Species-specific and Mutant MWFE Proteins
THEIR EFFECT ON THE ASSEMBLY OF A FUNCTIONAL MAMMALIAN MITOCHONDRIAL COMPLEX I*

Received for publication, February 28, 2002, and in revised form, March 27, 2002
Published, JBC Papers in Press, April 5, 2002, DOI 10.1074/jbc.M202016200

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The MWFE protein (76 amino acids) is highly conserved in evolution, but the human protein (80% identical to hamster) does not complement a null mutation in Chinese hamster cells. We have identified a small protein segment where significant differences exist between rodents and primates, illustrating very specifically the need for compatibility of the nuclear and mitochondrial genomes in the assembly of complex I. The segment between amino acids 39 and 46 appears to be critical for species-specific compatibility. Amino acid substitutions in this region were tested that caused a reduction of activity of the hamster protein or converted the inactive human protein into a partially active one. Such mutations could be useful in making mice with partial complex I activity as models for mitochondrial diseases. Their potential as dominant negative mutants was explored. More deleterious mutations in the NDUFAl gene were also characterized. A conservative substitution, R50K, or a short C-terminal deletion makes the protein completely inactive. In the absence of MWFE, no high molecular weight complex was detectable by Blue Native-gel electrophoresis. The MWFE protein itself is unstable in the absence of assembled mitochondrial encoded integral membrane proteins of complex I.

Proton-translocating, multisubunit NADH-quinone oxidoreductases (complex I) exist in mitochondria of most animals, plants, and fungi, as well as in prokaryotes (1–11). The complex is absent in some fungi such as the yeast Saccharomyces cerevisiae. The bacterial complex has a total of 14 subunits (9, 12, 13) for which orthologues can be identified in mammals and plants. These “core” subunits are essential for the dehydrogenase activity, electron transport, and proton translocation across the membrane (3). The complex I in mammals and higher plants has at least 43 subunits. Seven of these are encoded by the mitochondrial genome; they are made in the mitochondrial genome by Zhuchenko et al. (27). It is a small gene (~5 kb) with two introns (1.5 and 3 kb) encoding a peptide of 70 amino acids, the MWFE protein. This protein is imported into mitochondria and assembled in complex I without requiring proteolytic processing (22). It is listed among the ~29 accessory proteins for which no function has been established so far. Our results had shown that the MWFE protein is essential for a functional complex I in mammalian mitochondria.

The growing interest in complex I deficiencies related to human mitochondrial diseases (28–30) has been the impetus to map and characterize all the nuclear genes encoding subunits of this complex, because a number of human patients have been shown to suffer from partial complex I deficiencies due to nuclear mutations (31–34). Whereas severe mutations in mitochondrial structural genes can be tolerated in heteroplasmic patients, nuclear gene mutations in patients must be missense mutations causing a partial complex I deficiency. To model encoded proteins (in higher organisms). A second peripheral domain that may be further fractionated by mild dissociation conditions extends into the mitochondrial matrix. It includes the active site for the substrate NADH, a flavin mononucleotide (FMN), as the hydride acceptor, and several of the non-heme iron sulfur centers (9, 19). These two subcomplexes are connected by a narrower neck or hinge region (20). Functionally, the subdomain in the matrix is an NADH dehydrogenase, and the connecting fragment is related to multisubunit hydrogenases (8). Certain combinations of detergents and fractionation techniques appear to cleave the complex into three subcomplexes that may reflect this functional differentiation. There are indications from work in Neurospora crassa that subcomplexes appear to be assembled independently in the matrix and in the inner membrane, respectively, until they are joined to form the complete functional complex (10). From a comparison of the prokaryotic and eukaryotic peptides, ~29 proteins in the mitochondrial complex have been described as “accessory proteins” with functions yet to be defined (10, 21). They may assist in the assembly of the complex, contribute to its stability, have some role in regulation of activity, and may even carry out biochemical functions such as the acyl carrier protein (22).

The complex I in mammalian mitochondria.

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* This work was supported by grants from the California BioStar Program with matching contributions from MitoKor (San Diego, CA), grants from the Muscular Dystrophy Association, and Grant GM59909 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF495658, AF495659, and AF495660.
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This paper is available on line at http://www.jbc.org
complex I deficiencies, for example in the mouse, nuclear genes and specific mutations must be identified that affect complex I activity. In the present communication we have characterized a series of such mutations in the NDUF1 gene. Complementation of the hamster null mutant CCL16-B2 with such mutant alleles restores partial complex I activity.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—The parental Chinese hamster cell lines and respiration-deficient mutants (res−) derived from them have been described (see Ref. 35 for a review and Ref. 36). They were routinely cultured in Dulbecco's modified Eagle's medium with 4.5 mg/ml glucose (DME-Glu), 10% fetal calf serum, nonessential amino acids, gentamy- cin, and fungizone. Under these conditions even the respiration-deficient cell lines grow normally. To distinguish res− from res+ cell lines, the same medium was used with glucose replaced by 1 mg/ml galactose. It is referred to as DME-Gal (37). The mouse cell lines (3A-20-4, 4A) with defective NDUFA1 and ND6 mitochondrial genes, respectively, were obtained from Dr. A. Chomyn (18). Cells were harvested by trypsination after one wash with TD buffer (0.5% Triton, 0.8% NaCl, 0.038% KCl, 0.025% NaHPO4, 12H2O, brought to pH 7.4 with HCl).

**Plasmids and Genes**—The human NDUF1 gene was originally cloned and characterized by Zhuchenko et al. (27). The bovine and mouse sequences had also been published. This has permitted us to clone our own cDNAs from HSM and nurse cells of other primates by an approach based on reverse transcriptase-PCR using conserved oligonucleotide sequences (26). Crude RNA extracts from fibroblasts of lemur, chimpanzee, and gorilla were obtained from Dr. Oliver Ryder at the San Diego Zoo. The PCR products were cloned into pGEM-T vector (Promega) by T-A overhang cloning. The nucleotide sequence was determined by a multiplex dideoxy chain termination method (26). Primer pairs were designed to amplify the region of the res− phenotype by exposure to DME-Gal (23). The polycistronic constructs were transfected into cells using SuperFect reagent according to the manufacturer's instructions (Qiagen). The transfection mixture for CCL16-B2 (res−) cells constituted 0.5 μg of DNA and 5 μl of SuperFect; for CCL16-B1 (res+) cells it was 2 μg of DNA and 10 μl of SuperFect.

**Measurement of Respiratory Activities**—The respiratory chain activities of various cell lines were measured as described (25). The cells were harvested by trypsination, collected by centrifugation (350 g), and resuspended in 1× HSM buffer (20 mM Hepes, pH 7.1, 250 mM sucrose, 10 mM MgCl2) at density 2 × 10^7/ml. The cells were treated with 100 μg/ml digitonin until more than 90% of the cells are stained by trypan blue. After ~5 min of digitonin treatment at 4 °C, the cell suspension was centrifuged at 10-fold with HSM buffer, and the cells were harvested by centrifugation. Subsequently, the cells were washed once with the same medium and resuspended at 3 × 10^7 cells/ml. The total protein content was measured by Bradford microassay, and ~5–6 mg of cell suspension was used per assay. Oxygen consumption was measured polarographically in a 2.2 ml metabolic chamber with a water jacket maintained at 37 °C by using a Clark-type oxygen electrode. Substrates, inhibitors, etc. could be added via a capillary opening using microsyringes as described previously (26). Respiratory activity in 2,4-dinitrophenol-uncoupled mitochondria (3–4 μg/assay) was measured essentially as above.

**Isolation of Mitochondria and Mitochondrial Fractions**—Mitochondria were isolated from cells essentially according to Trounce et al. (42). Mitochondrial fractions were prepared as follows. Approximately 1 × 10^7 cells (CCL16-B1, CCL16-B2, and NDUF1-transfected cells) were washed twice with TD buffer and harvested by trypsination. The pellets were suspended in 5 ml of SM buffer (50 mM Tris-HCl, pH 7.4, 0.25 mM sucrose, 2 mM EDTA) and homogenized using a tightly fitting Dounce homogenizer (30–35 up/down strokes). The homogenate was centrifuged twice at 625 × g for 10 min at 4 °C in order to remove nuclei and unbroken cells and nuclei. The supernatant was centrifuged at 10,000 × g for 20 min at 4 °C. The mitochondrial pellet was suspended in 0.1 ml of the SM buffer. This fraction is designated as the mitochondrial fraction.

**Immunochromosomal Assays and Antibodies**—Cells were grown and harvested as above. The lysis of cells was carried out in buffer (50 mM Tris-HCl, pH 7.4, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 2% Triton X-100). Equal volume of Sigma X-galath we added in liquid nitrogen and 3 bursts of sonication. Protein samples (between 50 and 100 mg) were separated by SDS-PAGE and transferred to Immobilon-P (0.45 μm) or polyvinylidene difluoride (0.1 μm) membranes. Anti-MWFE and anti-ERK2 were used at 1:5000 dilution, and signals on the immunoblots were detected using enhanced chemiluminescence system (ECL + Plus from Amer sham Biosciences).

The antibody against the MWFE protein was generated by immunizing rabbits with two synthetic peptides corresponding to two major segments of the hydrophilic domain of the protein (amino acids 29–40 and 59–70; see Fig. 1A). Affinity-purified antibodies were prepared by a commercial manufacturer (HTI BioProducts, Inc. Ramona, CA). Antibodies specific to bovine complex I subunits (23 (TYKY) and 51 kDa) were generous gifts from Drs. Y. Hatafi and T. Yagi (The Scripps Research Institute) (1, 13, 43, 44). Dr. R. A. Capaldi (Institute of Molecular Biology, Eugene, OR) generously provided mouse monoclonal antibody 29-20 against the 29-20 mouse subunit complex I (44). Dr. M. David (Division of Biology, University of California, San Diego) generously gave anti-ERK2 antibody. Sources of other antibodies were as follows: anti-porin from Calbiochem, anti-cyto from BD Pharmingen, anti-HA from Covance Babco, and anti-mouse and anti-rabbit secondary antibodies from Bio-Rad and Amersham Biosciences, respectively.

**Blue Native-PAGE**—Blue native-gel electrophoresis of mitochondrial respiratory complexes was performed as described by Schagger (46).
The mitochondrial pellets equivalent to 400 μg of protein were solubilized with 800 μg of dodecyl-β-D-maltoside (Sigma) in 5 mM 6-aminohexanoic acid, 50 mM imidazole HCl, pH 7.0, and 10% glycerol. To the solubilized samples Coomassie Brilliant Blue G-250 (Serva) was added at a dye/detergent ratio of 1:5 g/g. A 3.5–13% acrylamide gradient gel was used for electrophoresis.

The histochemical assays on the BN-PAGE gels were performed according to Dabbeni-Sala et al. (47) by incubating the gel slices with 2 mM Tris-HCl, pH 7.4, 0.1 mg/ml NADH, and 2.5 mg/ml nitro blue tetrazolium (Sigma) for complex I. Complex V (ATPase) activity was measured by incubating the gel slices overnight in 35 mM Tris, 270 mM glycine, pH 7.8, 14 mM MgSO4, 0.2% Pb(NO3)2, and 8 mM ATP at room temperature.

**RESULTS**

**Mutant Alleles of NDUFA1 cDNA**—The MWFE protein from diverse mammalian species, from other vertebrates (Xenopus laevis), and even from plants (rice) is highly conserved (Fig. 1A). In all the species so far examined, the protein is precisely 70 residues long, with two distinct domains. A predicted transmembrane region consisting of the first 24 amino acids is followed by a highly charged domain of ~45 amino acids. Because there is no further processing (22, 48, 49), the N-terminal sequence must also act as the mitochondrial targeting sequence. The negative charge at position 4 is unusual; it is also highly conserved. The orientation of the protein in the membrane has not been firmly established, but preliminary results suggest that the hydrophilic domain of the MWFE protein extends into the intermembrane space.

Our previous studies have shown that human NDUFA1 cDNA does not complement the mutation in the hamster gene despite the high degree of sequence conservation in the protein (26). When additional primate and rodent sequences were determined and compared, significant differences between amino acids 39 and 46 were found to distinguish primates from rodents. Most notable is the difference in the spacing of the two positive charges in this segment; they are separated by one amino acid in the hamster and by three amino acids in primates. The mouse MWFE protein is missing a positive side chain in this region but mouse cDNA could complement the hamster mutation at a slightly lower efficiency than hamster cDNA (26) (Fig. 1B).

Several mutant alleles of the NDUFA1 gene were isolated during the original mutant selection in tissue culture (CCL16-B2, V79-G20, and V79-G14) and mutant proteins created by site-directed mutagenesis. The underlined short peptides were used in combination for the immunization of rabbits and the production of anti-MWFE antibody. Only the C-terminal peptide appears to be recognized by the antibody (see text).

The mitochondrial pellets equivalent to 400 μg of protein were solubilized with 800 μg of dodecyl-β-D-maltoside (Sigma) in 5 mM 6-aminohexanoic acid, 50 mM imidazole HCl, pH 7.0, and 10% glycerol. To the solubilized samples Coomassie Brilliant Blue G-250 (Serva) was added at a dye/detergent ratio of 1:5 g/g. A 3.5–13% acrylamide gradient gel was used for electrophoresis.

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**Other Reagents**—The transfection reagents were polyethyleneimine from Sigma and SuperFect from Qiagen (Germany). All other reagents were of the highest grade available.

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Our investigation of the complemented CCL16-B2 cells started with measurements of growth rates in DME-Gal medium, a condition requiring respiration and oxidative phosphorylation. The results show that the complemented cell lines B2-A42, B2-A41/42, and B2-A40/44 expressing mutated hamster MWFE protein grew slower compared with wild type CCL16-B1 cells in this medium (Fig. 2). The growth impairment was variable, depending on the type of mutation made in the MWFE protein (Fig. 2). In the “A” series, the most severe impairment (~50%) is due to the A41/42 double mutation in the hamster sequence. More interesting and less predictable was the observation that the inactive human MWFE protein could be turned into a partially complementing protein by the alteration of two amino acids (U41/42). In this protein there are three positively charged amino acid side chains in the small interval. Clearly, a detailed evaluation of the amino acid substitutions and their effect on activity must await the completion of the crystal structure of complex I.

Measurements of Complex I Activity—Measurements of respiration were made with digitonin-permeabilized cells and with isolated mitochondria. The successive addition of different substrates and specific inhibitors allowed measurements of the activity of individual complexes. Rotenone-sensitive, malate + glutamate-dependent respiration reflects complex I activity; complex II/III activity can be measured with succinate as substrate and the use of either malonate or antimycin as inhibitors, and TMPD/ascorbate can be used as substrates to measure complex IV activity. Results from the first series of transfectants with monocistronic transcripts are shown in Fig. 3. Compared with wild type cells, the CCL16-B2 cells had typically less than 10% complex I activity, and an inactive human protein could be modified at two positions to acquire partial activity (Fig. 3A); the mutants had intermediate activity levels that were roughly correlated with their relative growth rates in DME-Gal medium (Fig. 3B). The reduction in complex I activity was most severe in the double mutants B2-A41/42 and B2-U1/42. The reduction in complex I activity observed in whole cell assays was further confirmed by assaying the complex I activity in isolated mitochondria. Data from a representative cell line B2-A41/42 are shown in Fig. 3C. The activities were all normalized to total cellular protein or to total mitochondrial protein, respectively. By this means of normalization, some of the mutant cells had apparently elevated complex II/III levels. However, the significance of this variation is difficult to assess. These individual, selected populations of cells are not exactly diploid and isogenic. It is significant that the relative differences in complex I activity were observed in both whole cells and in isolated mitochondria, although a normalization to different protein subsets was employed in this comparison.

The Expression of MWFE Proteins and Complex I Activity in Various Mutants of Chinese Hamster Fibroblasts—A polyclonal anti-MWFE antibody was made in rabbits by immunization with two small peptides from the hydrophilic domain of the MWFE protein (see “Experimental Procedures” and Fig. 1B). To investigate the specificity and cross-reactivity of this antibody, whole cell lysates from hamster, mouse, and human cell lines were examined by Western blotting (Fig. 4A). The antibody cross-reacted with the corresponding mouse and human proteins, but the interaction appeared weaker with the human protein. Independent experiments established that all the antigenic protein found in whole cell lysates was membrane/mitochondria-associated, and it had the expected low molecular weight (results not shown).

Shown in Fig. 4B are Western blots with whole cell lysates from wild type CCL16-B1 cells, from various mutant cells of complementation group I (CCL16-B2, V79-G14, V79-G20, and V79-G42), and from additional mutant cell lines in different complementation groups (V79-G4 and V79-G7) (23). The V79-G7 mutant cells were included in this analysis because mitochondrial protein synthesis is absent in this mutant (36), and all seven mitochondrially encoded subunits of complex I are missing. The protein is found in V79-G4 cells (complementation...
tation group II). V79-G7 cells have a normal NDUF1 gene and can complement the CCL16-B2 mutant in somatic cell hybrids (23). The absence of a signal therefore indicates that the MWFE protein must be highly unstable when it is imported into mitochondria but not assembled in a complex I without mitochondrially encoded proteins. This observation also suggests that an excess of MWFE protein would not accumulate significantly in mitochondria as a free integral inner membrane protein, for example when overexpressed from a trans-

gene with a highly active promoter. The expression of MWFE from polycistronic pTRIDENT14-neo in V79-G7 cells is also not detectable on a Western blot (results not shown). From the characterization of the nature of the mutation (Fig. 1B), the absence of MWFE protein in the CCL16-B2 cells could be anticipated. The MWFE band was also missing from the three other mutant cell lines V79-G14, V79-G20, and V79-G42. The loss of activity due to the R50K mutation (in V79-G20) appears to be due to a failure of this mutant protein to be incorporated into complex I. The mutated site is outside of the regions for the two peptides used in the immunization. The deletion of four C-terminal amino acids (V79-G14) is equally deleterious for activity and for detection on the Western blot, although it is not possible to determine whether the truncated protein fails to be incorporated into the complex or whether the truncation has destroyed the major antigenic determinant at the C terminus. The precise alteration in the V79-G42 mutant allele has not yet been determined.

Western blots from CCL16-B2 cells complemented with different alleles of the NDUF1 cDNA are shown in Fig. 5. By using the lane with B2-MWFE (wild type) extracts as a basis for comparison, the MWFE protein levels in the CCL16-B2 cells could be estimated. The MWFE band was also missing from the three other mutant cell lines V79-G14, V79-G20, and V79-G42. The loss of activity due to the R50K mutation (in V79-G20) appears to be due to a failure of this mutant protein to be incorporated into complex I. The mutated site is outside of the regions for the two peptides used in the immunization. The deletion of four C-terminal amino acids (V79-G14) is equally deleterious for activity and for detection on the Western blot, although it is not possible to determine whether the truncated protein fails to be incorporated into the complex or whether the truncation has destroyed the major antigenic determinant at the C terminus. The precise alteration in the V79-G42 mutant allele has not yet been determined.

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membrane and to follow such a protein in transgenic cells (and animals), it was desirable to mark this protein with an epitope tag. An MWFE protein with the green fluorescent protein at the C-terminal yielded green mitochondria but did not completely stabilize the MWFE-HA protein could be achieved (Fig. 5B). Subsequently, several of the mutant alleles were also constructed with the HA tag at the C terminus. In these constructs the MWFE-HA protein was expressed from the pTRIDENT14-neo vector as the first cistron of a multicistronic mRNA (“Experimental Procedures”). In all cases, transfected cells were selected in DME-Glu in the presence of G418, and experiments were performed with stable, transformed cells.

The observation that the MWFE-HA protein could complement the CCL16-B2 mutant was interesting, because it suggested that the C-terminal carboxyl group was not absolutely required, although the results with the V79-G14 allele had indicated that a small deletion at that end could not be tolerated. A comparison of the wild type MWFE protein with the wild type MWFE-HA protein in CCL16-B2 cells revealed relatively small differences in complex I activity. The HA-tagged protein can be detected on Western blots, and the shift in mobility is as expected from the addition of 13 amino acids to the very small MWFE protein. However, it appears that the C-terminal addition completely destroyed the antigenic determinant(s) for the anti-MWFE antibody (Fig. 5B). The latter was raised against a mixture of two peptides (“Experimental Procedures”), one of which included the terminal 11 amino acids. It is therefore likely that the interior peptide is not recognized by this antibody.

The expression and function of the HA-tagged MWFE from a polycistronic vector was tested in hamster and human cells. As before, the mutant U41/42-HA allele and the mouse MWFE-HA yielded reduced complex I activities compared with the wild type hamster MWFE-HA (Fig. 6A). When complex I activity was observed, MWFE-HA could be detected on Western blots (Fig. 6B), with the human MWFE-HA protein being undetectable. When a series of the same constructs were transfected into human HT1080 cells, only the human MWFE-HA protein was stabilized (by incorporation into human complex I), but the hamster MWFE-HA, A41/42-HA, and U41/42-HA proteins were unstable (Fig. 6B). Two amino acid changes in the human protein made it active in hamster mitochondria but inactive in human mitochondria.

Overexpression of the MWFE Protein in Wild Type Cells—If a mutant MWFE protein causes a reduction in the specific activity of complex I, a mutant allele might act as a dominant
negative allele when overexpressed in a wild type background. Experiments were performed with the polycistrionic pTRIDENT14-neo constructs expressing HA-tagged mutant and wild type proteins in stable cell lines. Selections of stable transformed cell lines were made in DME-Glu with G418, and subsequently individual clones or pooled populations were characterized.

The analysis of individual clones revealed some surprises, and the results with wild type CCL16-B1 cells transfected with the A41/42-HA allele will serve as examples. Western blots with whole cell extracts and Northern blots with total RNA were made from a series of individual clones (Fig. 7A). Several conclusions stand out. 1) There is significant clonal variation in the Western blots with regard to complex I proteins; other mitochondrial proteins (porin) are present at comparable levels. Differences in the level of MWFE and MWFE-HA bands are particularly striking. It should be pointed out that whole cell extracts were used for these Western blots, and although the MWFE and MWFE-HA bands reflect the amount of assembled complex I, the 30- and 39-kDa bands include the unassembled fractions of these proteins that can also be seen in V79-G7 cells (results not shown) and in ρ0 cells (45). 2) The ratio of wild type MWFE from the endogenous gene to HA-tagged MWFE expressed from the transgene is also variable, but in all clones with wild type MWFE-HA the transgenic protein is higher, whereas the transgenic mutant A41/42-HA protein is relatively lower. This may reflect the relative stabilities or the relative rates at which these proteins are incorporated into complex I. Only relative signals can be compared, because two different non-cross-reacting antibodies were used for each protein species. 3) A difference in the HA-tagged MWFE proteins could be the result of a different dose of the transgene in the clones. On the other hand, different doses of transgene could not account for elevated or reduced levels of the endogenous MWFE protein. A Northern blot (Fig. 7B) reveals fairly constant levels of NDUF41 mRNA from the endogenous gene, as expected. Levels of the polycistrionic transcript were significantly higher (the same probe labeled both transcripts) and more variable. The mRNA for citrate synthase served as a loading control. The far right panels of Fig. 7B also show the citrate synthase and NDUF41 bands from untransfected wild type CCL16-B1 and mutant CCL16-B2 cells. As described before, the CCL16-B2 NDUF41 mRNA with an internal deletion is less stable and less abundant.

Three clones were selected for a more detailed analysis: B1-MWFE-HA.2, B1-A41/42-HA.3, and B1-A41/42-HA.8. Clones B1-MWFE-HA.2 and B1-A41/42-HA.8 were comparable with respect to transcript levels but very different in the level of protein. B1-A41/42-HA.3 had significantly more of either endogenous or transgenic protein, and the transcript levels appeared elevated from both the endogenous gene and transgene; citrate synthase mRNA levels were still comparable.

Growth rate comparisons in DME-Gal showed clone B1-A41/42-HA.8 to grow at half the rate compared with wild type protein overexpressing B1-MWFE-HA.2 cells, whereas a second clone, B1-A41/42-HA.3, grew almost twice the rate (Fig. 8A). The faster growing clone clearly had more MWFE and A41/42-HA proteins, and if these levels reflect the amount of complex I in mitochondria, the observed differences in growth rates in DME-Gal are due to differences in complex I activity in these cells.

It should be noted that in the faster growing clone the levels of both the endogenous and the HA-tagged MWFE protein are elevated, reflecting total complex I levels. BN-PAGE was used to verify these assumptions. Wild type CCL16-B1 and the null mutant CCL16-B2 cells were also investigated, yielding two novel results. A histochemical assay revealed activity in the
lane with wild type mitochondrial extracts associated with a band at ~900 kDa. This activity was not sensitive to rotenone, suggesting the nitro blue tetrazolium accepts electrons from a site unaffected by this inhibitor. No significant activity was found anywhere in the lane with mutant extracts. Several proteins (51, 39, and 23 kDa and others not shown) were found in the ~900-kDa band from wild type extracts, indicating an intact complex I, but these proteins were absent in the lane with mutant extract. A further analysis of the fate of these proteins in the mutant is in progress, because they are detectable on standard Western blots from mitochondrial membranes (26).

Mitochondria from wild type (CCL16-B1) and clones B1–41/42-HA.8 and B1–41/42-HA.3 were similarly analyzed by BN-PAGE, followed by a histochemical assay and Western blotting. As expected, clone B1–41/42-HA.3 had appreciably more activity compared with clone B1–41/42-HA.8. An assay for complex V (ATPase) on the same gel shows comparable activities, and from the staining and appearance of various other bands one can conclude that equivalent amounts of mitochondrial extracts were loaded in all lanes. Western analysis with anti-HA and anti-MWFE antibodies confirmed that both wild type and mutant MWFE proteins were associated with complex I. The signals from anti-HA, anti-MWFE, and anti-39-kDa antibodies indicated that the observed differences in complex I activity were reflected in the differences of the protein bands. Clearly, the clones differed greatly in the amounts of complex I (see “Discussion” for some further interpretations). In these Western blots the 39-kDa band represents only those subunits in the complete, assembled complex I, and hence the relative band intensities are different from those in Fig. 7A.

**DISCUSSION**

Previous work from this laboratory (26) and the present work demonstrate that the small integral membrane protein, MWFE, encoded by the X-linked NDUF1 gene, is absolutely required for activity of complex I. Here we demonstrate that MWFE is not only required for NADH-quinone oxidoreductase activity, but it is also absolutely required for stable assembly of an ~900-kDa complex with NADH dehydrogenase activity, measured on BN-PAGE with nitro blue tetrazolium as electron acceptor.

The MWFE protein is most likely anchored in the inner membrane with its N-terminal domain, leaving a short domain of ~45 amino acids with 12 positive and 5 negative charges for interactions with other subunits. The protein is highly conserved in evolution in animals as well as plants. At the same time it was striking that the human protein could not complement a null mutation in Chinese hamster cells. We believe that this is a specific example illustrating observations made over the past decades that mitochondria from one mammal cannot be functional in a cell with the nucleus from a distantly related mammal (38, 54, 55). In other words, the nuclear and the mitochondrial genomes must co-evolve to encode proteins that are compatible in the complexes of the electron transport chain. The MWFE protein is found in the membrane subcomplex, and hence it is probable that it interacts with one or more of the mtDNA-encoded proteins. Additional fractionations suggest that it is associated with the I1 subcomplex (47), and therefore the subunits ND1, ND2, ND3, and ND4L are likely candidates. Because the extra-membranous domain of MWFE is highly charged, it must interact with a hydrophilic, extra-membranous domain (a loop or terminal segment) of one or more of these integral membrane proteins.

An unsolved problem is to determine the orientation of the MWFE protein in the inner mitochondrial membrane. It should be noted that it is imported without further processing. The transmembrane domain may therefore serve both as the mitochondrial targeting sequence and as the membrane anchor. If the N terminus is inserted as in a normal import pathway for mitochondrial matrix proteins, one would predict the hydrophilic domain to extend into the intermembrane space. Preliminary results with a MWFE-GFP chimeric protein support such an interpretation, but this protein is not functionally active. The fully functional MWFE-HA proteins described in this report will be very useful in the determination of the orientation of this protein.

A detailed comparison of the hamster (rodent) protein with the human (primate) MWFE protein suggested a very small segment (amino acids 39–46) where significant sequence differences were observed. The human protein is not functional in a complementation test with a hamster null mutant. This segment must be involved in a critical interaction, making the human protein incapable of functioning with the other subunits encoded by the hamster mtDNA and nDNA. The finding that the human protein is highly unstable in the hamster mitochondria strengthens this conclusion (see below). The focus on this segment of the protein is further validated by our finding that a change of two amino acids in this segment can have a noticeable effect on complex I activity. First, when the hamster protein is altered in the direction toward the human sequence (e.g. A41/42), the activity is decreased relative to the wild type hamster protein. The A41/42 mutant protein also resembles the less active mouse protein with a reduced charge at position 42. Second, a change of two amino acids in the human protein (U41/42) can convert the totally inactive and unstable protein into a partially functional and more stable protein in hamster mitochondria. The U41/42 mutant protein has positively charged side chains at positions 40 and 42, like the hamster protein, and an additional positive charge at position 44. In the inverse experiment in human cells, hamster and mutated MWFE proteins are unstable, and two amino acid substitutions convert the stable human protein into an unstable protein.

The MWFE protein is stable at steady state levels when it is incorporated into complex I but is highly labile as an isolated subunit in mitochondria. The level of MWFE protein therefore may also reflect the level of assembled complex I. In a mutant (V79-G7) defective in mitochondrial protein synthesis the protein cannot be detected at steady state levels, although the V79-G7 mutant cells can complement the CCL16-B2 mutant in somatic cell hybrids. In the V79-G7 mutant all seven mtDNA-encoded subunits of complex I are absent. As expected, overexpressed MWFE is also undetectable in V79-G7. Similarly, in mouse mutant cells defective in single, mitochondrially encoded proteins ND5 or ND6, little or no complex I activity is detected (18). An inactive complex I is, however, assembled in the ND5 mutant. We detect correspondingly little or no MWFE protein at steady state in ND6 cells, but MWFE is present in ND5 cells. Other mutant alleles of the NDUF1 gene encode single amino acid substitutions (V79-G20) or small C-terminal deletions (V79-G14). In these mutants there is no complex I activity, and there is also no detectable MWFE protein (although in V79-G14 the antigenic determinant may have been destroyed). In this case all the other subunits of complex I are made, but the mutant protein presumably cannot become integrated and hence protected against degradation.

Several of the mutant forms of the MWFE protein were shown here to yield a reduced complex I activity when introduced into the CCL16-B2 null mutant. There are several possible explanations. The mutant MWFE protein could be incor-

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N. Yadava, unpublished data.
porated normally into complex I, but it could interfere partially with some of the conformational motions associated with complex I activity/turnover. The effect would be a lowering of the specific activity of complex I. Alternatively, the incorporation of a mutant MWFE protein into the complex could be slowed down. If assembly or degradation are alternative fates, degradation would be favored by a slower assembly. Results shown in Fig. 7 point in this direction. A lower activity would then be the result of less active complex being produced. One can also consider that the association of the MWFE protein with complex I is a dynamic one. The MWFE protein could diffuse in the plane of the membrane; its lateral association with a domain of the complex through interactions outside of the membrane would be critically dependent on charges and charge distributions and possibly the conformation of the extra-membranous domain. Mutant and wild type proteins can be expected to differ with regard to these parameters. Again, “free” MWFE protein would be subject to rapid degradation. This issue also becomes relevant in a discussion of how the MWFE protein is incorporated into the complex during or after its import into mitochondria. Is it first inserted into the membrane and then associated with an assembled or partially assembled complex I, or does the partially assembled complex constitute a direct target for import?

Western analyses and complex I activity measurements by polargraphy are unfortunately not sufficiently precise to distinguish between a lower specific activity and a reduced amount of complex I. The presence or absence of activity and/or MWFE proteins in the various mutants can be clearly established, but a precise correlation between differences of less than a factor of 2 is technically challenging.

The MWFE proteins were expressed from several different transgenes with strong constitutive promoters, yielding 5–10-fold elevated levels of mRNA relative to the endogenous NDUFA1 mRNA level. There is no corresponding increase in the level of MWFE protein detectable on Western blots. It is very plausible that all the excess MWFE protein that cannot be assembled is rapidly turned over by one or more mitochondrial proteases. These results also suggest that MWFE may not be a limiting factor in the assembly of active complex I. Based on Northern analysis, the wild type or mutant transgenes were expressed at significantly higher levels than the endogenous NDUFA1 gene. Nevertheless, complex I activity in transfected CCL16-B2 cells generally did not exceed the activity of wild type untransfected cells (see below), and when mutant alleles were expressed, the activity was reduced, depending on the mutation. It is therefore less likely that kinetic effects are responsible for reduced activity.

Some interesting and relevant observations were made when wild type and mutant HA-tagged MWFE protein was expressed in wild type cells, i.e. expression from the transgene contributed to the pool of proteins also expressed from the endogenous gene. One prediction was that a mutant allele should behave as a dominant negative mutation, leading to reduced complex I activity and slower growth on DME-Gal. Such clones were indeed found, but a generalization of this result was complicated by the observed clonal variations. Fast growing clones with relatively high complex I activity were present as well as slow growing clones with significantly less activity. These clones were picked up because with the polycistronic constructs the selections could be made in DME-Glu with the drug G418. However, under glucose deprivation or in DME-Gal one would expect a (pooled) population of cells to evolve in favor of the faster growing clones.

When wild type MWFE-HA was expressed as well as the endogenous MWFE, the signal ratio of the two proteins found on Western blots reflected the abundance of the HA-tagged protein from the more abundant transcript. In contrast, the A41/42-HA mutant protein appeared to be less abundant relative to the endogenous MWFE protein, even when the A41/42-HA transcript levels were much higher. Such a result suggests a differential stability or assembly rate, with the wild type protein favored over the A41/42-HA protein. It should be pointed out that these comparisons are all relative from a single blot such as that shown in Fig. 7. Because the antibodies are very different, it is not possible to deduce absolute amounts of the endogenous and HA-tagged MWFE proteins.

Clone B1-A41/42-HA.3 had the highest growth rate in DME-Gal, the highest complex I activity, and the greatest intensity of both protein bands on Western blots, whereas clone B1-A41/42.8 represented the other extreme (Fig. 8). The cells under consideration are pseudo-diploid established Chinese hamster cell lines. Different clones have shown significant variations in complex II/III activities which should have been unaffected by any of the various MWFE proteins being expressed. Are there adaptations in gene expression during prolonged culture? The formation of the mitochondrial complex I requires at least 36 nuclear genes scattered over many different chromosomes. How is their expression coordinated? It could also be that one of the mitochondrial gene products becomes limiting but variable among clones. Variations at any of these loci could account for some of the variations we have observed.

One rationale for using the poly-cistronic expression vector was to have a drug selection and maintenance of the transgene expression, whereas the cells could be grown in DME-Glu, where respiration is not essential. Nevertheless, glucose could become transiently low at higher population densities, favoring the growth of clones with higher respiratory capacity.

In conclusion, this paper highlights several aspects of the MWFE protein in complex I assembly and activity. A stable ~900-kDa complex detectable on BN-PAGE is not formed in the absence of the MWFE protein. The MWFE protein is detectable in mitochondria only when it is incorporated into the complex and protected from proteases in the presence of ND subunits. Species comparisons and mutagenesis experiments highlight a small segment of the protein that is involved in a critical interaction with other complex I proteins. Even though the protein is highly conserved in evolution, the protein is non-functional and unstable when the primate protein is expressed in rodent cells or when the rodent protein is expressed in human cells. If, as seems likely, the MWFE protein interacts with one or more mitochondrially encoded proteins, the present example is a highly specific illustration of the need for compatible nuclear and mitochondrial genes. Future experiments are aimed at identifying these proteins.

Acknowledgments—We are grateful to Drs. Y. Hatefi, T. Yagi, A. Matsuno-Yagi, R. Capaldi, and M. David for generously supplying various antibodies. Drs. Hatefi, Matsuno-Yagi, and Yagi deserve special thanks for numerous discussions and advice during the execution of this project. We also thank Drs. G. Attardi and A. Chomyn for sending mouse cell lines (3A-20-4, 4A) with defective ND5 and ND6 mitochondrial genes, respectively. D. Ream-Robinson made numerous contributions to the construction of various vectors, and E. Hoepchner provided valuable assistance with tissue culture, transfections, and other procedures. We thank MitoKor and staff (Dr. A. Murphy) for financial as well as technical support and encouragement.

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