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Mitochondrial Mutations Impair Signal Transduction in *Dictyostelium discoideum* Slugs

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Subpopulations of mutant mitochondria appear to play important roles in degenerative processes associated with aging and are characteristic of many mitochondrial diseases. We have generated mutants carrying plasmid insertions in the *Dictyostelium discoideum* mitochondrial genome and have shown that phototaxis and thermotaxis in these mutants is more sensitive than growth and division to the presence of a subpopulation of defective mitochondria. This could result from direct impairment of a mitochondrial role in signal transduction, or indirectly from the effects of energy depletion. Either way, signal transduction may be the first cellular activity to be compromised by the accumulation of defective mitochondria in age-related tissue dysfunction and in mitochondrial disease.

The mitochondria of modern eukaryotic organisms have ceded almost all of their putative primeval genetic functions to the nuclear genome and are functionally fully integrated into the cells that carry them. They can exchange with other cellular compartments not just the intermediates of energy metabolism and the necessary imported proteins, but also RNA (1-4) and DNA (5-7). Neither are the known exchanges between mitochondria and the rest of the cell limited to energy metabolites and macromolecules. Information is also exchanged in the form, for example, of signals passing in both directions between mitochondria and nucleus to coordinate gene expression in both compartments (8-10), or of interactions between Ca^{2+} signals in the mitochondria and the cytosol (11-15). Mitochondria may thus be fully integrated into the eukaryotic cell's signalling systems as well as its metabolism. If this is so, then intracellular signal transduction should be impaired by mitochondrial mutations. The presence of subpopulations of mutant mitochondria is believed to contribute to the ageing process and is characteristic of mitochondrial cytopathies, degenerative diseases that primarily affect central nervous system, heart and muscle tissues (16-18). Age-related tissue dysfunction and the pathology of mitochondrial diseases could both be explained partly by defective signal transduction arising either indirectly from energy depletion or from direct impairment of a mitochondrial role in intracellular signalling. In support of this view, we report here that disruption of the large subunit rRNA gene in a subpopulation of mitochondria impairs photosensory and thermosensory signal transduction in the multicellular ("slug") stage of the life cycle of *Dictyostelium discoideum*.

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MATERIALS AND METHODS

Strains. The *Dictyostelium discoideum* mutant strain HPF231 (19) was constructed by random insertion of the *D. discoideum* transformation vector pDNeo2 (20) into the genome of AX2 (21), a derivative of the wildtype strain NC4. HPF266-HPF270, HPF272 and HPF273 were isolated in targeted disruption experiments using cloned fragments of the mitochondrial large ribosomal RNA gene to target insertions to the mitochondrial genome.

Phototaxis. Amoebae were transferred by toothpick to non-nutrient charcoal agar plates (1.0% agar, 0.5% activated charcoal) from the edges of isolated colonies of each mutant growing on lawns of the bacterial food source *Klebsiella aerogenes*. After incubation with a lateral light source at 21°C for 24-48h the starving amoebae had aggregated to form slugs which had migrated over the agar surface leaving behind the usual trail of collapsed slime sheath. The plates were inspected and scored for normal or defective phototaxis as previously (22). Trails of slugs were transferred to PVC discs, stained and digitized as previously(22), then plotted from a common origin.

Thermotaxis. For thermotaxis amoebae were transferred by sterile toothpick from colonies on bacterial lawns to water agar plates (22). Plates were incubated in light-tight PVC boxes on the surface of an insulated heat bar that provided a temperature gradient at the agar surface of about 0.2°C/cm and a temperature at the centre of the plate of about 22°C (22). After 48 hours slugs and their trails were transferred by contact onto the surface of a clear PVC disc, stained and the directions of travel digitized (22). Statistical analysis of the directions of travel yielded estimates of the accuracy of thermotaxis as measured by the concentration parameter (κ) of the von Mises distribution, an analogue for directional data of the Gaussian distribution.

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Growth experiments. Generation times were measured during exponential growth of AX2 and mitochondrial mutants. Cultures (50 ml HL-5 medium in Costar 100ml flat tissue culture bottles) were shaken at 150 rpm at 21°C. Cell counts were performed using a haemocytometer and generation times were estimated by linear regression analysis of the growth curves in log-linear plots during the exponential phase of growth.

Pulsed-field gel electrophoresis and Southern blot analysis. For PFGE the cells were prepared as described previously (23). After being washed in phosphate buffer (17 mM Na/K phosphate, pH 6.0) cells were mixed with an equal volume of 2% (w/v) InCert phosphate buffered agarose (FMC Bioproducts) which had been precooled to 39°C. The final cell density was $4\text{-}5\times10^8$ cells/ml. Mitochondria were released by mechanical rupture of cells either with a Potter Homogenizer or by vortexing with 425-600 μm diameter glass beads (Sigma). They were purified by differential and Percoll gradient centrifugation from total cell extract prior to embedding in agarose (24). The solidified agarose blocks ($\sim30\mu$l) were incubated in digestion buffer (0.5M EDTA, pH 8.0; 2% sodium lauroyl-sarcosinate; 2 mg/ml Proteinase K) for 48h at 50°C. For restriction enzyme digestion the agarose plugs were repeatedly washed in TE (the first washes containing 1 mM Phenylmethylsulfonyl fluoride) and incubated overnight at 37°C in 50U of restriction enzyme. PFGE was performed using a BioRad CHEF apparatus (Contour-clamped Homogeneous Electric Fields) (25). CHEF gels were 1% agarose in 0.5×TBE running buffer. PFGE was for 22h, 60-120s ramped switching time, at 200V and 14°C. Southern transfer was by acid depurination and alkaline transfer to nylon membranes (Hybond, Amersham). The blots were hybridized using as a DIG-labelled probe either pUC19 DNA or a 785 bp DIRS1 fragment from AX2 genomic DNA that was amplified by PCR (using primers dirs1a [tcgattcggtatcaatgaagc] and dirs1b [ttgtatgcgattgaacacttcc]).

RESULTS AND DISCUSSION

We recently isolated a series of phototaxis deficient mutants of the cellular slime mould *Dictyostelium discoideum* by nontargeted or random integration of plasmids (RIP) into genes important for photosensory transduction (19). The resulting insertions normally contain multiple, sometimes rearranged copies of the inserted plasmid. A portion of the disrupted gene responsible for the phototaxis deficiency in one of the mutants, HPF231, was cloned by replicon rescue, a novel strategy in which a tetracycline resistance cassette is used to selectively clone fragments from the insertion boundary that carry a plasmid origin of replication (26). The cloned fragment was shown to carry part of a gene important for phototaxis by means of targeted disruption experiments in which cells were transformed with either the original clone or a fragment subcloned into the *Dictyostelium* shuttle vector pDNeo2 (20). In all experiments 25-40% of the transformants were deficient in phototaxis, compared to the normal frequency for nontargeted insertions of less than 0.2%. These results showed that the cloned sequences facilitated the targeted insertion of the plasmid into a gene important for phototaxis (26). Such targeted insertions in *Dictyostelium* occur by homologous recombination and provide a routine means of verifying the biological function of cloned genes (27). Seven of the putative targeted disruptants (HPF266-270, HPF272, HPF273) were retained for further study. Their phototaxis phenotypes are illustrated in Fig. 1.

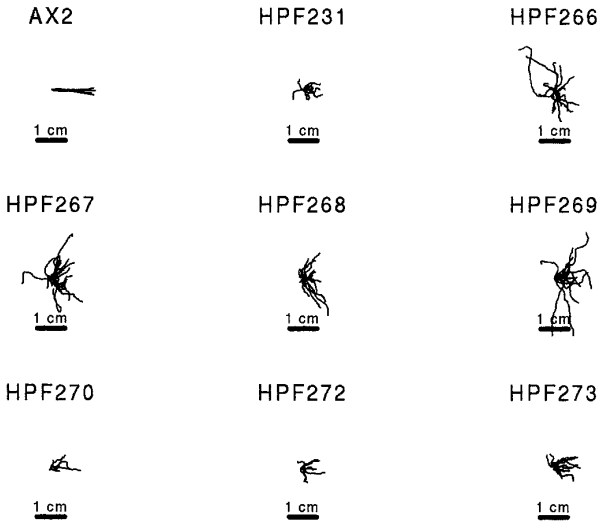

FIG. 1. Phototaxis deficient phenotypes of mitochondrial insertion mutants of *Dictyostelium discoideum*. The light source was to the right. Whereas slugs of the parental strain AX2 migrated directly towards the light source, all of the mitochondrial mutant strains were highly disoriented. HPF231 is the original nontargeted disruptant whereas HPF266-270, HPF272 and HPF273 are targeted disruptants.

To determine the identity of the cloned gene(s) important for phototaxis, the recovered fragment was subcloned and sequenced. Searches through the Genbank and EMBL data bases revealed a strong homology to a variety of bacterial, mitochondrial and chloroplast large ribosomal RNA genes. Southern blotting against pulsed field gels of whole cell, nuclear and mitochondrial preparations confirmed a mitochondrial location for the cloned fragment (results not shown). It was thus identified as the partial sequence of the *D. discoideum* mitochondrial large ribosomal RNA gene (here designated *rnlA* by agreement with Dr. Y. Tanaka, University of Tsukuba) and was submitted to Genbank under the accession number U21880. The subsequent release of a 13.2 kb mitochondrial DNA sequence by Angata *et al.* (28,29) confirmed the identity of the cloned fragment. Comparison of the sequence with that of the *E. coli rrnB* gene (30,31) maps the apparent position of the pDNeo2 insertion in the *Dictyostelium* mitochondrial gene to the region of rRNA most strongly implicated in ribosomal peptidyl transferase activity (31). Presumably an insertion at this site would interfere with mitochondrial protein synthesis resulting in a defective mitochondrion that is unable to perform respiratory electron transport.

To confirm that plasmid molecules had indeed inserted into the mitochondrial genome, Southern blots from pulsed field gels containing *Avr*II digested DNA of the *Dictyostelium* wildtype strain AX2 and the mutant

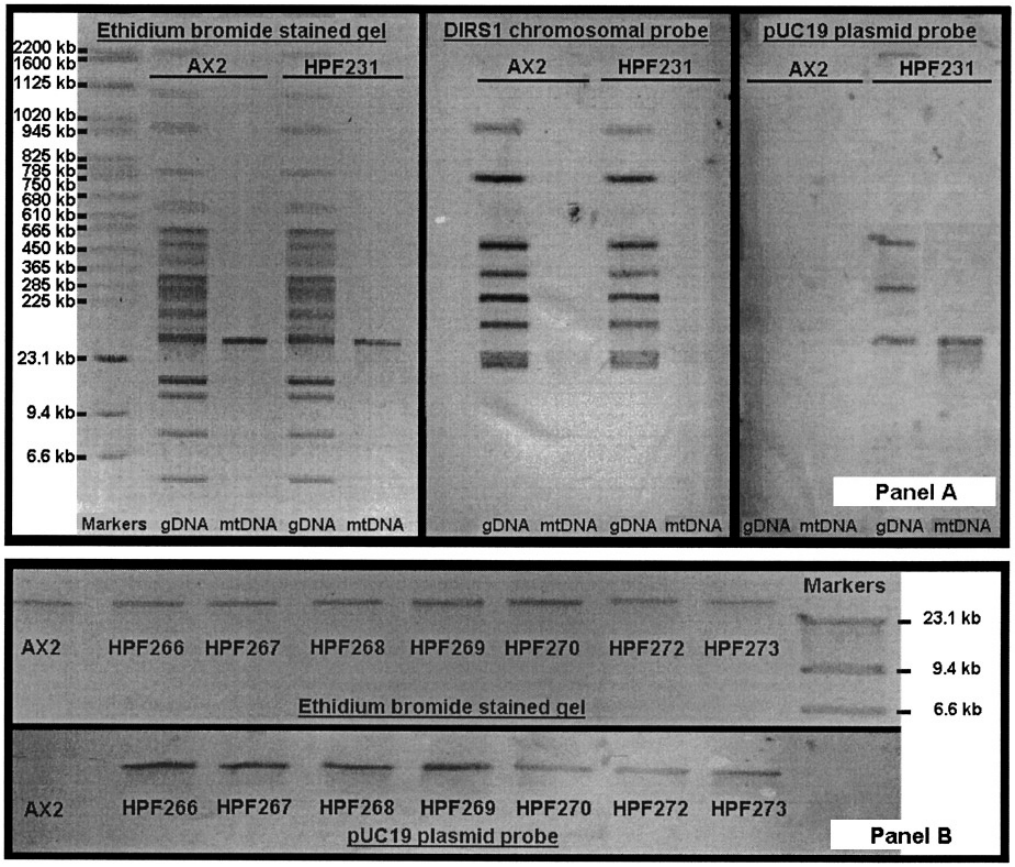

FIG. 2. Pulsed field gel electrophoresis (PFGE) mapping of plasmid insertions in mitochondrial disruptants of wild-type strain AX2. DNA of whole cell (gDNA) and mitochondrial (mtDNA) preparations of AX2 and the nontargeted disruptant HPF231 (Panel A) and mitochondrial DNA of 7 targeted disruptants HPF266-270,HPF272,HPF273 (Panel B) were digested with *Avr*II and separated by PFGE. Shown are ethidium bromide stained gels and Southern blots using either plasmid pUC19 or a DIRS1 fragment as a probe. pUC19 forms the bacterial plasmid backbone of the shuttle vector pDNeo2 which is present in the insertions. The plasmid insertions contain no *Avr*II sites so upon complete digestion each band corresponds to a separate insertion site. The 550 kb band hybridizing to pUC19 in HPF231 gDNA and the 950 kb bands hybridizing to DIRS1 in AX2 and HPF231 gDNA may be partial digestion products.

HPF231 were probed with plasmid (pUC19) DNA. The results revealed the presence of plasmid sequences in the mtDNA band in both whole cell and purified mitochondrial preparations from the mutant (Fig. 2, Panel A). In order to exclude possible chromosomal and therefore plasmid DNA contamination of the mitochondrial preparations, a 785 bp probe for the chromosomal inverted repeat sequence DIRS1 (32) was amplified by PCR from genomic DNA of the untransformed parent strain AX2 (preparation of the DIRS1 probe in this way excluded any possible plasmid contamination of the probe itself). In Southern blots of *Avr*II digests of whole cell and mitochondrial preparations in pulsed field gels, the DIRS1 probe hybridized to 7 bands corresponding to its known 7 chromosomal locations in *Dictyostelium* (33), but did not hybridize at all to the mitochondrial DNA preparation of HPF231 (Fig. 2, Panel A). Since about 40 copies of DIRS1 are present in the nuclear genome (32), the lack of hybridization of the DIRS1 probe to the mtDNA confirmed that nuclear contamination of the mitochondrial preparations could not explain the strong hybridization of plasmid to them. Similar experiments with the 7 targeted disruptants (HPF266-270,HPF272,HPF273) confirmed that each of them contained plasmid sequences inserted into the mitochondrial genome (Fig. 2, Panel B). Other transformants in our collection, phototaxis deficient or otherwise, do not exhibit hybridization of plasmid to the mitochondrial genome in Southern blots of pulsed field gels (not shown).

As well as the mitochondrial DNA band, HPF231 (Fig. 2, Panel A) and all targeted disruptants (not shown) contained additional bands in the whole cell preparations that hybridized to plasmid. We presume these additional chromosomal insertion(s) to be a result of selection for transformants on the basis of G418 (geneticin) resistance conferred in the vector pDNeo2 by the *Tn903* neomycin phosphotransferase gene under the control of the *Dictyostelium* actin 6 gene promoter (20). This nuclear promoter would presumably be inac-

tive in the mitochondria. It remains theoretically possible that in any given mutant the phototaxis deficient phenotype is conferred by a chromosomal insertion. However this hypothesis cannot explain why the mitochondrial gene should target such insertions so effectively to phototaxis genes, nor why the resulting deficiencies in phototaxis (Fig. 1) should always be accompanied by plasmid insertions into mitochondrial DNA.

Mitochondrial cytopathies are a subclass of human oxidative phosphorylation diseases that arise from mtDNA mutations of various kinds (insertions, deletions, base substitutions) in a variety of different genes, including the large and small subunit rRNA genes (16-18). In most cases they are characterized by heteroplasmy, the state in which each cell contains at least two populations of mitochondria, mutant and normal, with the defective mitochondrial genomes being in the minority. We showed that our mutants are heteroplasmic for plasmid insertions by demonstrating that in ethidium bromide stained gels, the wild-type pattern of the mtDNA bands was present. It is characteristic of heteroplasmic mtDNA mutations that the phenotype is variably expressed depending on the proportion of defective mitochondria (16-18). Consistent with this, we noticed during screening of transformants for impaired phototaxis that the severity of the deficiency varied significantly amongst the mutants.

To determine whether the signal transduction defect caused by disruption of the mitochondrial genome was specific to phototaxis, for example via deranged photoreceptor synthesis, we also examined the mutant phenotypes in thermotaxis. *D. discoideum* is positively thermotactic (migrates towards warmth) with maximum sensitivity at temperatures close to the growth temperature (22). HPF231 and all 7 targeted disruptants were severely disabled in thermotaxis (Table 1), showing that the mitochondrial defects impair signal processing downstream of the previously demonstrated convergence (22) between the photosensory and thermosensory transduction pathways.

A plasmid insertion into the mitochondrial genome could cause deranged signal transduction by direct impairment of a mitochondrial role in intracellular signalling, as also implied by recent work demonstrating interactions between mitochondrial and cytosolic Ca^{2+} signals (11-13). An alternative explanation is that signal transduction might be impaired indirectly by ATP depletion of the more active anterior cells that control behaviour in the multicellular ("slug") stage of the *Dictyostelium* life cycle. In either case, photosensory and thermosensory responses could be more sensitive to the presence of defective mitochondria than other energy dependent cellular activities, such as growth and division. We therefore measured the generation times of HPF231, the 7 targeted disruptants and the parental strain AX2. HPF231 and one of the targeted disruptants (HPF272) grew slightly more slowly than wild type (Table 1). Of the other disruptants, three showed markedly slower growth than AX2 and three showed normal growth rates (Table 1). Phototaxis and thermotaxis by all mutants were severely deficient, showing that their responses to extracellular signals are more sensitive than growth to impairment by mtDNA defects. Whether the effects are direct or are pleiotropic consequences of ATP depletion, the results suggest that deranged signal transduction may indeed be the phenotype most sensitive to the presence of defective mitochondria. This hypothesis could also explain the "bang-sensitive" behaviour conferred by the *tko* mutation in *Drosophila*, which involves a defect in the mitochondrial ribosomal protein S12 (34). The tissues most commonly affected by mitochondrial cytopathies (central nervous system, muscle and heart) have in common not only high energy demands, but also a heavy dependence on transduction of extracellular signals for regulation of their normal activities. An obvious inference is that disturbances in signal transduction arising from the presence of defective mitochondria could play an important role in the pathology of mitochondrial disease.

TABLE 1

Thermoxtaxis and Generation Times of Phototaxis-Deficient Mitochondrial Mutants

Strain	Accuracy of thermotaxis (κ)	Generation time (h)
AX2	10.5 (5.6, 16.9)	13.0 (10.8, 16.2)
HPF231	0.55 (0.04, 1.10)	16.7 (16.1, 17.3)
HPF266	1.32 (0.80, 1.91)	29.6 (26.6, 33.4)
HPF267	1.14 (0.39, 2.00)	13.2 (11.8, 15.1)
HPF268	0.67 (0.17, 1.21)	26.3 (24.3, 28.7)
HPF269	0.11 (0, 0.49)	34.3 (27.8, 44.8)
HPF270	0.97 (0.51, 1.47)	12.9 (10.8, 16.2)
HPF272	0.02 (0, 0.39)	15.6 (13.5, 18.6)
HPF273	0.47 (0.06, 0.91)	12.8 (11.2, 15.1)

Note. AX2 is the wildtype parental strain. HPF231 is the original nontargeted disruptant, while strains HPF266-HPF270, HPF272 and HPF273 were isolated in targeted disruption experiments. κ is the concentration parameter of the Mises distribution (see Materials and Methods) and ranges from 0 in the case of no orientation (all directions equally probable) to infinity in the case of perfect orientation (all directions identical). Parentheses include lower and upper 90% confidence intervals. A lower confidence limit of zero indicates no significant thermotactic orientation. The generation times refer to doubling time during exponential growth in HL-5 medium shaken at 150 rpm at 21°C. Parentheses include lower and upper 95% confidence limits.

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