Journal of Bioenergetics and Biomembranes, Vol. 33, No. 3, 2001

Mitochondrial Genetic Control of Assembly and Function of Complex I in Mammalian Cells

Anne Chomyn[1]

Sixteen years ago, we demonstrated, by immunological and biochemical approaches, that seven subunits of complex I are encoded in mitochondrial DNA (mtDNA) and synthesized on mitochondrial ribosomes in mammalian cells. More recently, we carried out a biochemical, molecular, and cellular analysis of a mutation in the gene for one of these subunits, ND4, that causes Leber's hereditary optic neuropathy (LHON). We demonstrated that, in cells carrying this mutation, the mtDNA-encoded subunits of complex I are assembled into a complex, but the rate of complex I-dependent respiration is decreased. Subsequently, we isolated several mutants affected in one or another of the mtDNA-encoded subunits of complex I by exposing established cell lines to high concentrations of rotenone. Our analyses of these mtDNA mutations affecting subunits of complex I have shown that at least two of these subunits, ND4 and ND6, are essential for the assembly of the enzyme. ND5 appears to be located at the periphery of the enzyme and, while it is not essential for assembly of the other mtDNA-encoded subunits into a complex, it is essential for complex I activity. In fact, the synthesis of the ND5 polypeptide is rate limiting for the activity of the enzyme.

KEY WORDS: Mitochondrial translation; mitochondrial diseases; LHON; ND subunits; mitochondrial gene expression; complex immunoprecipitation.

Complex I, the rotenone-sensitive NADH dehydrogenase or NADH:ubiquinone oxidoreductase, is the first enzyme complex of the respiratory chain in most eukaryotic organisms. In mammalian cells, it is a large enzyme complex, consisting of ~43 proteins, lipids, flavin mononucleotide (FMN), and iron–sulfur clusters (Hatefi *et al.*, 1979; Walker, 1992; Skehel *et al.*, 1998). This paper will review the work that the Attardi laboratory has carried out that concerns this important and interesting complex.

STRUCTURE AND SITES OF SYNTHESIS

The sequencing of human mitochondrial DNA was completed in 1981 in Fred Sanger's laboratory in Cambridge (Anderson *et al.*, 1981). This was the first mitochondrial genome to be sequenced in its entirety and, taken together with the fine mapping of the mtDNA transcripts performed in Attardi's laboratory, and published at the same time (Montoya *et al.*, 1981; Ojala *et al.*, 1981), the sequence revealed that this small genome, ~16,569 nucleotides, contained 37 genes. There were 13 open reading frames, in addition to genes for all the other RNA components required to synthesize the mtDNA-encoded proteins: 22 tRNAs and 2 ribosomal RNAs.

The Sanger group identified, by similarity with known yeast and *Neurospora crassa* proteins, the genes encoding three subunits of cytochrome *c* oxidase, subunit 6 of the H^+ATPase, and cytochrome *b.* However, there remained eight unidentified reading frames (URFs). We know now that seven of these encode subunits of complex I (Chomyn *et al.*, 1985, 1986), and the eighth encodes the ATPase subunit 8 (Attardi *et al.*, 1984).

Most of the URFs were matched to their translation products, in collaboration with R. F. Doolittle and his group, by immunoprecipitation with peptide-specific antibodies (Chomyn *et al.*, 1983; Mariottini *et al.*, 1983, 1986), an approach pioneered by Walter and Doolittle (Walter *et al.*, 1980). Ching and Attardi (1982) had

[1] California Institute of Technology, Division of Biology 156-29, Pasadena, California 91125. e-mail: chomyn@caltech.edu

0145-479X/01/0600-0251$19.50/0

previously resolved, by two-dimensional high-resolution SDS–polyacrylamide gel electrophoresis, as many as 26 mitochondrial translation products from HeLa cells. In some cases, as with ND4, the antibodies directed against the NH_2-and/or COOH-terminal peptide of the protein immunoprecipitated a polypeptide that ran as two bands on a gel, suggesting that the protein had structural heterogeneity (Mariottini *et al.*, 1986). In other cases, the antiserum immunoprecipitated a polypeptide of the correct size and a second polypeptide, having a much smaller size, corresponding to a breakdown product (Chomyn *et al.*, 1983; Mariottini *et al.*, 1983). By this approach, most of the 26 polypeptides were accounted for. The URF5 polypeptide was identified on the basis of its size and by tryptic peptide fingerprinting (Mariottini *et al.*, 1986).

Since two of the four mitochondrial respiratory complexes of eukaryotic cells were already represented in the human mitochondrial genome, we thought that perhaps one or both of the other two complexes might also be at least partly encoded in human mtDNA. The guess turned out to be correct. Six of the human URF translation products, those of URF1, URF2, URF3, URF4L, URF4, and URF5, could, in fact, be immunoprecipitated by specific antibodies directed against bovine complex I, under conditions in which multimeric complexes remained intact (Fig. 1) (Chomyn *et al.*, 1985). This identification of complex I subunits was supported by an experiment in which a fractionation of human heart mitochondrial proteins was carried out. A small amount of human mitochondrial translation products labeled with [^{35}S]methionine in the presence of cycloheximide, an inhibitor of cytosolic protein synthesis, had been added to the mitochondria preparation. The fractions that were enriched for complex I enzyme activity were also found to be enriched for six [^{35}S]methionine labeled URF translation products (Chomyn *et al.*, 1985). Thereafter, the last URF, URF6, was soon identified as yet another subunit of complex I (Chomyn *et al.*, 1986). With the assignment of the URF6 product, the functional identification of all thirteen mitochondrial reading frames of human mtDNA was complete. URFs 1, 2, 3, 4L, 4, 5, and 6 were renamed ND1, ND2, ND3, ND4L, ND4, ND5, and ND6 (Chomyn *et al.*, 1985, 1986).

Complex I was known at the time to be a very large protein complex of the size of a bacterial ribosome (Walker, 1992). Chaotropic agents could be used to fractionate the bovine complex into subcomplexes: the flavoprotein fragment, the iron–protein fragment, and the hydrophobic residue (Galante and Hatefi, 1978). SDS–polyacrylamide gel electrophoresis of the different fragments had allowed the assignment of several subunits to one or another of the subcomplexes (Heron *et al.*, 1979).

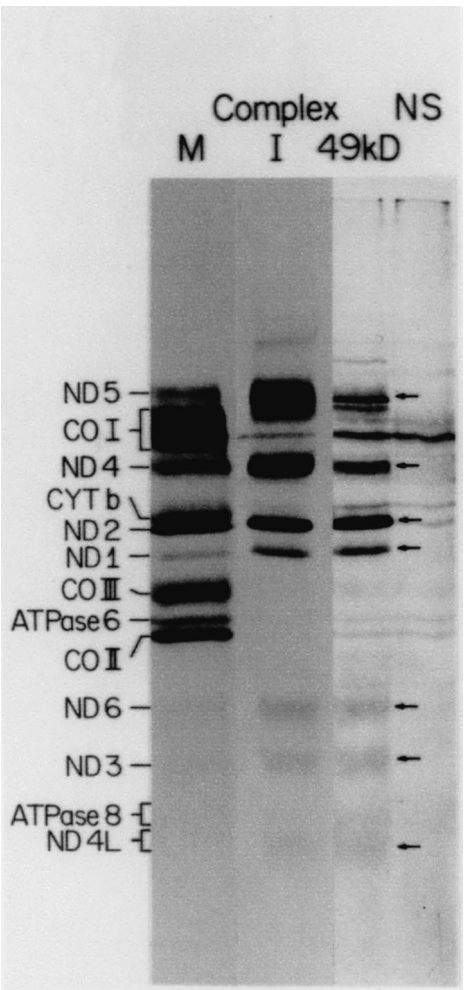

Fig. 1. Immunoprecipitation of complex I. Cells were exposed to chloramphenicol for 22 h, washed, labeled in methionine-free medium with [^{35}S]methionine in the presence of cycloheximide for 2.5 h, washed, and grown for a further 18 h in complete medium in the absence of inhibitors. Mitochondria were isolated, lysed in K deoxycholate or Triton χ-100, and mixed with antiserum directed against bovine complex I, antiserum directed against the 49-kDa subunit of complex I, or normal serum (NS). The immunoprecipitates were electrophoresed on a 15–20% SDS–polyacrylamide gel. Taken from Chomyn *et al.* (1986).

To determine whether any of these subunits corresponded to an mtDNA-encoded subunit, immunoprecipitation experiments were carried out using antibodies that had been prepared by C. I. Ragan and colleagues against the flavoprotein fragment or against individual subunits of the flavoprotein fragment and of the iron–protein fragment (cited in Chomyn *et al.*, 1988). These experiments were carried out with HeLa cell proteins labeled *in vivo* with [^{35}S]methionine under three conditions: in the absence of any inhibitor, in the presence of chloramphenicol, an inhibitor of mitochondrial protein synthesis, and

in the presence of emetine or cycloheximide, inhibitors of cytosolic protein synthesis. These experiments showed that none of the subunits investigated were labeled in the presence of emetine or cycloheximide, but they were labeled in the presence of chloramphenicol. Therefore, the 75-, 49-, 30-, and the 13-kDa polypeptides of the iron–protein fragment and the 51- and 24-kDa polypeptides of the flavoprotein fragment were all synthesized on cytosolic ribosomes and were thus encoded in the nucleus (Chomyn *et al.*, 1988). Since then, the cDNAs for these and all the rest of the subunits of complex I have been cloned (Walker, 1992; Skehel *et al.*, 1998). The model of the complex that we now have, based on electron microscopy of two-dimensional crystals of the *Neurospora* enzyme (Hofhaus *et al.*, 1991) and on electron cryomicroscopy (Grigorieff, 1998) and cryoelectron crystallography of bovine complex I (Sazanov and Walker, 2000), is that of an L-shaped complex, consisting of a "peripheral arm," which is a hydrophilic domain extending into the mitochondrial matrix and a "membrane arm," which lies within the inner mitochondrial membrane at right angles to the peripheral arm.

MUTANTS

Several mutations in the ND subunits have been associated with Leber's hereditary optic neuropathy (LHON) (Wallace *et al.*, 1988, 1992), LHON with CNS disturbance (Howell *et al.*, 1991), LHON with dystonia (Jun *et al.*, 1994), a LHON/MELAS overlap syndrome (Pulkes *et al.*, 2000), and diabetes (Tawata *et al.*, 2000). The effects of the G11778A ND4 LHON mutation (Wallace *et al.*, 1988) on complex I function and on cell growth were analyzed in collaboration with D. Johns and O. Hurko (Hofhaus *et al.*, 1996). For this work, mitochondria were transferred from patient-derived fibroblasts into a human ρ^0 (mtDNA-less) cell line (143B.TK$^-$ $\rho^0$206) (King and Attardi, 1989) by cytoplast-ρ^0 cell fusion, in order to control for any possible contribution of the nucleus to the phenotype.

The G11778A mutation, which changes an arginine codon to a histidine codon, caused the transmitochondrial cells (the ρ^0 cell transformants) to grow very poorly or not at all in medium in which galactose replaced glucose, indicating that there was a defect in oxidative phosphorylation. Polarographic analysis, performed on digitonin-permeabilized cells, showed that the rate of complex I-dependent respiration was decreased in mutant cells, as expected. However, enzyme assays on submitochondrial particles showed that the rate of rotenone-sensitive NADH oxidation in mutant cells was not different from that in control cells. These apparently conflicting results could be reconciled by two possible explanations. The first one assumes that a supercomplex, consisting of complex I with other dehydrogenases, as proposed by Srere (Sumegi and Srere, 1984; Srere, 1991), was disrupted by the ND4 mutation, resulting in decreased channeling of electrons from substrates, like glutamate, to complex I. In this case, the respiration rate would be decreased in intact mitochondria, but the spectrophotometric assay, in which one exposes the enzyme to an excess of NADH, would show no decrease in activity. The second explanation is that the ND4 mutation causes a dismutation of ubisemiquinone, the free radical intermediate between ubiquinone and ubiquinol. The result is that the enzyme would accept electrons from NADH at a normal rate, but not all of the electrons would be passed down the respiratory chain (i.e., energy would not be conserved) (Degli Esposti *et al.*, 1994) and the rate of oxygen consumption would be decreased. A decreased rate of quinone reduction would support this second explanation, and has, in fact, been observed (Brown *et al.*, 2000).

Another mutation, which causes nonsyndromic deafness, affects the expression of another ND gene, the *ND6* gene. This mutation, T7445C (Reid *et al.*, 1994), however, occurs outside the *ND6* gene, just 3′ to the tRNA$^{ser(UCN)}$ gene, which is encoded in the light mtDNA strand. This mutation, analyzed in lymphoblastoid cell lines, has the property of reducing the efficiency of processing of the large precursor transcript from which the mature tRNA$^{Ser(UCN)}$ and ND6 mRNA derive (Guan *et al.*, 1998). The level of ND6 mRNA, ND6 polypeptide synthesis, and malate/glutamate-dependent respiration rate were all substantially affected in cells carrying this mutation.

To obtain additional complex I mutants, we set out to isolate them from an established cell line. Rotenone was used as a selective agent. Since ND1 had been proposed to be involved in rotenone binding (Earley *et al.*, 1987), it was expected that ND1 mutants would be isolated. Mutants resisitant to high concentrations of rotenone, $\geq$0.4 μM, were successfully isolated from the human cell line VA_2B. No mutagenesis had been carried out, so presumably these mutations had occurred spontaneously, before the selection was applied. Several of the rotenone-resistant clones were analyzed by polarography, and six were found to have a specific complex I defect (Hofhaus and Attardi, 1993, 1995).

When the mitochondria were transferred from these rotenone-resistant VA_2B clones into ρ^0 cells, the complex I defect was also transferred in all six cases (Hofhaus and Attardi, 1993, 1995). Interestingly, the rotenone resistance was not transferred with the mitochondria, suggesting that the rotenone resistance was nucleus encoded. It is possible that the rotenone resistance was due to an overexpression or amplification of the genes encoding the

P-glycoproteins, the cell membrane-associated drug efflux pumps that are responsible for multidrug resistance (Endicott and Ling, 1989). The cells carrying the mtDNA mutations would have adapted themselves to derive their energy from glycolysis rather than from oxidative phosphorylation. Perhaps this adaptation gave the mutant cells a growth advantage during the exposure of the culture to rotenone and thus allowed their selection.

The clues as to which mitochondrial gene was affected in these mutants came from an SDS–polyacrylamide gel electrophoresis analysis of the mitochondrial translation products in the cybrids. The first mutant showed an absence of synthesis of the ND4 polypeptide (Hofhaus and Attardi, 1993). Sequencing of the *ND4* gene revealed an insertion of a C in the run of C's at positions 10947–10952. This frameshift mutation would lead to the production of a very small polypeptide, 113 amino acids in length, rather than the 459 amino acid-long ND4 polypeptide. Polarographic measurements showed that there was no malate/glutamate-dependent respiration in the mutant cells. Hofhaus and Attardi also carried out an immunoprecipitation experiment using antibodies directed against the 49-kDa polypeptide subunit of the complex I iron–protein fragment. These antibodies had previously been shown to precipitate, from a Triton X-100 mitochondrial lysate of HeLa cells labeled with [^{35}S]methionine in the presence of cycloheximide, a complex, i.e., complex I or a part thereof, that contained all of the mtDNA-encoded subunits of complex I (Fig. 1) (Chomyn *et al.*, 1986). No such complex could be immunoprecipitated from the ND4 mutant cells with the 49 kDa-specific antibodies. This showed that ND4 is essential for the assembly of complex I.

The second mutant analyzed by Hofhaus and Attardi also carried a frameshift mutation: the run of eight A's beginning at position 12417 was elongated by an additional A (Hofhaus and Attardi, 1995). Immunoprecipitation experiments with the 49 kDa-specific antibodies showed that all of the mtDNA-encoded subunits of complex I, except ND5, were assembled into a precipitable complex. Thus, ND5 is not required for assembly of the mtDNA-encoded subunits into a complex. However, because the bands in the gel corresponding to the other ND subunits had lower intensities than in control cells, it appeared that the absence of the ND5 polypeptide affected the efficiency of the assembly process or affected the stability of the complex. It should be noted, however, that despite complex I's being assembled in the mutant cells, it was inactive. These observations on ND5 suggest that this subunit is at a rather peripheral location in complex I and may be the last of the mtDNA-encoded subunits to be assembled into the complex. Such a location is consistent with the findings of Sazanov and colleagues, that ND5 tends to become detached from the complex when the complex is subfractionated (Sazanov *et al.*, 2000; Sazanov and Walker, 2000).

It is interesting that all of the rotenone-resistant mutants were complex I-deficient mutants. No complex III- or complex IV-deficient mutants were isolated. This is very likely due to the fact that the selection medium contained no added pyrimidine (Hofhaus and Attardi, 1995). Complex III- or complex IV-deficient mutants would be unable to synthesize pyrimidines, because of the lack of the oxidized form of ubiquinone to serve as electron acceptor for the dihydroorotate dehydrogenase, essential in pyrimidine biosynthesis.

Rotenone was also used to isolate several mutants from the established mouse cell line A9 (Bai and Attardi, 1998). These also showed a decrease in complex I-dependent respiration rate. Two clones were chosen for further investigation (Bai and Attardi, 1998; Bai *et al.*, 2000). These were enucleated and fused with the mtDNA-less mouse cell line, ρ^0 LL/2-m21, also isolated in our laboratory. The complex I defect was transferred with mitochondria in both cases. One original rotenone-resistant clone, designated 4A, and the ρ^0 cell transformant derived from it, 4AT1, exhibited a decrease in NADH:Q_1 oxidoreductase activity, but not in NADH:$K_3Fe(CN)_6$ oxidoreductase activity (Bai and Attardi, 1998). This result suggested that the mutation affected a protein in the membrane arm of the complex. This membrane arm is where most or all of the mtDNA-encoded subunits reside (Sazanov *et al.*, 2000). SDS–polyacrylamide gel electrophoretic analysis of the mitochondrial translation products from 4A and in 4AT1 showed the absence of synthesis of the ND6 polypeptide. The *ND6* gene in the mutant cells was amplified by PCR and sequenced, revealing a C insertion in the run of C's at positions 13879–13884 (Bibb *et al.*, 1981). The resulting frameshift introduces a stop codon 17 codons downstream of the insertion, creating a 79-codon long reading frame, whereas the wild-type gene encodes a 172-amino acid long polypeptide. Immunoprecipitation experiments with antisera capable of precipitating complex I, in this case, anti-49 kDa and anti-ND4L antisera (Chomyn *et al.*, 1985, 1986), showed that no such complex could be precipitated from mutant cells. Thus ND6 is essential for complex I assembly.

The second complex I-deficient mutant investigated, 3A, was also enucleated and the cytoplasts, fused with mouse ρ^0 cells. 3A and its derivatives were found to have a deficiency in ND5 synthesis associated with their complex I defect (Bai *et al.*, 2000). Sequencing analysis revealed a C to A transversion at position 12081 in the *ND5* gene. This mutation creates a stop codon, truncating the 607 codon-long reading frame to 115 codons. In this

mutant, a complex containing the other mtDNA-encoded subunits, but lacking ND5, was assembled, as determined by immunoprecipitation experiments, as had been previously found for the human mutant cells lacking ND5 polypeptide.

The *ND5* mutation occurred in heteroplasmic form, that is, mutant mitochondrial genomes coexisted in the cell with wild-type genomes (Bai *et al.*, 2000). Furthermore, every ρ^0 transformant clone had a different percentage of mutant *ND5* gene. This variety of heteroplasmic clones proved to be very useful for the analysis of the control of *ND5* gene expression and its relationship to complex I activity, as will be discussed below.

REGULATION OF EXPRESSION OF ND GENES

Human, like all mammalian mtDNAs, has a unique mode of transcription. The promoters lie in the main control region of the genome, near the origin of heavy-strand mtDNA synthesis. Transcription initiates at three sites in this small control region: the upstream heavy-strand initiation site, used for transcribing the two rRNA genes; the less active, downstream heavy-strand initiation site, used for transcribing the entire heavy strand; and the light-strand initiation site, used for transcribing all of the genes encoded on the light strand (Montoya *et al.*, 1982) and for the synthesis of the primer for heavy-strand mtDNA synthesis (Chang and Clayton, 1985). Mitochondrial RNAs are synthesized as polycistronic transcripts, destined to be processed at the tRNA sequences by RNase P (Doersen *et al.*, 1985; Puranam and Attardi, 2001), a 3′-endoribonuclease, a poly(A) polymerase, and CAA-adding enzymes (Attardi, 1985; Ojala *et al.*, 1981). Thus, the expression of mitochondrial genes can be regulated at the level of initiation of transcription in only a global way.

Another step in the formation of the mature RNAs, i.e., processing from the polycistronic transcripts, could conceivably be modulated to regulate the expression of mitochondrial genes differentially. There is some evidence that the levels of heavy strand- and light strand-encoded tRNAs are regulated in this way (King and Attardi, 1993; Puranam and Attardi, 2001), but there is no evidence yet for such regulation of the mRNAs. The mRNAs do have different stabilities (Gelfand and Attardi, 1981), and, conceivably, the stability of an mRNA could be regulated.

Last, and the most likely in the list of possible sites of regulation, is the translation of the mRNAs. In fact, a striking example of this regulation was the observation, in ^{35}S-methionine *in vitro* labeling experiments carried out on rat brain synaptosomes and on rat skeletal muscle, that the ND5 polypeptide was not synthesized, whereas all of the other mitochondrial translation products were (Attardi *et al.*, 1989; Loguercio Polosa and Attardi, 1991). The ND5 mRNA, on the other hand, was as abundant, relative to other mitochondrial mRNAs, in these tissues as in the R2 rat fibroblast cell line, which *did* synthesize the ND5 polypeptide. This phenomenon can be taken as evidence for an ND5 mRNA-specific translation factor. In light of what we know about ND5 being required for complex I activity, it seems likely that *in vivo* the ND5 polypeptide is synthesized in brain synaptic endings and skeletal muscle and the lack of ND5 synthesis that we observed was the result of the particular conditions used for labeling mitochondrial translation products *in vitro*, in particular, of the possible instability of the putative translation factor.

A very detailed analysis on ND5 gene expression was carried out by using a series of mitochondrial transformant clones which differed, as mentioned above, in the proportion of mtDNA molecules carrying the ND5 mutation at position 12081. This mutation, which produced a loss of a ClaI site, caused a truncation of the ND5 reading frame and a loss of complex I activity (Bai *et al.*, 2000). The heteroplasmic clones had proportions of mutant DNA ranging from 0 to 96%. It was found that the proportion of mutant ND5 mRNA, as determined by reverse transcription PCR, very closely paralleled the proportion of mutant *ND5* DNA. Thus, no degradation of mRNA activated by a nonsense mutation, as occurs in the nucleocytosolic compartment (Maquat, 1988), was evident. The rate of ND5 polypeptide synthesis started decreasing linearly if the proportion of wild-type mRNA dropped below 60% (Fig. 2a). This means that, in wild-type cells, the ND5 mRNA is present in only ~twofold excess over what is needed for the maximum rate of ND5 protein synthesis. Furthermore, measurements of the malate/glutamate-dependent oxygen consumption rates in the various clones revealed that ND5 polypeptide synthesis was rate-limiting for complex I activity (Fig. 2b). Since this activity is normally rate limiting for overall respiration, these experiments indicated that there was no excess of ND5 protein synthesis at all over what was needed to support the wild-type rate of respiration. Perhaps an ND5-specific translation factor determines the maximum rate of ND5 polypeptide synthesis in wild-type cells, and, consequently, the rate of complex I-dependent respiration.

Mutations, which do not involve ND genes, can have a specific effect on ND gene expression. For example, the $tRNA^{Lys}$ gene mutation at position 8344, that causes the MERRF syndrome (myoclonic epilepsy and ragged red fibers) (Shoffner *et al.*, 1990) would be expected to affect translation of any mitochondrial reading frame containing one or more lysine codons. In fact, a very strong

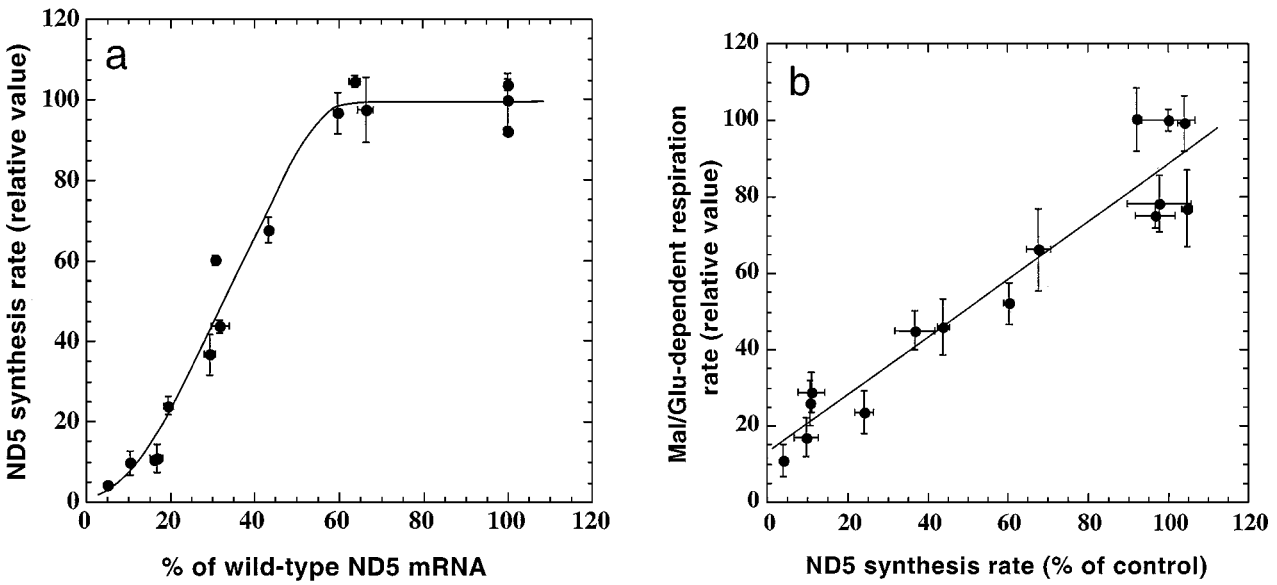

Fig. 2. (a) ND5 synthesis rate versus the amount of wild-type ND5 mRNA. The synthesis rate of ND5 polypeptide, expressed relative to the rate in one of the wild-type mitochondrial transformant clones, was determined in several clones that were wild-type or heteroplasmic for the ND5 mutation at position 12108, by labeling mitochondrial proteins with [^{35}S]methionine in the presence of emetine, and electrophoresing the mitochondrial lysates on an SDS–polyacrylamide gel. The bands corresponding to ND5 were quantified by laser densitometry or PhosphorImager analysis. These data were plotted versus the proportion of wild-type ND5 mRNA in the same clones, determined by reverse transcriptase PCR and ClaI digestion. (b) Malate/glutamate-dependent respiration rate versus ND5 synthesis rate. Malate/glutamate-dependent respiration rates in the clones used in the experiments of (a) were measured by polarography carried out on digitonin-permeabilized cells. These data were plotted against the ND5 polypeptide synthesis rates determined in (a). Taken from Bai *et al*. (2000).

negative (exponential) correlation was observed in mutant cells between the residual rate of translation of a given reading frame and the number of lysine codons in that reading frame (Enriquez *et al.*, 1995). The reading frame with the highest number of lysine codons is ND5, with 21 lysine codons, and its translation rate in cells carrying the mutation in 99% of their mitochondrial genomes is decreased to virtually zero. On the other hand, the complex IV subunit reading frame with the most lysine codons is CO I, with ten lysine codons. Its translation rate is decreased to 10% of the normal rate in the same cells.

Given these results, one would expect complex I activity to be more affected in these mutant cells than complex IV activity and, in fact, this is the case (Villani and Attardi, 1997). The difference in enzyme deficiencies was shown by a KCN titration assay in which the respiration rate in intact cells was measured with incremental additions of KCN to the oxygraph chamber. Because this respiration rate measures actual cytochrome *c* oxidase activity, but is driven by endogeous substrates feeding into the respiratory chain upstream of cytochrome *c* oxidase, it is referred to as "cytochrome *c* oxidase (cox) integrated step." This respiration rate, under these assay conditions, is limited by complex I. The results of this KCN titration were then compared with the results of a KCN titration of the rate of complex IV-dependent respiration in intact cells, driven by the membrane permeant electron carrier *N, N, N′, N′*-tetramethyl-*p*-phenylenediamine (TMPD). The TMPD is reduced outside the cells by ascorbate. This latter respiration measurement is referred to as "cox isolated step." In control cells, KCN inhibited endogenous substrate-dependent respiration (i.e., complex I-dependent respiration) at a higher concentration than was needed to inhibit complex IV-dependent respiration to the same degree. This indicated that there was an excess of complex IV activity over what was needed to support the endogenous substrate-dependent respiration. In the cells carrying the G8344A mutation in the $tRNA^{Lys}$ gene, this difference in KCN concentrations was much greater, indicating that there was a much greater excess of complex IV activity over complex I activity in these cells than in control cells.

PERSPECTIVES

There is still much we do not know about the mtDNA-encoded subunits of complex I. The isolation of more ND gene mutants, using the approach discussed above, would be important for learning which subunits are essential for

assembly of the enzyme complex and what the role of each subunit in the complex is. Furthermore it would be interesting to determine whether the synthesis of any other ND polypeptides besides ND5 were rate limiting for complex I-dependent respiration and whether there is a control at the level of translation by specific factors. Last, it is expected that the ND gene mutations present in the mouse rotenone-resistant cell lines can be transferred to mouse embryos to produce mouse models of mitochondrial disease.

ACKNOWLEDGMENTS

I thank Giuseppe Attardi for helpful comments on the manuscript. I thank also the National Institutes of Health (Grant GM-11726 to Giuseppe Attardi) for support.

REFERENCES

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J., Staden, R., and Young, I. G. (1981). *Nature (London)* **290**, 457–465.

Attrdi, G., Chomyn, A., and Mariottini, P. (1984). In *H^+ATPase (ATP synthase): Structure, Function, Biogenesis. The F_0F_1 Complex of Coupling Membranes* (Papa, S., Altendorf, K., Ernster L., and Packer, L., eds.), Bari, Adriatica Editrice, pp. 25–40.

Attardi, G. (1985). *Intern. Rev. Cytol.* **93**, 93–145.

Attardi, G., Chomyn, A., and Loguercio Polosa, P. (1989). In *Advances in Myochemistry* (Benzi, G., ed.), Montrouge, John Libbey Eurotext Ltd., pp. 55–64.

Bai, Y., and Attardi, G. (1998). *EMBO J.* **17**, 4848–4858.

Bai, Y., Shakeley, R. M., and Attardi, G. (2000). *Mol. Cell. Biol.* **20**, 805–815.

Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W., and Clayton, D. A. (1981). *Cell* **26**, 167–180.

Brown, M. D., Trounce, I. A., Jun, A. S., Allen, J. C., and Wallace, D. C. (2000). *J. Biol Chem.* **275**, 39831–39836.

Chang, D. D., and Clayton, D. A. (1985). *Proc. Natl. Acad. Sci. USA* **82**, 351–355.

Ching, E., and Attardi, G. (1982). *Biochemistry* **21**, 3188–3195.

Chomyn, A., Mariottini, P., Gonzalez-Cadavid, N., Attardi, G., Strong, D. D., Trovato, D., Riley, M., and Doolittle, R. F. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 5535–5539.

Chomyn, A., Mariottini, P., Cleeter, M. W., Ragan, C. I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R. F., and Attardi, G. (1985). *Nature (London)* **314**, 592–597.

Chomyn, A., Cleeter, M. W., Ragan, C. I., Riley, M., Doolittle, R. F., and Attardi, G. (1986). *Science* **234**, 614–618.

Chomyn, A., Patel, S. D., Cleeter, M. W., Ragan, C. I., and Attardi, G. (1988). *J. Biol. Chem.* **263**, 16395–16400.

Degli Esposti, M., Carelli, V., Ghelli, A., Ratta, M., Crimi, M., Sangiorgi, S., Montagna, P., Lenaz, G., Lugaresi, E., and Cortelli, P. (1994). *FEBS Lett.* **352**, 375–379.

Doersen, C. J., Guerrier-Takada, C., Altman, S., and Attardi, G. (1985). *J. Biol. Chem.* **260**, 5942–5949.

Earley, F. G., Patel, S. D., Ragan, I., and Attardi, G. (1987). *FEBS Lett.* **219**, 108–112.

Endicott, J. A., and Ling, V. (1989). *Annu. Rev. Biochem.* **58**, 137–171.

Enriquez, J. A., Chomyn, A., and Attardi, G. (1995). *Nat. Genet.* **10**, 47–55.

Galante, Y. M., and Hatefi, Y. (1978). *Methods Enzymol.* **53**, 15–21.

Gelfand, R., and Attardi, G. (1981). *Mol. Cell Biol.* **1**, 497–511.

Grigorieff, N. (1998). *J. Mol. Biol.* **277**, 1033–1046.

Guan, M. X., Enriquez, J. A., Fischel-Ghodsian, N., Puranam, R. S., Lin, C. P., Maw, M. A., and Attardi, G. (1998). *Mol. Cell. Biol.* **18**, 5868–5879.

Hatefi, Y., Galante, Y. M., Stiggall, D. L., and Ragan, C. I. (1979). *Methods Enzymol.* **56**, 577–602.

Heron, C., Smith, S., and Ragan, C. I. (1979). *Biochem. J.* **181**, 435–443.

Hofhaus, G., and Attardi, G. (1993). *EMBO J.* **12**, 3043–3048.

Hofhaus, G., and Attardi, G. (1995). *Mol. Cell Biol.* **15**, 964–974.

Hofhaus, G., Weiss, H., and Leonard, K. (1991). *J. Mol. Biol.* **221**, 1027–1043.

Hofhaus, G., Johns, D. R., Hurko, O., Attardi, G., and Chomyn, A. (1996). *J. Biol. Chem.* **271**, 13155–13161.

Howell, N., Kubacka, I., Xu, M., and McCullough, D. A. (1991). *Amer. J Human Genet.* **48**, 935–942.

Jun, A. S., Brown, M. D., and Wallace, D. C. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 6206–6210.

King, M. P., and Attardi, G. (1989). *Science* **246**, 500–503.

King, M. P., and Attardi, G. (1993). *J. Biol. Chem.* **268**, 10228–10237.

Loguercio Polosa, P., and Attardi, G. (1991). *J. Biol. Chem.* **266**, 10011–10017.

Maquat, L. E. (1988). *RNA* **1**, 453–465.

Mariottini, P., Chomyn, A., Attardi, G., Trovato, D., Srong, D. D., and Doolittle, R. (1983). *Cell* **32**, 1269–1277.

Mariottini, P., Chomyn, A., Riley, M., Cottrell, B., Doolittle, R. F., and Attardi, G. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 1563–1567.

Montoya, J., Ojala, D., and Attardi, G. (1981). *Nature (London)* **290**, 465–470.

Montoya, J., Christianson, T., Levens, D., Rabinowitz, M., and Attardi, G. (1982). *Proc. Natl. Acad. Sci. USA* **79**, 7195–7199.

Ojala, D., Montoya, J., and Attardi, G. (1981). *Nature (London)* **290**, 470–474.

Pulkes, T., Siddiqui, A., Morgan-Hughes, J. A., and Hanna, M. G. (2000). *Neurology* **55**, 1210–1212.

Puranam, R. S., and Attardi, G. (2001). *Mol. Cell Biol.* **21**, 548–561.

Reid, F. M., Vernham, G. A., and Jacobs, H. T. (1994). *Human Muta.* **3**, 243–247.

Sazanov, L. A., and Walker, J. E. (2000). *J. Mol. Biol.* **302**, 455–464.

Sazanov, L. A., Peak-Chew, S. Y., Fearnley, I. M., and Walker, J. E. (2000). *Biochemistry* **39**, 7229–7235.

Shoffner, J. M., Lott, M. T., Lezza, A. M., Seibel, P., Ballinger, S. W., and Wallace, D. C. (1990). *Cell* **61**, 931–937.

Skehel, J. M., Fearnley, I. M., and Walker, J. E. (1998). *FEBS Lett.* **438**, 301–305.

Srere, P. A. (1991). *J. Theoret. Biol* **152**, 23.

Sumegi, B., and Srere, P. A. (1984). *J. Biol. Chem.* **259**, 15040–15045.

Tawata, M., Hayashi, J. I., Isobe, K., Ohkubo, E., Ohtaka, M., Chen, J., Aida, K., and Onaya, T. (2000). *Diabetes* **49**, 1269–1272.

Villani, G., and Attardi, G. (1997). *Proc. Natl. Acad. Sci. USA* **94**, 1166–1171.

Walker, J. E. (1992). *Quart. Rev. Biophys.* **25**, 253–324.

Wallace, D. C., Singh, G., Lott, M. T., Hodge, J. A., Schurr, T. G., Lezza, A. M., Elsas, L. J. D., and Nikoskelainen, E. K. (1988). *Science* **242**, 1427–1430.

Wallace, D. C., Lott, M. T., Shoffner, J. M., and Brown, M. D. (1992). *J. Inherit. Metab. Dis.* **15**, 472–479.

Walter, G., Scheidtmann, K. H., Carbone, A., Laudano, A. P., and Doolittle, R. F. (1980). *Proc. Natl. Acad. Sci. USA* **77**, 5197–5200.