Tyr Thr Gly Thr Tyr Asn Als Tyr e Ile Ile Als Thr Thr Glu Ser Phe Ser Thr Lau Trp Lys Gly Thr Thr Pro Asn Lau Val () 181 Leu Ary Asn Val Ile Ile Asn Cys Val Glu Leu Val Thr Tyr Asp Leu Met Lys Gly Ala Net Thr 201 Lau Val Asn Asn Gin 11e Lau Ala Asp Asp Val Pro Cys His Lau Lau Ser Ala Phe Val His His Arg Glu Lau Ile 221 Ala Gly Fhe Cys Thr Thr Fhe Leu Ala Ser Pro Ala Asp Val Val Lys Thr Arg Fhe Ile Leu Val Leu Val 

 241 Ann Ser Leu Pro Gly Gln Tyr Pro Ser Val Pro Ser Cys Ala Het Thr Het Leu Thr (Lys Tyr

 Pro
 Ala

 Asn

 C

 261 Glu Gly Pro Thr Ala Phe Phe Lys Gly Phe Val Pro/Ser Phe Leu Ary

 Leu Ala

 Ala

 Ala

 Que Cly Comparison

 Cly Cly

 Ala

 Cly Cly

281 Asn Val Ile Met Phe Val Cys Phe Glu Gln Leu Lys Lys Glu Leu Ser Lys Ser Arg Gln Het Lys Gly Met Arg G 301 Thr Val Asp Cys Thr Thr 210

Fig. 23. Alignment of hamster, rat and rabbit UCP sequences. Hamster UCP (Aquila <u>et al</u>., 1985) is the upper, rat UCP (Ridley <u>et al</u>., 1986b) the middle and rabbit UCP the lower sequence in each set of three lines. Numbers correspond to residues of the mature hamster protein. Only amino acid differences from hamster UCP are shown. Membranous regions (Aquila <u>et al</u>., 1985) are bounded by []; the most highly conserved region is bounded by (); the proposed nucleotide binding region is bounded by {}; ---- respresents one less amino acid;  $\bigcirc$  indicates negatively charged residues and  $\oplus$  indicates positively charged residues. Positively charged regions possibly involved in targeting (Ridley <u>et al</u>., 1986b,c) are underlined. Inversions are boxed.

25 changes within the membrane result in substitution with similar amino acids. Eleven (39.3%) of the 28 changes outside the membrane result in substitution with similar amino acids.

It should be pointed out that there are four amino acid changes in rabbit UCP compared to rat and hamster UCP in the region proposed to span the membrane as a  $\beta$ -strand. The changes at residues 46 (glycine to phenylalanine) and 49 (serine to threenine) result in the conservation of hydrophobic and hydrophilic residues respectively. The first significant change occurs at position 40 which is leucine (hydrophobic, hydrophobicity value of 1.06; Eisenberg et al., 1984 a,b) in rat and hamster UCP and glutamine in rabbit UCP (hydrophilic, hydrophobicity value of -0.85). The second significant change is from a glutamine in rat and hamster UCP to a proline (hydrophobicity value of 0.12) in rabbit UCP at position 47. These changes make the region of residues 37 to 45 more hydrophilic, i.e. six hydrophilic versus three hydrophobic residues, and the presence of the proline would introduce a kink in the  $\beta$ -strand. These changes raise the possibility that this region lies outside the membrane as has been proposed in a recent model of the ADP/ATP carrier (Dalbon et al., 1988). This model was based on the binding of a photoaffinity analogs of ATP (Dalbon et al., 1988) and atractyloside (Boulay et al., 1983) and on the N-ethylmaleimide modification (Boulay et al., 1984) of the carrier.

#### 5.5 STRUCTURE/FUNCTION RELATIONSHIPS OF THE UCPS

Sequence analysis was performed on the three UCP sequences in order to determine conserved regions that may represent common functional domains. Plots were drawn showing the number of amino acid differences between each pair of proteins per 11 amino acid window over the entire length of the proteins. Points were plotted at the 6th amino acid of each window which was moved over by one amino acid each time. The plot for the hamster-rat comparison is shown in Fig. 24a, that for the hamster-rabbit comparison in Fig. 24b and that for the rat-rabbit comparison in Fig. 24c. To get a better idea of the conservation of amino acid residues amongst the three proteins the number of amino acid differences/window at each amino acid were averaged from the three comparisons and a plot of the average number of amino acid differences per 11 amino acid window generated, as shown in Fig. 25. Over the entire length of UCP there is an average of  $1.27 \pm 0.92$  (s.d) amino acid changes/11 amino acid window. Only those regions in which at least eleven consecutive amino acids are conserved in all three proteins appear as zero on this plot. An analysis of the minima reveals several regions of at least eleven consecutive amino acids that are completely conserved amongst all three UCPs, as shown in Table 5.

A closer examination of Fig. 25 and Table 5 indicates two long regions of conservation within each of the first two tripartite segments of UCP. The conservation amongst species of residues 52 to 69 within the first tripartite segment is paralleled by a conservation amongst species of residues 150 to 161 within the second tripartite segment.

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Fig. 24. Plots of amino acid differences per 11 amino acid window for each pair of UCPs. a) hamster vs. rat, b) hamster vs. rabbit, c) rat vs. rabbit.

b)

a)

C)



Fig. 25. Plot of average number of amino acid differences per 11 amino acid window for the three UCPs. The horizontal line represents the average of 1.27 differences per 11 amino acid window over the length of the entire protein with a standard deviation of  $\pm$  0.92.

Table 5. Regions of conservation amongst three UCPs and three mammalian ADP/ATP carriers. UCP amino acid residues of mature UCP that are conserved; ADP/ATP carrier (AAC/UCP), the conserved regions of the ADP/ATP carrier when aligned with UCP (Aquila <u>et al.</u>, 1987) - the number of residues in a direct comparison of UCP with AAC/UCP may not directly correspond due to insertion of gaps in either sequence to get maximal alignment; ADP/ATP carrier (mature), amino acid residues of the ADP/ATP carrier that are conserved; Basic regions, regions of high positive charge in mature UCP with no negatively charged residues (Ridley <u>et al.</u>, 1986b).

Uncoupling Protein	ADP/ATP Carrier		Basic Regions
	AAC/UCP	Mature	
10-21			
29–39	26-48	21-44	
52 <del>-</del> 69	52 <del>-</del> 68	48 <del>-</del> 64	53-66
82-100	71 <del>-</del> 100	67-96	72-91
129–139	111–145	107-145	
150-161			137-161
190-202	171-192	171–192	174-182
206-218	194-208	194-206	
279–289	265-294	267-295	268–272

These regions are homologous to one another in an alignment of the tripartite segments of hamster UCP (Aquila <u>et al.</u>, 1987). The conservation amongst species of residues 82 to 100 of the first tripartite segment is paralleled by conservation amongst species of residues 190 to 202 in the second tripartite segment. These regions are also homologous to one another in an alignment of the tripartite segments of hamster UCP (Aquila <u>et al.</u>, 1987). These could possibly represent targeting signals within each of the first two tripartite segments of UCP. It is interesting to note that these longer regions of

conservation amongst species do not appear in a comparable position in the third tripartite segment.

## 5.5.1 Targeting of UCP to Mitochondria

UCP does not contain an amino-terminal presequence as determined by sequencing of the full length cDNA clone of rat UCP but must contain a mitochondrial targeting sequence within the mature protein sequence (Ridley <u>et al.</u>, 1986b). One of several positively charged regions in the protein may be involved in targeting: residues 53 to 66, 72 to 91, 137 to 161, 174 to 182, and 268 to 272 (Ridley <u>et al.</u>, 1986b,c). This is based on the fact that positive charge is important in the targeting function of mammalian mitochondrial presequences (Horwich <u>et al.</u>, 1985; 1986; 1987; Chu <u>et al.</u>, 1987). Two targeting signals of UCP have been localized (Liu <u>et al.</u>, 1988). The first signal, located between residues 12 and 104, functions in targeting and membrane insertion, whereas the second signal, located downstream of residue 100 functions in targeting (Liu <u>et al.</u>, 1988).

The two longest regions of conservation amongst the three UCPs are residues 52 to 69 and 82 to 100 of the mature protein (see Table 5). These two regions correspond to two of several positively charged regions in the protein (Table 5) that could possibly function in targeting. The first of these regions, residues 52 to 69, is located on the matrix side of the inner mitochondrial membrane in the membrane disposition model of the hamster protein (Aquila <u>et al.</u>, 1985). This represents the region to which mitochondrial targeting signals would be expected to be localized.

Evidence for these two regions as possible targeting domains comes from our laboratory (S. Reichling, personal communication). A fusion protein consisting of amino acids 52 to 104 of rat UCP joined to the carboxyl terminal 176 amino acids of rat OCT was directed to mitochondria. This narrows down the region of one targeting signal from residues 12 to 104 obtained by Liu <u>et al</u>. (1988). This result indicates that this region may function as at least part of the intracellular targeting signal for UCP.

There is no primary consensus sequence for mitochondrial targeting sequences although it is known that positive charge (Chu et al., 1987; Horwich et al., 1987) and amphiphilicity (Roise et al., 1988) are important in their function. It has been suggested that amphiphilic  $\alpha$ -helices could be involved in presequence targeting function (von Heijne, 1986; Roise et al., 1986; Epand et al., 1986). A helical wheel diagram of residues 52 to 67 was plotted rather than plotting residues 52 to 69 because residue 68 is glutamic acid and negative charges are found infrequently in mitochondrial targeting sequences. This plot reveals a possible amphiphilic  $\alpha$ -helix (Fig. 26a). A similar plot was made for residues 72 to 92 rather than plotting residues 82 to 100 because residue 72 is the start of a positively charged region and because the change at residue 81, isoleucine to leucine, is both similar and conservative. This plot also suggests amphiphilc  $\alpha$ -helix formation (Fig. 26b). Positively charged regions that may be involved in the targeting of UCP may be conserved, however, these regions may also be involved in carrying out UCP's other functions. These functions include purine nucleotide and free fatty acid binding

and transport of H<sup>+</sup> and possibly Cl<sup>-</sup> ions.



Fig. 26. Helical wheel diagrams of two regions possibly involved in the targeting of UCP. These corresond to residues a) 52 to 69 and b) 72-92 of the mature proteins.  $\cdot$ , hydroxylated residue; +, basic residue. Numbers refer to position in mature UCP.

The hydrophobic moment and maximum hydrophobicity values were determined for these two regions according to normalized consensus hydrophobicity scale of Eisenberg <u>et al.</u>, (1984 a,b). A hydrophobic moment greater than 7.3 and a maximum hydrophobicity greater than 4.5 characterize surface seeking peptides. The sixteen residue peptide from amino acids 52 to 69 was calculated to have a hydrophobic moment of 3.4 and a maximum hydrophobicity of 5.41 over seven adjacent residues. The twenty-one residue peptide corresponding to amino acids 72 to 92 was calculated to have a hydrophobic moment of 5.87 and a maximum hydrophobicity of 5.21 over 10 adjacent residues. Thus it would appear that these two peptides do not fit the criteria for surface seeking peptides. When the hydrophobic moment and maximum hydrophobicity are

averaged over the length of each peptide it is found that the sixteen residue peptide has an average hydrophobic moment of 0.21 and an average maximum hydrophobicity of 0.08. The corresponding values for the twentyone residue peptide are 0.21 and 0.13 respectively. In a plot of hydrophobic moment versus hydrophobicity these two peptides appear in the globular protein region (eisenberg et al., 1984b). Thus again these data do not support a surface seeking peptide. However, a peptide corresponding to residues 1-27 of OCT has an average hydrophobic moment of 0.136 over residues 6-23 (Epand et al., 1986) yet can function as a targeting sequence. In addition the first 18 residues of the yeast 70 kDa protein do not fit the criteria of a surface seeking peptide (von Heijne, 1986), yet residues 1-12 of the protein target dihydrofolate reductase to mitochondria (Hurt et al., 1985b). Thus there may be subtleties associated with mitochondrial targeting sequences and the outer mitochondrial membrane such that the above criteria are not a true reflection of targeting potential.

### 5.5.2 Nucleotide Binding Domain of UCP

Both UCP and the ADP/ATP carrier are known to bind purine nucleotides and a binding site has been proposed based on a comparison of proteins that bind adenine nucleotides (Walker <u>et al.</u>, 1982; Higgins <u>et al.</u>, 1985). The nucleotide binding domain of UCP was proposed to be residues 273-293 of the mature rat protein (272-292 of the mature rabbit protein) which correspond to residues 275-297 of the bovine heart ADP/ATP carrier (Bouillaud <u>et al.</u>, 1986; Ridley <u>et al.</u>, 1986b). This region would form a hydrophobic pocket (Bouillaud <u>et al.</u>, 1986) and is

referred to as a Rossman fold (Walker <u>et al</u>., 1982). A consensus sequence has been proposed for ATP binding domains (Walker <u>et al</u>., 1982; Higgins <u>et al</u>., 1985). The consensus sequence is shown below (Fig. 27) (bold type) together with the corresponding sequences from the three UCPs and three mammalian ADP/ATP carriers. Additional residues that are conserved in at least five of the six proteins are indicated as well.

hamster UCP	S FL R LASWNVIMFVCFEQLKK	273-293
rat UCP	S FL R LGSWNVIMFVCFEQLKK	273-293
rabbit UCP	S FL R LGSWNVIMFVCFEKLKG	272-292
bovine heart AAC	SNVL REMEGAFVLVLYD EI KKFV	275-297
human fibroblast AAC	SNVL AGMGGAFVLVLYD EI KKFV	275-297
human skeletal AAC	SNVL REMGGAFVLVLYD EI KKFV	274-296

#### CONSENSUS

S--L-R---G---HHHH---E--KK

Fig. 27. Alignment of the proposed nucleotide binding sequences of three UCPs and three mammalian ADP/ATP carriers. The single letter amino acid code is used. Consensus sequence (bold type) is presented below (Walker <u>et al.</u>, 1982; Higgins <u>et al.</u>, 1985) with additional residues conserved in at least five of the six proteins indicated in regular type. H, hydrophobic residue. D, aspartic acid; E, glutamic acid; and K, lysine residues following consensus sequence are thought to play an important role in binding (Walker <u>et al.</u>, 1982).

In the membrane disposition model of UCP (Aquila <u>et al.</u>, 1985) this region is found mostly within the inner mitochondrial membrane extending outwards toward the cytosolic side of the inner membrane which correlates with the binding of nucleotides at the outer face of the inner mitochondrial membrane (Heaton <u>et al.</u>, 1978). There is some variation between nucleotide binding regions of the UCPs and the ADP/ATP carriers (Fig. 27) but this is to be expected since the ADP/ATP carrier binds only adenine nucleotides whereas UCP binds adenine and guanine nucleotides. In addition UCP binds purine nucleotides as a regulator of proton transport whereas the ADP/ATP carrier binds adenine nucleotides as a substrate for translocation. Cysteine 287 of UCP was implicated in nucleotide binding as sulfhydryl reagents inhibited GDP binding (Rial and Nicholls, 1987). Interestingly this residue is conserved in all three UCPs (Fig. 27). Arginines and lysines have also been proposed to be involved in nucleotide binding (Klingenberg, 1984; 1987). The arginine within the nucleotide binding domain is conserved in all three UCPs (Fig. 27). There are two lysines within the hydrophobic pocket and they are largely conserved in all six proteins (Fig. 27). It is interesting to note however that the positions of the lysine residues within the nucleotide binding domain of rabbit UCP differ slightly from those in the rat and hamster proteins. It would be interesting to determine whether this change in the relative positions of these two lysines in rabbit UCP affects the purine nucleotide binding characteristics of the rabbit protein.

# 5.5.3 PROTON TRANSPORT BY UCP

Cysteine residue 304, which has been implicated in proton transport (Jezek, 1987), is conserved in all three UCPs. Ion channels are proposed to span the phospholipid bilayer as amphiphilic  $\alpha$ -helices with serine, threenine or cysteine residues forming the polar face in some (Lear <u>et al.</u>, 1988). Recently it has been found that a 21 residue model peptide containing two serines per seven amino acid repeat formed proton selective ion channels (Lear <u>et al.</u>, 1988). An examination of the membrane disposition model of UCP (Fig. 5a, page 25) and the helical wheel diagram (Fig. 26a) indicate the presence of three serine residues on the same face of helix B. As Fig. 23 reveals these three serine residues are conserved in all three UCPs.

#### 5.5.4 Homology of UCP with the ADP/ATP Carrier

The sequences of the ADP/ATP carrier and UCP have been compared (Aquila et al., 1985) and there appears to be a great deal of structural similarity between the two proteins. A search of the NBRF data bank for sequences homologous to rabbit UCP was performed using the program of Lipman and Pearson (1985). Homology to the bovine heart ADP/ATP carrier was evident from this search demonstrating the tripartite nature and the conservation of this structure between the two proteins. The initial alignment of rabbit UCP with the bovine heart ADP/ATP carrier resulted in an alignment of the first tripartite segment of UCP with the second tripatite segment of the ADP/ATP carrier. From the matrix protein alignment of these two proteins (Runswick et al., 1987) these are the most identical tripartite segments between these two proteins. The degree of sequence identity and conservation of residues between rabbit UCP and five ADP/ATP carriers is presented in Table 6. The sequence data for the bovine heart and Neurospora ADP/ATP carriers were from the NBRF data bank, whereas the sequences for human fibroblast (Battini et al., 1987) and human skeletal muscle (Neckelman et al., 1987) ADP/ATP carriers were from the literature, and the yeast sequence was from Microgenie.

Table 6. Identity, similarity and conservation between rabbit UCP and five ADP/ATP carriers. % similarity, percentage of identical and similar amino acids; % conservation, percentage of identical and conservative amino acid replacements that are likely to have occured during evolution, according to Lipman and Pearson (1985) - including similar amino acids.

ADP/ATP Carrier	% Identity	<pre>% Similarity</pre>	% Conserved	
Sacchromyces	21.3	38.2	70.4	
Neurospora	18.8	N.D.	67.4	
Bovine heart	20.9	N.D.	69.1	
human fibroblast	22.6	39.0	66.8	
human skeletal	21.0	39.7	64.8	

Regions corresponding approximately to those conserved in all three UCPs are also conserved in three mammalian ADP/ATP carriers: bovine heart (Aquila et al., 1982), human fibroblast (Battini et al., 1987) and human skeletal muscle (Neckelman et al., 1987) as shown in The highest degree of conservation amongst the UCPs and Table 5. amongst the ADP/ATP carriers occurs for the two positively charged regions previously mentioned. Residues 48 to 64 are conserved in the ADP/ATP carriers and correspond to residues 52 to 69 in the UCPs. Residues 67 to 96 conserved in the ADP/ATP carriers correspond to residues 82 to 100 of the UCPs. These regions are homologous to one another in an alignment of the tripartite segments of hamster UCP and bovine heart ADP/ATP carrier (Aquila et al., 1987). This may indicate a common role for these regions, perhaps including targeting. Both these regions are within the first 115 amino acids of the protein and correlate well with the presence of a targeting signal in the yeast ADP/ATP carrier in this region (Adrian et al., 1986).

Although the two proteins are only 20% identical 68% identity was found between residues 260 to 276 of rat UCP and residues 262 to 279 of the bovine heart ADP/ATP carrier as shown in Fig. 28 (Ridley <u>et al</u>., 1986b). The most highly conserved region between the UCPs and five ADP/ATP carriers is presented below in Fig. 28.

hamster UCP	K EGPTAFFKGFVPSF LR	260-276
rat UCP	K EGPAAFFKGFAPSF LR	260-276
rabbit UCP	K EGPTAFFKGFVPSF LR	259-275
bovine heart AAC	KDEGPKAFFKG AWSNVLR	262-279
human fibroblast AAC	RDEGGKAFFKG AWSNVLA	262-279
human skeletal AAC	KDEGAKAFFKG AWSNVLR	261-278
Neurospora AAC	K EGVKSLFKG AGANILR	269-285
Sacchromyces AAC	K EGAYSLFKG CGANIFR	269 <b>-</b> 285
CONSENSUS	K-EGAFFKG-A-SIR	

Fig. 28. Alignment of the most highly conserved region amongst three UCPs and five ADP/ATP carriers. The single letter amino acid code is used. The consensus sequence is presented below and indicates the conservation of the residue in at least five of the eigth sequences.

#### 5.6 Molecular Evolution of the Uncoupling Protein

The amino acid sequence of hamster UCP (Aquila <u>et al.</u>, 1985) together with the deduced amino acid sequences of rat (Ridley <u>et al.</u>, 1986b) and rabbit UCP allows the application of principles of molecular evolution to this protein. Sequence alignments can be quantitated according to the average number of amino acid substitutions per site (Kaa) according to the formula of Kimura (1983)

 $[Kaa = -ln (1 - p_d - 1/5 p_d^2)]$ 

in which p<sub>d</sub> is the fraction of amino acid differences between the two proteins being compared.

Such a comparison was performed between hamster and rat UCP, between hamster and rabbit UCP, and between rat and rabbit UCP. Hamster and rat UCP are both 306 residues in length with 26 differences between the two proteins. The percentage sequence difference is 8.5% and the value for Kaa is 0.090. A comparison of hamster and rabbit UCP reveals 45 amino acid differences, including the one less amino acid in rabbit UCP. This represents a percentage sequence difference of 14.7% and gives a Kaa value of 0.164. A comparison between rat and rabbit UCPs reveals 42 amino acid differences, including the one less amino acid in rabbit UCPs reveals 42 amino acid differences, including the one less amino acid in rabbit UCPs reveals 42 amino acid differences, including the one less amino acid in rabbit UCPs reveals 42 amino acid differences, including the one less amino acid in value is 0.151.

In order to determine the absolute rate of evolution of a protein one must take into consideration the phylogenic relationship amongst the species of interest. The absolute rate of evolution UCP with respect to number of amino acid substitutions per site per year (kaa) is given by the relationship [ kaa = Kaa/2T ] where T is the elapsed time since the divergence of the species under consideration from a common ancestor (Doonan et al., 1986). It deduced from the fossil record that the divergence of rabbits (Order Lagomorpha, family Leporidae) (Eisenberg, 1981; Nowak and Paradiso, 1983) and rodents from their common ancestor occured approximately 80-90 million years ago (Doonan et al., 1986). Rats, mice and hamsters all belong to the Order Rodentia and superfamily Muroidea (Eisenberg, 1981; Nowak and Paradiso, 1983). This superfamily diverged into two families, Muridae, to which the rats and mice belong, and Cricetidae, to which the hamsters belong (Eisenberg, 1981). Rats and mice belong to the subfamily Murinae, whereas hamsters belong to the tribe Cricetini of the subfamily Cricetinae. If the families Muridae and Cricetidae diverged shortly after the mammalian radiation and rats diverged from mice approximately

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30 million years ago (Lanave <u>et al.</u>, 1985: Li <u>et al.</u>, 1987) it would not be unreasonable to assume the hamster-rat divergence occured 60 million years ago. The absolute rate of divergence between hamster and rat UCP is therefore  $0.75 \times 10^{-9}$  per year, that between hamster and rabbit UCPs  $0.96 \times 10^{-9}$  per year, and that between rat and rabbit UCPs  $0.89 \times 10^{-9}$ per year, giving an average value of  $0.87 \times 10^{-9}$  per year. Thus UCP has evolved at about the same rate as the average protein which has a kaa of  $0.88 \times 10^{-9}$  (Li <u>et al.</u>, 1985). The Kaa and kaa values for the three UCPs are presented in Table 7.

Table 7. Molecular evolution of UCP according to the method of Kimura (1983). Kaa, number of amino acid substitutions per site; D, divergence of species from common ancestor in millions of years; kaa, number of amino acid substitutions per site per year.

UCP	% difference	Kaa	D	kaa (x10 <sup>-9</sup> )
hamster/rat	8.5	0.090	60	0.75
hamster/rabbit	14.7	0.164	85	0.96
rat/rabbit	13.7	0.151	85	0.89
average		0.135		0.87

A similar analysis was performed on three mammalian ADP/ATP carriers: bovine heart, human fibroblast, and human skeletal muscle. The results are summarized in Table 8. The bovine/human divergence is assumed to have occurred 80 million years ago whereas that of fibroblast and skeletal muscle tissue is assumed to have occurred 275 million years ago (Neckelman <u>et al</u> ., 1987). As heart is also muscle tissue it was assumed that the heart and fibroblast divergence occured 275 million years ago.

ADP/ATP Carrier	% difference	Kaa	D	kaa (x10 <sup>-9</sup> )
bovine heart/ human fibroblast	10.4	0.113	275	0.24
bovine heart/ human skeletal	6.7	0.060	80	0.38
human fibroblast/ human skeletal	11.8	0.128	275	0.23
average				0.28

Table 8. Molecular evolution of the ADP/ATP carrier. Legend is as in Table 7.

A comparison of the absolute rate of evolution of UCP, average kaa =  $0.87 \times 10^{-9}$ , with the ADP/ATP carrier, average kaa =  $0.28 \times 10^{-9}$ , indicates that UCP is a faster evolving protein than the ADP/ATP carrier.

The application of the above criteria must be taken with caution however as there is no weighting of conservative versus nonconservative substitutions. The above values may therefore be an overestimation of divergence as many of the substitutions in both the UCPs and the ADP/ATP carriers are conservative in that replacement of amino acids are by similar amino acids. A more accurate determination of the molecular evolution of UCP can be carried out by comparing the nucleotide sequences of rat and rabbit UCP cDNAs by the method of Perler <u>et al</u>. (1980). Two types of substitutions can occur at the nucleotide level. The first type of substitution results in an amino acid change and is known as a replacement substitution. The second type of substitution results in a synonomous amino acid codon and is known as a silent substitution. Calculations of sequence divergence on the basis of replacement substitutions provides an excellent evolutionary clock as shown for preproinsulin (Perler <u>et al.</u>, 1980) and for the globins (Efstratiadis <u>et al.</u>, 1980). The accumulation of mutations is proportional to the divergence time (Perler <u>et al.</u>, 1980). The unit evolutionary period is defined as the time in millions of years required for the fixation of 1% divergence in terms of replacement substitutions between two sequences (Perler <u>et al.</u>, 1980).

The method of Perler <u>et al</u>.,(1980) was used to determine the unit evolutionary period of UCP. First the number of potential silent or replacement substitution sites in each of three categories was determined and averaged. The sequences were then compared nucleotide by nucleotide within each codon and each substitution was categorized according to the type of site in which it occured. The percentage of silent or replacement substitutions in each category was determined. The percentage change in each category was corrected for multiple substitution events and the overall divergence calculated. The results of this analysis are presented in Table 9.

The value for corrected percentage of silent substitutions for UCP, 82.6%, compares with values of 82% for the  $\alpha$ -globins and 64% for the  $\beta$ -globins (Perler <u>et al.</u>, 1980). The value for corrected percentage of replacement substitutions for UCP, 8.2%, compares with values of 11% for the  $\alpha$ -globins and 12% for the  $\beta$ - globins.

A comparison of the corrected percentage divergence of replacement substitutions with evolutionary divergence times allows a calculation of the unit evolutionary period for UCP. As rat and rabbit sequences diverged approximately 85 million years ago and the corrected percentage divergence was determined to be 8.2% for replacement substitutions the unit evolutionary for UCP is 10.4 million years. This compares with unit evolutionary periods of 10 million years for globin (Perler <u>et al.</u>, 1980), and 8 million years for carbonic anhydrase I (Fraser and Curtis, 1986).

Table 9. Molecular evolution of UCP according to the method of Perler <u>et al</u>. (1980). Silent refers to substitutions giving rise to synonomous codons. Replacement refers to substitutions giving rise to nonsynonomous codons. Category refers to number of substitutions that a site can afford. Potential sites refers to the number of nucleotides at which a particular category of substitution could occur. Average is between rat and rabbit UCP. Actual sites is the number of nucleotides at which a particular category of substitution has occured in a comparison of the two nucleotide sequences. Percentage refers to actual sites as a fraction of average potential sites. Corrected percentage is based on an arithmetic calculation that takes into account multiple substitutions.

Substitution		Silent	Replacement			
Category	1	2	3	1	2	3
potential sites rat UCP	145	18	164	18	145	594
potential sites rabbit UCP	146	18	166	18	146	588
potential sites average	145.5	18	165	18	145.5	591
actual sites	35.5	6.5	76	0.5	6.5	50
percentage	24.4	36.1	46.1	2.8	4.5	8.5
corrected %	100	77	71	8.6	6.9	9.0
average corrected %		82.6			8.2	

#### 5.7 FUIURE RESEARCH

The work carried out in this thesis leaves some questions unresolved and suggests possible further research. The first question arises out of the difference in the relative positions of the lysine residues in the nucleotide binding site of rabbit UCP as compared with rat and hamster UCP (Fig. 27). This change in the relative positions of the lysine residues might result in the nucleotide binding characteristics of rabbit UCP differing from those of rat and hamster UCP. This could be tested by isolating rabbit and rat BAT mitochondria and characterizing their binding of adenine versus guanine nucleotides, and of mono- versus di- versus tri-phosphates.

Does the threenine to alanine change at the carboxyl-terminus of rabbit UCP account for the 25% cross-reactivity of rabbit UCP with the first bleed of the anti-rat UCP antibody? An attempt was made to answer this question during the course of this thesis. Translation and immunoprecipitation were standardized for competetion of peptides, corresponding to the carboxyl-terminal ten amino acids of rat and rabbit UCP, with radiolabelled rat UCP. Unfortunately the peptides obtained were impure and the competetion studies could not be carried out. Work related to this portion of the thesis is found in the Appendix.

A further question is whether the various bleeds of the anti-rat UCP antibody react with different regions of UCP. With respect to this last question a number of constructs are available for testing the specificity of the various bleeds for specific regions of UCP. Among the constructs that should be tested are the following: UCP - as a control;  $(\Delta l-12)$  UCP - to test for the presence of amino-terminal antigenic determinants;  $(\Delta 35-104)$  UCP-OCT and either  $(\Delta 1-101)$  UCP or  $(\Delta 1-101)$  UCP-OCT to test for antigenic determinants in the second and third tripartite segments.

Finally, in order to resolve the particularly high hybridization of the 3'- end of rabbit UCP cDNA to a 2000 nucleotide rat BAT mRNA the following hybrid select translation and immunoprecipitation experiment should be performed to definitively determine whether this mRNA is for UCP. The 241 nucleotide long Hinc II/Pst I fragment of rabbit UCP cDNA, containing the extreme 3'- end of the sequence, should be bound to nitrocellulose. This would be followed by hybridization of rat BAT mRNA to the bound fragment, elution of the bound mRNA and translation of this mRNA with and without immunoprecipitation by rat UCP antibody. If UCP is precipitated as a result of this experiment then the the 3'- ends of rabbit UCP cDNA and the rat UCP gene would be homologous.

### 6 APPENDIX

#### ANALYSIS OF THE CARBOXYL-TERMINAL ANTIGENIC DETERMINANT OF UCP

6.1 Translation/Immunoprecipitation

6.1.1 <u>Materials</u>

L-[<sup>35</sup>S]methionine: NEN - 1150 Ci/mmol

Nuclease treated rabbit reticulocyte lysate: Promega

Amino acid mixture (1 mM, minus methionine): Promega

Soybean trypsin inhibitor: Cooper Biomedical - 5 mg/ml

Protein A-Sepharose CL-4B: Pharmacia

Adjusting buffer: 0.3 M NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl, pH 7.2

Striping solution: 600  $\mu$ l 10% SDS, 125  $\mu$ l stacking gel buffer, 100  $\mu$ l  $\beta$ -mercaptoethanol

Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8

Separating gel buffer: 1.5 M Tris-HCl, pH 8.8

30% acrylamide, 0.75% bis-acrylamide solution: 9 g acrylamide, 0.24 g bis-acrylamide to 30 ml

Reservoir buffer: 2 M glycine, 0.25 M Tris, pH adjusted to 8.3 with HCl Separating gel (12.5%): 3.75 ml water, 3.00 ml separating gel buffer, 5.00 ml 30% acrylamide, 0.75% bis-acrylamide solution, 120  $\mu$ l 10% SDS, 24  $\mu$ l 50% glycerol and 4  $\mu$ l TEMED (for two minigels)

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Stacking gel: 2.36 ml water, 1.00 ml stacking gel buffer, 0.76 ml 30% acrylamide, 0.75% bis-acrylamide solution, 20  $\mu$ l 10% SDS, 8  $\mu$ l 50% glycerol and 4  $\mu$ l TEMED (for two minigels)

Sample buffer: 600  $\mu$ l 10% SDS, 125  $\mu$ l stacking gel buffer, 100  $\mu$ l 50% glycerol, 100  $\mu$ l 0.1% bromophenol blue, 50  $\mu$ l  $\beta$ -mercaptoethanol Destain solution: acetic acid/ethanol/water (240:600:1560, v/v/v) Rat UCP mRNA prepared by S. Reichling by transcription of rat UCP cDNA (Ridley <u>et al</u>., 1986b) from the vector pSPT18 using T<sub>7</sub> RNA polymerase was used for the translation and immunoprecipitation experiments. Peptides: Biosearch Inc.

rat peptide: NH2-KSRQTV(ABA)TT-COOH

rabbit peptide: NH2-KSRQTV(ABA)AT-COOH

Densitometric scans were done on a GS-300 densitometer from Hoeffer Scientific, San Francisco, CA, using GS-350 Data Analysis Software.

# 6.1.2 Methods

Translation of rat UCP mRNA was carried out as described by Freeman <u>et al</u>. (1983). A reticulocyte lysate mixture was made containing 35  $\mu$ l of Promega nuclease treated-reticulocyte lysate, 1  $\mu$ l of 1 mM amino acid mixture (minus methionine) and 7  $\mu$ l H<sub>2</sub>O. A control translation with no added mRNA contained 12  $\mu$ l reticulocyte lysate mixture, 1  $\mu$ l H<sub>2</sub>O, and 2.5  $\mu$ l of L-[<sup>35</sup>S]methionine. Rat UCP mRNA (2  $\mu$ l) was heated at 70°C for 3 min quickly put on ice and to this was added 24  $\mu$ l of reticulocyte lysate mixture, and 5  $\mu$ l of L-[<sup>35</sup>S]methionine. Two 1  $\mu$ l samples of each mixture were spotted onto Whatman 3 MM paper. The samples were incubated at 30°C for 30 min and two more 1  $\mu$ l samples of each mixture were spotted on 3 MM paper and allowed to dry for 30 min. The polysomes were pelleted by centrifugation at 4°C for 15 min and the supernatant containing the radioactive UCP removed for immunoprecipitation.

The filters were washed as follows: boiling for 10 min in 5% trichloroacetic acid containing 1 mg methionine/ml; 10 min at room temperature in 5% trichloroacetic acid; 10 min at room temperature in ethanol/ether (1:1, v/v); and 10 min at room temperature in ether. The filters were dried and counted in 3 ml toluene containing 4 g omnifluor/l.

Immunoprecipitation was performed by adding 50,000 cpm of the translation to  $5 \,\mu$ l of soybean trypsin inhibitor ( $5 \,\text{mg/ml}$ ), an appropriate amount of monospecific antibody  $(0.1-20 \mu l)$ , 0-10 ug of peptide and bringing the volume to 1 ml with adjusting buffer. The sample was rotated overnight at 4°C. Twenty microliters of swelled settled protein A-Sepharose CL-4B was added and the sample rotated for 3 hr at 4°C. The samples were centrifuged briefly to precipitate the antigen-antibody-protein A-Sepharose complex, and the supernatant removed and saved. One milliter of adjusting buffer containing 10 mM methionine was added to the pellet, shaken briefly and centrifuged for This wash was repeated six more times or until 50 µl of 15 sec. supernatant from the wash added to 3 ml of ACS contained less than 100 cpm. After the washes were complete the sample was centrifuged again and final traces of the supernatant removed. The antigenantibody-protein A-Sepharose complex was dissociated by adding 100 µl of striping buffer and rotating at room temperature for 1 hr. The sample

was centrifuged for 1 min, and the supernatant transferred to a fresh Eppendorf microfuge tube. Two 10  $\mu$ l samples were counted in 3 ml ACS.

Samples of the immunoprecipitation were separated by SDS-PAGE (Laemmli, 1970) using a Bio-Rad minigel apparatus and the results visualized by fluorography (Bonner and Laskey, 1974). Briefly the separating gel solution was deserated for 5 min after which 90  $\mu$ l of 10% ammonium persulfate solution was added and the gel was poured immediately. Water was layered on top of the gel which polymerized for The stacking gel was prepared by deaerating the stacking gel 1 hr. solution for a few minutes, adding 25  $\mu$ l of 10% ammonium persulfate, and pouring immediately ensuring that the combs were in place. Polymerization was for 30 min. Samples (10 µl) from the immunoprecipitation were diluted to 20  $\mu$ l with sample buffer and loaded Electrophoresis was for 1 hr at 150 V. onto the gel. After electrophoresis was complete the gel was placed in destain solution for 30 min. The gel was washed in first wash dimethyl sulfoxide for 15 min, fresh dimethyl sulfoxide for 15 min and then in 22.5% PPO in dimethyl sulfoxide for 1.5 hr. The gel was flooded with water and then left in water for 30 min. The gel was dried for 30 min and exposed to Kodak Xomat AR5 film for 3 to 4 days.

A Vydac  $C_{18}$  analytical column was used for peptide separation. Buffer A was 0.05% TFA/H<sub>2</sub>0 and buffer B was 0.05% TFA/ CH<sub>3</sub>CN. The gradient used for separation was 5% buffer B for 3 min and 5 to 15% buffer B over the next 30 min.

# 6.1.3 Results

Radiolabelled rat UCP was immunoprecipitated with varying amounts of rat UCP antibody (A4 - terminal bleed) (Freeman et al., 1983) to obtain a standard curve which would be used to determine the appropriate amount of antibody to be used in the peptide competition studies. The immunoprecipitates were counted directly and run on SDS-PAGE followed by densitometric scanning of the autoradiogram of the gel (see Fig. 29). The results of several immunoprecipitation experiments are presented in Table 10. It was determined from the standard curves (not shown) that 2 to 5  $\mu$ l of a 1 in 10 dilution of the A4 (terminal bleed) antibody (0.2 to 0.5  $\mu$ l) would be optimal for peptide competition studies as these were in the linear portion of the curves. With low levels of antibody (0 to  $0.5 \ \mu$ ) it was found that the scintillation counts and values obtained from densitometric scanning increased directly with increasing amounts of antibody. At higher levels of antibody it was found that the values obtained by densitometric scanning began to drop off whereas the scintillation counts continued to increase. It should be noted that in Fig. 29 bands appear at the stacking gel/separating gel interface and this may account for the decrease in values obtained by scanning. These may represent aggregates of immunoprecipitated UCP that did not enter the gel. This immunoprecipitated UCP would therefore be considered in the scintillation counting but not in the densitometric scanning. Thus the scintillation counting appears to be accurate reflection of the immunoprecipitation of UCP.

Fig. 29. Autoradiogram of SDS-PAGE showing immunoprecipitation of radiolabelled rat UCP with various amounts of rat UCP antibody. Rat UCP mRNA was translated and immunoprecipitated as described in the methods. A 10 µl sample of the immunoprecipitation was run on SDS-PAGE which was followed by fluoragraphy as described in the methods. Lanes 1 and 9 represent <sup>14</sup>C-labelled molocular mass markers including chymotrypsinogen A (26 kDa), carbonic anhydrase (30 kDa), aspartate transcarbamoylase (34 kDa), ovalbumin (43 kDa) and bovine serum albumin (68 kDa). Lanes 2 and 10 represent  $^{14}C_{-}$ labelled rat UCP. Lanes 3 and 11 represent immunoprecipitation of translation of nuclease-treated reticulocyte lysate. Lanes 4 to 8 and 12 to 15 represent immunoprecipitations of translation of rat UCP mRNA. Lane 4, 0 µl Ab; lane 5, 0.1 µl Ab; lane 6, 0.2 µl Ab, lane 7, 0.5  $\mu$ l Ab; lanes 8 and 12, 1.0  $\mu$ l Ab; lane 13, 2.0  $\mu$ l Ab; lane 14, 5.0 µl Ab; lane 15 10.0 µl Ab. Ab, rat UCP antibody. This gel corresponds to immunoprecipitation experiment 3 (Table 10).



Table 10. Radioactive counts from various immunoprecipitation expreiments. Ab, amount of rat UCP antibody used. S, scintillation counts of total immunoprecipitation; D, values obtained from densitometric scanning.

Experiment	1		2		3		4
Ab (µl)	S	S	D	S	D	S	D
0	0	0	0	0	0	0	0
0.1		439	167	437	566	470	991
0.2		813	265	857	1125	760	1812
0.5		1181	475	1417	1656	2150	4030
1.0	2462	1544	768	1825	2173	3250	6109
2.0	2266	2088	571	1725	1596	4880	7147
5.0	2936			2454	1539	4180	5067
10.0	2458			2140	778	6120	4740
20.0	2037						

Peptide competition was performed with the 10 amino acid peptide corresponding to the carboxyl-terminus of rat UCP. The results of the competition experiments were inconclusive. Numerous attempts to dissolve the peptide in various solvent systems were unsuccessfull. The two peptides were extracted twice with two volumes of ethylacetate followed by lyophilization in attempt to remove possible organic contaminants. The peptides were now readily soluble in both water and adjusting buffer. Peptide competition was performed with this treated rat peptide but was still inconclusive.

An amino acid analysis was therefore performed on the two treated peptides (not shown). These analyses showed 1/3 of the expected amount of both lysine and arginine and 7/10 of the expected amount of serine for both peptides. There may therefore have been a problem with the coupling of the three amino-terminal amino acids during the peptide synthesis, as synthesis occurs from carboxyl- to amino-terminus, with the result being a mixture of peptides. It was therefore decided that the treated peptide mixture would be run on the same Vydac C18 column/ solvent system as that used in its synthesis in an attempt to separate the various peptides. The peptides were finally separated on a 5 to 15% gradient. This separation revealed that the single major peak was in fact two peaks and that there were several additional peaks. The two major peaks could not be separated further and it was not possible to determine which of the numerous peaks in fact represented the desired The peptide competition experiments could therefore not be peptide. completed. It was therefore not possible to determine if the threonine to alanine change from rat to rabbit UCP was responsible for the reduced reactivity of the rat UCP antibody with rabbit UCP (Afong et al., 1985).

## 6.1.4 Discussion

There is an antigenic determinant within the carboxyl-terminal 11 amino acids of UCP (Ridley <u>et al.</u>, 1986a). This was demonstrated by the immunological screening of a rat BAT cDNA library in <u>Escherichia</u> <u>coli</u> with a rabbit antibody preparation against rat UCP (Freeman <u>et al.</u>, 1983) which revealed five possible clones (Ridley <u>et al.</u>, 1986a) each of which encoded a minimum of the carboxyl-terminal 11 amino acids of UCP. Using a rabbit antibody preparation against rat UCP (Freeman <u>et al.</u>, 1983) it was found that there was complete cross reactivity of the

antibody preparation with rat, mouse and hamster UCP but only 25% cross reactivity with rabbit UCP (Afong et al., 1985). If the carboxylterminal antigenic determinant is the dominant determinant in the antibody preparation the complete identity of the carboxyl-terminal 11 amino acids in the rat and hamster proteins would explain the complete cross reactivity of the two proteins. This antigenic determinant may be less highly conserved among more distantly related mammalian species such as rabbit the lesser reactivity of the rabbit protein may be due to amino acid difference(s) in the carboxyl-terminal 11 residues of rat and rabbit UCP. Subsequent to this it was demonstrated that the carboxylterminal 10 amino acids of UCP are found to be on the cytosolic side of the inner mitochondrial membrane (Eckerskorn and Klingenberg, 1987). The presence of this carboxyl-terminal portion on the outer face of a protein detergent micelle correlates well with the antibody detection of a cDNA clone encoding the carboxyl-terminal 11 amino acids of rat UCP as a rat UCP-detergent micelle was used to immunize rabbits (Ridley et al., 1986a).

An analysis of the carboxyl-terminal 11 amino acids of rat and rabbit UCP (see Fig. 23, p 97) reveals two amino acid differences between the two proteins. Lysine in rat UCP is replaced by arginine in rabbit UCP at position 297. This represents a similar amino acid substitution and would likely not affect an antibody reaction. Threonine in rat UCP is replaced with alanine in rabbit UCP at position 305. This is a conservative amino acid substitution according to lipman and Pearson (1985). The threonine to alanine replacement in rabbit UCP means the loss of the hydrogen bonding hydroxyl group of threonine and
its replacement with a methyl group. This loss of a hydrogen bond could decrease the binding of rabbit UCP to the rat UCP antibody relative to the binding of rat UCP. In contrast, the change in hydrophilicity at this position is relatively minor, threonine having a hydrophilicity value of -0.400 whereas that of alanine is -0.500 (Hopp and Woods, 1981).

To test whether the threeonine to alanine change was responsible for the difference in cross-reactivity of the rat and rabbit proteins with the antibody preparation peptides corresponding to the carboxylterminal 10 amino acids of the two proteins were synthesizeed. Both peptides contained lysine at the amino-terminus as the lysine to arginine change was not expected to affect the antibody reaction. Cysteine was replaced by  $\alpha$ -aminobutyric acid in both peptides to prevent reaction of the peptides with each other to form disulphide bonded dimers. The two peptides differed only in that the rat peptide had threeonine at position 9 whereas the rabbit peptide had alanine at the same position. Unfortunately the peptides were not sufficiently pure to be used in the peptide competition experiments (see Results 6.1.3).

Consequently at this point one cannot say for certain that the threenine to alanine change is responsible for the lesser reaction of rabbit UCP with rat UCP antibody (Afong <u>et al.</u>, 1985). This can be tested by obtaining a purer peptide and trying to perform the competition experiments again. It would be wise however to first ethylacetate extract and lyophilize the sample to remove organic impurities. This would have to be followed by separation of the peptide from incorrect peptides by HPLC followed by amino acid analysis and

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sequencing. In addition it might be wise to just do the competition studies with Al (first bleed) antibody as this was the bleed that showed differential cross-reactivity. Later bleeds may not be as specific for the carboxyl-terminus.

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