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Molecular Genetics of the Mammalian NADH–Ubiquinone Oxidoreductase

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A serendipitous observation led to the first characterization of a respiration-deficient Chinese hamster mutant cell line. It has guided the design of an enrichment scheme for the isolation of additional mutant cell lines. Several complementation groups were identified with mutations affecting complex I. The X-linked *NDUFA1* gene encoding the MWFE protein represents one group. Several mutant alleles isolated independently are described that yield very low activities and demonstrate that the MWFE protein is essential for activity. A phylogenetic sequence analysis of this highly conserved protein has directed attention to species-specific differences that make the primate MWFE protein inactive in hamster cells. Based on such comparisons, mutant alleles made by site-directed mutagenesis were expressed in a null mutant and reduced complex I activities were observed, with the mutant protein assembled into the complex. These and other mutants promise to be valuable for structure–function analyses, especially in conjunction with a high-resolution structure to be expected in the future. The possibility for transgenic and knock-in mice as models for mitochondrial diseases is being explored.

KEY WORDS: Mitochondria; complex I mutants; Chinese hamster cells; nuclear genes.

INTRODUCTION

Our understanding of the molecular machinery associated with respiration and oxidative phosphorylation has reached new plateaus with the elucidation of the crystal structures of ATP synthase (complex V), cytochrome oxidase (complex IV), ubiquinone–cytochrome *c* oxidoreductase (complex III), and fumarate reductase (closely related to succinate–ubiquinone oxidoreductase/complex II). The key enzyme at the beginning of the mitochondrial electron transport chain, NADH-ubiquinone oxidoreductase (complex I), continues to be a major challenge for the future, with several as yet unanswered questions.

In prokaryotes a functionally similar complex I has 14 subunits (Yagi *et al.*, 1998), while the complex in mammalian mitochondria has at least 42 subunits. In view of the evolutionary history of mitochondria, it is no longer surprising to find 14 subunits of the mammalian enzyme to have orthologs in bacteria. Of those "core" subunits, seven are encoded by the mitochondrial genome and seven by nuclear genes. The role played by most of the 28 "accessory" proteins in the mammalian enzyme is still completely unknown (Walker, 1992). Questions that can be raised include: Are they scaffolding proteins needed for assembly, are they regulatory subunits, or are they intimately involved in electron transport or proton pumping? No crystal structure is available for guidance and only a rough topological map is available for locating some of these subunits within the overall shape and domains of the complex, as determined from electron microscopy (Guénebaut *et al.*, 1997; Videira, 1998; Finel, 1998).

In the past, genetic approaches have made significant contributions to elucidating mitochondrial biogenesis and function. First, genes defined by mutations have identified components of a functional complex. Second, powerful molecular–genetic techniques have permitted the study of complexes with modified protein subunits (obtained from site-directed mutagenesis) to elucidate structure–function relationships. Such studies have made highly informative

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contributions in prokaryotic systems as well as in yeast (*Saccharomyces cerevisiae*), and, to a limited extent, in *Neurospora*. With respect to complex I, prokaryotic systems have been useful (Takano *et al.*, 1996; Yagi *et al.*, 1993, 1998; Yano and Yagi, 1999), but the lack of an equivalent complex has eliminated *Saccharomyces cerevisiae* as a powerful model system. In this and some other yeasts, NADH oxidation is accomplished by simple enzymes associated with the outer or inner surface of the inner mitochondrial membrane. Ubiquinone is the electron acceptor, but no protons are pumped across the membrane. Complex I mutants in other eukaryotic model systems are relatively rare. A recent example is the knock out of the acyl carrier protein in *Neurospora* (Schneider *et al.*, 1995). Perhaps unexpectedly, the activity/assembly of the whole complex was severely affected in this mutant. Another system in which complex I mutants have been identified and analyzed in part are Chinese hamster fibroblasts in tissue culture (Scheffler, 1986, 1999). A brief review of their isolation and initial characterization will be followed by a more detailed description of molecular genetic studies with a null mutant in the *NDUFA1* gene encoding the MWFE protein.

ISOLATION OF COMPLEX I MUTANTS IN TISSUE CULTURE

Mutant Isolation

A detailed description of the isolation of respiration-deficient mammalian cell mutants was published some time ago (Ditta *et al.*, 1976; Scheffler, 1986) and only some key concepts will be summarized here. A serendipitous observation during attempts to isolate temperature-sensitive mutants led to the characterization of a Chinese hamster cell mutant as an auxotroph for carbon dioxide. A further investigation revealed that the Krebs cycle was completely inactive in such cells, but the problem turned out to be feedback inhibition of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase by elevated levels of NADH in mitochondria. The electron transport chain was inactive and initial experiments identified an inactive NADH–ubiquinone oxidoreductase. In the absence of Krebs cycle activity insufficient CO_2 was produced intracellularly to sustain such mutant cells in bicarbonate-free medium. A second phenotype of such mutants was their dependence on aspartate and asparagine in the medium, two amino acids normally considered to be nonessential. In wild-type fibroblasts, the biosynthesis of these amino acids occurs from glutamine/glutamate via the operation of the Krebs cycle (α-ketoglutarate to oxaloacetate, followed by transaminations). Most significantly, it was established that this mutant cell line could not grow in DME medium in which glucose was substituted by galactose (DME-Gal), because such respiration-deficient cells became dependent on a high rate of glycolysis. The Leloir pathway, required for galactose utilization, is too slow to sustain the required glycolysis rate with this carbon source (DeFrancesco *et al.*, 1976; Ditta *et al.*, 1976; Scheffler, 1974).

An understanding of the biochemical properties of the first mutant permitted the design of an enrichment procedure and an efficient screen for the isolation of additional mutants. Approximately fifty such mutant cell lines were isolated from CCL16 Chinese hamster fibroblasts and from another Chinese hamster cell line, V79. All such mutants were unable to grow in DME-Gal and all were shown to be severely respiration-deficient by polarographic measurements (Breen and Scheffler, 1979; Soderberg *et al.*, 1980).

Genetic Analysis

Pairwise fusions and the testing of the phenotype of the intraspecies cell hybrids permitted their sorting into seven complementation groups (Soderberg *et al.*, 1979). All mutations (with one curious exception, see below) were recessive, and assumed to be nuclear mutations. The latter assumption was explicitly proved in most cases, as discussed below.

The parental Chinese hamster cells were pseudodiploid cells and the observation of recessive mutations raised the issue of ploidy, potential heterozygosity, and even the possibility that epigenetic events were observed. A single mutant with a severe defect in mitochondrial protein synthesis (Burnett and Scheffler, 1981; Ditta *et al.*, 1977) could later be shown to be due to a mutation on an autosomal chromosome, where the second allele was most likely silenced by hypermethylation (Au and Scheffler, 1997). Another single mutant with a defect in complex II (and succinate dehydrogenase) was defective in the *SDHC* gene mapped on human chromosome 1 (1q21) (Elbehti-Green *et al.*, 1998; Oostveen *et al.*, 1995; Soderberg *et al.*, 1977), but it was not clear whether the hamster cells were heterozygous at this locus to start with or whether they were hemizygous for a portion of the corresponding hamster autosome.

Of considerable interest was the finding that our enrichment/isolation procedure yielded independent mutants in the same complementation groups and from two different Chinese hamster parental cell lines (Soderberg *et al.*, 1979). Since heterozygosity or hemizygosity at the same autosomal locus were less likely in that case, the

localization of some of the defective genes on the X chromosome became a possible explanation. Intraspecies cell hybrids do not lose chromosome readily and, hence, the mapping by chromosome segregation from such hybrids is more involved. Briefly, one can quite readily introduce a drug-resistance marker (thioguanine-resistance, $hprt^-$) on the X chromosome with the res^- mutation and complement these mutations with a normal X chromosome (RES^+ $HPRT^+$). As expected, such hybrid cells grow in DME-Gal and in HAT medium. A subsequent selection in thioguanine yields hybrids the majority of which have lost the entire normal X chromosome and one can demonstrate that the X-linked *HPRT* gene and the wild-type gene complementing the res^- mutation segregate together. A more conventional mapping technique at the time required interspecies hybrids and a mouse X chromosome was shown to complement the mutation in two complementation groups of hamster cells (Day and Scheffler, 1982). All attempts to make human–hamster hybrids with mutants from these complementation groups were frustrated, although hamster–human hybrids were readily obtained in which the complex II (*SDHC*) mutation was complemented by human chromosome 1 (Mascarello *et al.*, 1980). Our speculations at the time already included hypotheses about the incompatibility of a nuclear-encoded human gene product with mtDNA-encoded complex I proteins from the hamster.

Biochemical Characterization

Detailed biochemical characterizations of many of these mutant cell lines have been published (Breen and Scheffler, 1979). In addition to the inability to grow in DME–Gal, and the lack of respiration by whole cells, it was demonstrated that a variety of ^{14}C-labeled precursors could not be metabolized to $^{14}CO_2$, indicative of a severe block in the Krebs cycle. Several complementation groups were proposed to have defects in complex I, based on measurements of respiration with isolated mitochondria in the presence of different substrates and specific inhibitors. That is, rotenone-sensitive respiration driven by β-hydroxybutyrate (or malate/glutamate) was severely reduced, while succinate-driven respiration was normal or near normal (except in the sdh^- mutant and in the mutant defective in mitochondrial protein synthesis) (Breen and Scheffler, 1979).

The original mutant in this series, CCL16-B2, has since become the focus of much of our attention, as will be elaborated below. Here it is appropriate to describe an experiment that established (among other interesting conclusions), that the downstream portion of the electron transport chain from ubiquinone to oxygen was perfectly intact in these mutants, i.e., the defect in complex I was the only defect. It has been mentioned that yeast does not have a complex I, but NADH oxidation in the mitochondrial matrix is accomplished by an enzyme consisting of a single polypeptide chain encoded by the *NDI1* gene. This protein is (believed to be) attached to the inner membrane on the matrix side. The coding sequence for Ndi1p was incorporated into a mammalian expression vector. CCL16-B2 cells were transfected and stable cell lines were selected. The results were unambiguous: the wild-type phenotype of the CCL16 cells was completely restored (Seo *et al.*, 1998). Cells were able to grow in DME–Gal and respiration in permeabilized cells driven by malate/glutamate was restored. As expected from the properties of the yeast enzyme, this activity was not sensitive to rotenone, but instead was sensitive to flavone. The yeast enzyme was imported into mammalian mitochondria, as demonstrated by immunocytochemical methods. Ndi1p has a noncovalently bound FAD and no iron–sulfur centers, but can transfer electrons from NADH to ubiquinone in bacteria as well as in mammalian mitochondria (Kitajima-Ihara and Yagi, 1998; Seo *et al.*, 1998).

Mutants with Defective *NDUFA1* Genes

In the period from 1975–1990, the number of subunits in the mammalian complex I almost doubled, with a final number at 42 or 43. The subunits were characterized after electrophoretic fractionation, starting with purified bovine complex I. Sufficient quantities were isolated to permit N-terminal sequencing and the design of degenerate probes to isolate the corresponding cDNAs from bovine cDNA libraries (Fearnley and Walker, 1992; Pilkington *et al.*, 1993; Walker, 1992; Walker *et al.*, 1992, 1995). At the same time, interest in human mitochondrial diseases exploded. Although much excitement was derived from the discovery of mitochondrial DNA mutations, the possibility of nuclear mutations causing partial defects in complex I also had to be considered (Loeffen *et al.*, 1998, 1999; Triepels *et al.*, 1998; Van den Heuvel *et al.*, 1998). Thus, there was a motivation to clone the corresponding human cDNAs, and, at the same time, their mapping on human chromosomes became a goal.

The human *NDUFA1* gene encoding the MWFE protein was cloned and mapped by Zhuchenko and colleagues a few years ago (Zhuchenko *et al.*, 1996). It was (and still is) the only known X-linked gene encoding a complex I subunit. An obvious experiment was to test the wild-type *NDUFA1* cDNA in our CCL16-B2 hamster mutant cells for its ability to complement the mutated hamster gene. The previous experience with a human X chromosome

made it advisable to clone the corresponding hamster cDNA for a definitive test and, indeed, it was clearly shown that the normal hamster *NDUFA1* cDNA could rescue the CCL16-B2 cells, while the human *NDUFA1* cDNA was inactive (Au *et al.*, 1999). The mouse *NDUFA1* cDNA restored activity at a reduced level. The result proves that the MWFE protein is absolutely essential for complex I activity.

The MWFE protein is one of several very small subunits (with a total of 70 amino acid residues) of the mammalian complex I belonging to the group of ~28 "accessory" proteins in search of a function. In the CCL16-B2 mutant, the *NDUFA1* mRNA has a 65 nt internal deletion, creating a premature stop codon and, if translated, it would yield a mutant peptide with 13 amino acids from the N-terminus, followed by 16 amino acids from a different reading frame. It is effectively a null mutation. If the genomic structure of the gene in the hamster is similar to that in humans, the deletion is in exon I.

Is complex I assembled in the absence of MWFE subunits? An immunoblotting analysis reveals clearly that eight of the peripheral membrane proteins of complex I (FP 51K, FP 24K, IP 75K, IP49K, IP 30K, IP13K, TYKY, PSST) were associated with mitochondrial membranes in the CCL16-B2 mutant cells at normal or near normal levels (Au *et al.*, 1999). Thus, no gross deficiency in assembly of the complex can be observed. A partial reduction in the intensity of some signals on the Western blot (FP 51K and IP75 K) could not account for the complete loss of activity and may be within experimental error.

The hamster *NDUFA1* cDNA was also cloned from other complex I mutants in our collection by an RT-PCR-based protocol. The mutants V79-G20 and V79-G14 yielded sequences with alterations compared to the wild-type sequence, confirming the assignment of these mutants to the same complementation group. In the V79-G20 mutant the only change was a R50K substitution. Such a conservative change would not necessarily have been expected to be so detrimental and, hence, we verified the existence of the mutation in several independently isolated PCR clones. The same mutation was also introduced into the wild-type cDNA by site-directed mutagenesis with the same result. Thus, the highly conserved arginine at position 50 (see below) must play a crucial role. The precise nature of its function will have to await the completion of a crystal structure of this complex. In the V79-G14 mutant, a small insertion in exon III leads to the formation of a MWFE protein that is truncated (66 amino acids, with the last two being different from the original sequence).

Significantly, the *NDUFA1* cDNA cloned from the V79-G4 mutant had the wild-type coding sequence, confirming that this mutant belonged to a different complementation group. This mutant also could not be complemented by wild-type *NDUFA1* cDNA. It is noteworthy, however, that the V79-G4 mutation was also mapped on the X chromosome, while no other X-linked gene for complex I has, thus far, been identified. A possible explanation is that a specific assembly factor required for complex I formation is encoded on the X chromosome. The corresponding protein may be absent from the final, functional complex.

While the V79-G20 mutant can be complemented with the wild-type *NDUFA1* cDNA as expected from the sequencing analysis, we continue to be puzzled by our earlier observations that in somatic cell hybrids between the V79-G4 mutant and the V79-G20 mutant respiration is not restored (Soderberg *et al.*, 1979). On the other hand, the V79-G20 mutation does not behave as a dominant negative mutation in other intraspecies hybrids.

The MWFE protein is a very small protein with only 70 amino acids. The bovine, mouse, and human cDNA sequences had been published at the time when the hamster clone was characterized. Using the same strategy and the same oligonucleotide primers, we cloned and sequenced the cDNAs from the rat (another rodent) and from chimpanzee, gorilla, and lemur (primates). Sequences from *Xenopus laevis* and a plant (*Oryza*, rice) have recently been added to the data base. It is clear that this protein is highly conserved in evolution, with a precise length of 70 amino acids in each species (Fig. 1). Twenty five amino acids at the N-terminus stand out as a highly hydrophobic domain, with the exception of the conserved glutamate at position 4. The remaining domain of 45 amino acids is quite hydrophilic, with twelve positive and five negatively charged side chains (in mammals). The protein is synthesized and imported into mitochondria without further proteolytic processing (Walker, 1992). One can predict that the N-terminus acts as a transmembrane anchor and it also appears that this sequence is sufficient for targeting the protein to mitochondria for import (see below). The significance of the conserved glutamate at position 4 was investigated by replacing it with alanine or serine by site-directed mutagenesis. Either of these proteins was capable of restoring complex I activity in the CCL16-B2 null mutant (E. N. Smith, 2000).

The MWFE protein was found in the integral membrane subcomplex of complex I in the fractionation scheme of Walker *et al.* (1995), making it plausible that it also interacts with at least one of the integral membrane proteins encoded by mtDNA. Another problem is to determine its orientation in the membrane. An initial approach to this problem was to make a chimeric protein with a green fluorescent protein (GFP) attached via a short linker

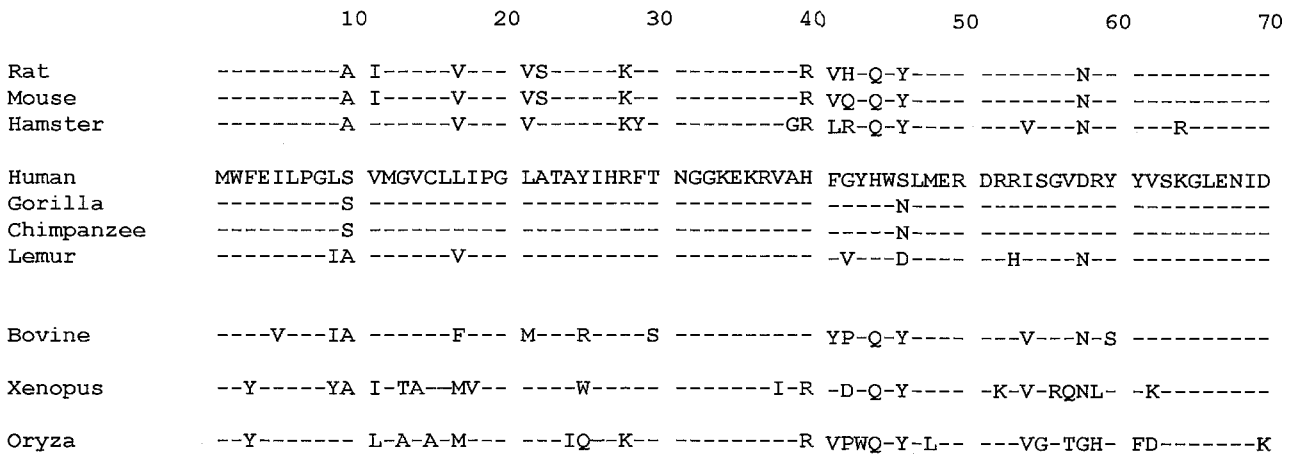

Fig. 1. Amino acid sequence comparison for the MWFE protein.

to the C-terminus of the MWFE protein. Import into mitochondria was achieved (unpublished), but this chimeric protein was unable to complement the null mutation in the CCL16-B2 cells. Steric interference with the assembly of a functional complex is a likely explanation. The orientation of the MWFE-GFP protein in the mitochondrial inner membrane was investigated in mitoplasts by measuring its suceptibility to protease digestion. A preliminary experiment suggested that the GFP (C-terminus) was on the outside facing the intermembrane space (results not shown). Another chimeric protein with the hydrophobic N-terminal region of MWFE fused to GFP was also imported into mitochondria, confirming the role of the N-terminal domain in targeting and as a membrane anchor. A second approach was to use a significantly smaller epitope tag at the C-terminus. With a monomeric HA tag (12 amino acids) added, the resulting protein was capable of restoring complex I activity in the CCL16-B2 mutant (Yadava *et al.*, in preparation). Experiments aimed at determining the orientation of the HA-tagged protein are in progress.

COMPLEX I ASSEMBLY AND STABILITY OF THE MWFE PROTEIN

A polyclonal antiserum against the MWFE protein was made in rabbits by immunizing with two peptides of 12 amino acids (positions 29–40 and 59–70). The affinity-purified antiserum produced a clear signal on Western blots with proteins from whole cell lysates of wild-type cells and, subsequently, several other res$^-$ mutants in our collection were tested. The results can be summarized in the following small table, where a plus sign indicates a normal signal, and a minus sign indicates the total absence of a signal (Table I).

The absence of a signal in the CCL16-B2 mutant is consistent with the null mutation and the signal is clearly restored in the mutant complemented with wild- type *NDUFA1* cDNA. The V79-G4 mutant has the MWFE protein, as expected from the molecular-genetic analysis. The absence of a signal from the V79-G7 cells is interesting, but perhaps not surprising. There is no mitochondrial protein synthesis in such mutants (Au and Scheffler, 1997) and, hence, no synthesis of the complex I subunits ND1, ND2, ND3, ND4, ND4L, ND5, and ND6. It is very likely that complex I cannot be assembled on the membrane and, although the MWFE protein is made and imported, it is highly unstable and, therefore, not accumulated in the mitochondria. Therefore, *excess* MWFE proteins in transfected cells capable of assembling complex I are also likely to be quickly removed by proteolysis. This will become relevant in a discussion to follow. The complete absence of signals in V79-G20 and V79-G14 cells may have two different causes. In the V79-G14 mutant there is a small terminal deletion in the protein and two different amino

Table I. Presence or Absence of the MWFE Protein in Various Mutant Cell Lines

CCL16 wild type	CCL16-B2	V79-G20	V79-G14	V79-G4	V79-G7	CCL16-B2 + wt cDNA
+	−	−	−	+	−	+

acids are substituted at the new C-terminus. We speculate that the C-terminal peptide of the MWFE protein was the dominant antigenic determinant in eliciting the immune response in the rabbit and thus the antigenic determinant in the V79-G14 protein is destroyed. Consistent with this idea is the observation that in the presence of the HA tag at the C-terminus, the anti-MWFE antiserum no longer reacts with the protein on a Western blot. If only the terminal peptide was strongly antigenic, then the absence of a signal in the V79-G20 mutant suggests that the protein with the R50K mutation is not assembled into complex I. *A priori* this would have been impossible to predict, but the significance of the arginine at this position is emphasized by the conservation of this amino acid at this position in all species examined so far, including plants (rice) and humans. Experiments with the R50K mutation in a chimeric, HA-tagged protein may answer this question.

Interspecies Complementation (Nuclear–Mitochondrial Interactions)

Observations with interspecies hybrids and cybrids (xenomitochondrial cybrids) have been interpreted from the earliest available results to indicate a certain level of incompatibility between nuclear gene products and mitochondrial gene products, when the phylogenetic separation between species was beyond a certain range. Thus, we had interpreted the failure of the human X chromosome to complement the CCL16-B2 mutant in terms of an incompatibility of the human nuclear-encoded protein with mtDNA-encoded proteins of the hamster. Other examples for such incompatibility include the xenomitochondrial cybrids between humans and more distant primates and, more recently, in mouse–rat cybrids (Barrientos *et al.*, 1998; Dey *et al.*, 2000).

The failure of the human MWFE protein to function in the CCL16-B2 hamster mutant must be explained in relation to amino acid sequence differences between the two species (Fig. 1). Despite a high degree of sequence conservation, there is a striking divergence in the segment including amino acids 40–45. Most evident is the increased spacing of two positively charged side chains in the human and all primate proteins. In the mouse protein, one of the charged side chains is missing, although it is present in the rat. The only other position with a significant difference is at position 58 with a negatively charged aspartate replaced by asparagine in rodents.

To test the significance of these differences, a series of mutant MWFE proteins were expressed from cDNAs modified by site-directed mutagenesis (Fig. 2). In the "A" series, changes were made in the hamster protein, changing one or two amino acids to those found in the human protein. In U41/42, two amino acids in the human protein were replaced with those found in the hamster protein. When the CCL16-B2 mutant cells were complemented with these altered proteins, partial activity in complex I was restored to a level that depended on the mutation. An initial test involved growth in DME–Gal. The two double mutants A41/42 and U41/42 exhibited the slowest growth rate (25–30% of wild type), the single mutation (A42) caused a ~50% reduction in growth rate, and the double mutant A40/44 was least affected. A measurement of rotenone-sensitive complex I activity in permeabilized cells roughly reflected these relative growth rates: activity was reduced ~30% in A40/44, ~55% in A42, ~60% in A41/42, and ~50% in U41/42. The antimycin-sensitive succinate-driven respiration (complex II and III) was normal or even elevated in these clones (Yadava *et al.*, in preparation). MWFE protein levels were restored in all complemented cells to levels that were comparable to the

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                    10         20         30         40         50         60         70
                  -                    ++      +-++  +  +      -+ -++     +     +  -  -
Hamster     MWFEILPGLA VMGVCLVIPG VATAYIHKYT NGGKEKRVGR LRYQWYLMER DRRVSGVNRY YVSRGLENID

CCL16-B2    ---------- ---xxxxxxxxxxxxxxx
V79-G20     ---------- ---------- ---------- ---------- ---------K ---------- ----------
V79-G14     ---------- ---------- ---------- ---------- ---------- ---------- ----IR

A42         ---------- ---------- ---------- ---------- -G-------- ---------- ----------
A41/42      ---------- ---------- ---------- ---------- FG-------- ---------- ----------
A40/44      ---------- ---------- ---------- ---------H ---H------ ---------- ----------
U41/42      ---------S ------L--- L------RF- --------AH ---HWS---- ---I---D-- ---K------

Human       MWFEILPGLS VMGVCLLIPG LATAYIHRFT NGGKEKRVAH FGYHWSLMER DRRISGVDRY YVSKGLENID
              -                    ++      +-++  +    +    -+ -++    -+     +  -  -
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Fig. 2. Mutant forms of MWFE peptide.

level observed with wild-type *NDUFA1* cDNA. The signals from other complex I proteins on the same Western blots were not distinguishable from wild-type levels in the mutant in which the MWFE protein was absent, and similar analyses are in progress with the complemented cells. A preliminary conclusion is that the missense mutations explored so far are interfering with complex I activity, but not with the assembly of this subunit in complex I.

A thorough interpretation of these results will not be possible in the absence of a crystal structure. What are the crucial interactions involving this domain of MWFE? Is the deleterious effect of these mutations due to an effect on the rate of electron transfer from NADH to ubiquinone, or is it due to an effect on proton pumping? It is unlikely that the mutations affect binding of the substrate NADH; the binding site(s) for ubiquinone have not been sufficiently well defined in relation to individual subunits, such as the MWFE protein. The path of protons is still a complete mystery.

Dominant-Negative Alleles of the *NDUFA1* Gene

If mutant MWFE proteins can be readily incorporated into complex I, where they will then lower its activity, one can predict that they would act as dominant negative mutations. In a complex I with a single MWFE subunit, a mutant protein would compete with a wild-type protein. The degree of inhibition would be expected to depend on the nature of the mutation and on the efficiency with which the wild-type protein is replaced by mutant protein. There could be an intrinsic difference in the assembly rates, but one would also expect this efficiency to depend on the relative levels of expression of the two genes. Exploratory experiments with mutant *NDUFA1* cDNAs expressed in wild-type CCL16-B1 hamster cells have suggested that these expectations are realized.

FUTURE PROSPECTS

In the absence of yeast mutants and with only a limited number of complex I mutants in other eukaryotic model systems, the hamster mutant cell lines promise to serve as good model systems in the investigation of structure–function relationships and the mechanism of electron transport and proton pumping of complex I. The characterization of the *NDUFA1* mutant has already emphasized that the MWFE protein has more than an "accessory" function. It is essential for activity. Specific mutations in this protein, first characterized in tissue culture, can be exploited to make transgenic mice with dominant negative alleles, or knock-in mutations yielding partially active complex I. Hence, mouse models for mitochondrial diseases can be created and investigated.

It will, in principle, be very easy to clone cDNAs for expression in vectors and testing of complementation of the other complex I mutants in our collection. Thus, additional genes and their gene products will be identified. Most may be expected to represent already known proteins, but it may also be possible to identify true accessory proteins that may play only a transient role in complex I assembly, similar to a variety of genes/factors already identified in yeast for the assembly of complexes IV (cytochrome oxidase) and V (ATP synthase) (Glerum *et al.*, 1997; Hell *et al.*, 2000; Tzagoloff *et al.*, 1994).

The most powerful application of these mutants will become possible when the crystal structure of complex I has been resolved. An inspection of the structure will then dictate very precise questions about the role of specific amino acid side chains in mechanisms related to assembly, electron transport, and proton pumping. Predictions can be tested by substituting altered proteins with specific changes made by site-directed mutagenesis. These complementing and partially complementing proteins can be expressed in hamster cells in tissue culture (null mutants) with the very severe defects of the type described for our mutants.

It will also be interesting to identify precisely the postulated interaction of the MWFE protein with one or more mtDNA-encoded subunits and shed light on the incompatibility of nuclear genes with mitochondrial genes from phylogenetically more distant mammals. The observations with xeno-mitochondrial cybrids can then be interpreted on a structural level.

REFERENCES

Au, H. C., and Scheffler, I. E. (1997). *Somatic Cell Mol. Genet.* **23**, 27–35.

Au, H. C., Seo, B. B., Matsuno-Yagi, A., Yagi, T., and Scheffler, I. E. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 4354–4359.

Barrientos, A., Kenyon, L., and Moraes, C. T. (1998). *J. Biol. Chem.* **273**, 14210–14217.

Breen, G. A. M., and Scheffler, I. E. (1979). *Somat. Cell Genet.* **5**, 441–451.

Burnett, K. G., and Scheffler, I. E. (1981). *J. Cell Biol.* **90**, 108–115.

Day, C., and Scheffler, I. E. (1982). *Somat. Cell Genet.* **8**, 691–707.

DeFrancesco, L., Scheffler, I. E., and Bissell, M. J. (1976). *J. Biol. Chem.* **251**, 4588–4595.

Dey, R., Barrientos, A., and Moraes, C. T. (2000). *J. Biol. Chem.* **275**, 31520–31527.

Ditta, G. S., Soderberg, K., and Scheffler, I. E. (1976). *Somat. Cell Genet.* **2**, 331–344.

Ditta, G. S., Soderberg, K., and Scheffler, I. E. (1977). *Nature London* **268**, 64–67.

Elbehti-Green, A., Au, H. C., Mascarello, J. T., Ream-Robinson, D., and Scheffler, I. E. (1998). *Gene* **213**, 133–140.

Fearnley, I. M., and Walker, J. E. (1992). *Biochim. Biophys. Acta* **1140**, 105–134.
Finel, M. (1998). *Biochim. Biophys. Acta* **1364**, 112–121.
Glerum, D. M., Muroff, I., Jin, C., and Tzagoloff, A. (1997). *J. Biol. Chem.* **272**, 19088–19094.
Guénebaut, V., Vioncentelli, R., Weiss, H., and Leonard, K. R. (1997). *J. Mol. Biol.* **265**, 409–418.
Hell, K., Tzagoloff, A., Neupert, W., and Stuart, R. A. (2000). *J. Biol. Chem.* **275**, 4571–4578.
Kitajima-Ihara, T., and Yagi, T. (1998). *FEBS Lett.* **421**, 37–40.
Loeffen, J., Smeets, R., Smeitink, J., Ruitenbeek, W., Janssen, A., Mariman, E., Sengers, R., Trijbels, F., and Van den Heuvel, L. (1998). *J. Inherit. Metab. Dis.* **21**, 210–215.
Loeffen, J., Smeets, R., Smeitink, J., Triepels, R., Sengers, R., Trijbels, F., and Van den Heuvel, L. (1999). *J. Inherit. Metab. Dis.* **22**, 19–28.
Mascarello, J. T., Soderberg, K., and Scheffler, I. E. (1980). *Cytogenet. Cell Genet.* **28**, 121–135.
Oostveen, F. G., Au, H. C., Meijer, P.-J., and Scheffler, I. E. (1995). *J. Biol. Chem.* **270**, 26104–26108.
Pilkington, S. J., Arizmendi, J. M., Fearnley, I. M., Runswick, M. J., Skehel, J. M., and Walker, J. E. (1993). *Biochem. Soc. Trans.* **21**, 26–31.
Scheffler, I. E. (1974). *J. Cell. Physiol.* **83**, 219–230.
Scheffler, I. E. (1986). In *Carbohydrate Metabolism of Cultured Cells* (M. J. Morgan, ed.), Plenum, New York, pp. 77–109.
Scheffler, I. E. (1999). *Mitochondria.* Wiley, New York.
Schneider, R., Massow, M., Lisowsky, T., and Weiss, H. (1995). *Curr. Genet.* **29**, 10–17.
Seo, B. B., Kitajima-Ihara, T., Chan, E. K. L., Scheffler, I. E., Matsuno-Yagi, A., and Yagi, T. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 9167–9171.
Soderberg, K., Ditta, G. S., and Scheffler, I. E. (1977). *Cell* **10**, 697–702.
Soderberg, K., Mascarello, J. T., Breen, G. A. M., and Scheffler, I. E. (1979). *Somat. Cell Genet.* **5**, 225–240.
Soderberg, K., Nissinen, E., Bakay, B., and Scheffler, I. E. (1980). *J. Cell. Physiol.* **103**, 169–172.
Takano, S., Yano, T., and Yagi, T. (1996). *Biochemistry* **35**, 9120–9127.
Triepels, R., Van den Heuvel, L., Loeffen, J., Smeets, R., Trijbels, F., and Smeitink, J. (1998). *Human Genet.* **103**, 557–563.
Tzagoloff, A., Yue, J., Jang, J., and Paul, M. -F. (1994). *J. Biol. Chem.* **269**, 26144–26151.
Van den Heuvel, L., Ruitenbeek, W., Smeets, R., Gelman-Kohan, Z., Elpeleg, O., Loeffen, J., Trijbels, F., Mariman, E., de Bruijn, D., and Smeitink, J. (1998). *Amer. J. Human Genet.* **62**, 262–268.
Videira, A. (1998). *Biochim. Biophys. Acta* **1364**, 89–100.
Walker, J. E. (1992). *Quart. Rev. Biophys.* **25**, 253–324.
Walker, J. E., Arizmendi, J. M., Dupuis, A., Fearnley, I. M., Finel, M., Medd, S. M., Pilkington, S. J., Runswick, M. J., and Skehel, J. M. (1992). *J. Mol. Biol.* **226**, 1051–1072.
Walker, J. E., Skehel, J. M., and Buchanan, S. K. (1995). *Methods Enzymol.* **260**, 14–34.
Yadava, N., *et al.* (2001). in preparation.
Yagi, T., Yano, T., and Matsuno-Yagi, A. (1993). *J. Bioenerg. Biomembr.* **25**, 339–345.
Yagi, T., Yano, T., Di Bernardo, S., and Matsuno-Yagi, A. (1998). *Biochim. Biophys. Acta* **1364**, 125–133.
Yano, T., and Yagi, T. (1999). *J. Biol. Chem.* **274**, 28606–28611.
Zhuchenko, O., Wehnert, M., Bailey, J., Sun, Z. S., and Lee, C. C. (1996). *Genomics* **37**, 281–288.