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In Vitro Analysis of Mutations Causing Myoclonus Epilepsy with Ragged-Red Fibers in the Mitochondrial tRNA^{Lys} Gene: Two Genotypes Produce Similar Phenotypes

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Cytoplasts from patients with myoclonus epilepsy with ragged-red fibers harboring a pathogenic point mutation at either nucleotide 8344 or 8356 in the human mitochondrial tRNA^{Lys} gene were fused with human cells lacking endogenous mitochondrial DNA (mtDNA). For each mutation, cytoplasmic hybrid (cybrid) cell lines containing 0 or 100% mutated mtDNAs were isolated and their genetic, biochemical, and morphological characteristics were examined. Both mutations resulted in the same biochemical and molecular genetic phenotypes. Specifically, cybrids containing 100% mutated mtDNAs, but not those containing the corresponding wild-type mtDNAs, exhibited severe defects in respiratory chain activity, in the rates of protein synthesis, and in the steady-state levels of mitochondrial translation products. In addition, aberrant mitochondrial translation products were detected with both mutations. No significant alterations were observed in the processing of polycistronic RNA precursor transcripts derived from the region containing the tRNA^{Lys} gene. These results demonstrate that two different mtDNA mutations in tRNA^{Lys}, both associated with the same mitochondrial disorder, result in fundamentally identical defects at the cellular level and strongly suggest that specific protein synthesis abnormalities contribute to the pathogenesis of myoclonus epilepsy with ragged-red fibers.

Myoclonus epilepsy with ragged-red fibers (MERRF) is a maternally inherited disorder characterized by myoclonus, myoclonus epilepsy, ataxia, and mitochondrial myopathy with ragged-red fibers (indicative of mitochondrial proliferation in muscle). Recent work has associated MERRF with two different point mutations, both in the mitochondrial DNA (mtDNA)-encoded tRNA^{Lys} gene: an A→G transition at nucleotide (nt) 8344 (45, 60) and a T→C transition at nt 8356 (47, 62) (mtDNA numbering is according to Anderson et al. [1]). The nt 8344 mutation alters a highly conserved base in the TΨC loop of the tRNA; the nt 8356 mutation interrupts a highly conserved base pair in the TΨC stem of the tRNA. The MERRF nt 8344 (MERRF-8344) mutation has been analyzed previously in muscle cells (6) and in cytoplasmic hybrid cells (cybrids [12]) and has been associated with severe defects in protein synthesis and respiratory chain activity. No similar analyses have been performed on the MERRF-8356 mutation.

We have used the ρ^0 cell culture system to address further the mechanisms of pathogenesis of the point mutations associated with MERRF. This system is based upon the repopulation of human cells which are completely devoid of mtDNA (ρ^0 cells) with exogenous mitochondria containing the mtDNA of interest (30). We repopulated ρ^0 cells with mtDNA from two unrelated MERRF patients harboring each of the two different tRNA^{Lys} point mutations and created clonal cybrids. Cybrids containing either 100% mutated or 100% wild-type mtDNAs were selected for molecular genetic, biochemical, and morphological analyses.

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MATERIALS AND METHODS

Cell culture. The 143B and 143B206 cell lines, which have been described previously (30), were grown in Dulbecco's modified Eagle's medium containing 4.5 mg of glucose per ml and 110 μg of pyruvate per ml (DMEM) supplemented with 100 μg of 5-bromodeoxyuridine per ml and 5% fetal bovine serum (FBS). The medium for the 143B206 cell line was additionally supplemented with 50 μg of uridine per ml. Our patient 1 was patient 3 of Graf et al. (20), and our patient 2 was patient 5 of Silvestri et al. (47). Fibroblasts from patient 1 (nt 8344 mutation; a gift from M. Sumi) and patient 2 (nt 8356 mutation; a gift from S. Oh) were grown in DMEM supplemented with 15% FBS. Transformation of the 143B206 cell line with patient mitochondria by cytoplast fusion was performed as described previously (30). Cybrids were replated 24 h after fusion in DMEM supplemented with 100 μg of 5-bromodeoxyuridine per ml and 5% dialyzed FBS (selective medium). For cybrids derived from patient 1, independent cell clones were isolated by trypsinization in glass cloning cylinders 13 to 18 days after fusion. For patient 2, cybrids were replated at a low cell density in selective medium to pick cell clones. Cybrid cell clones derived from patient 2 were independent on the basis of previously established criteria (32). Cells were subsequently cultured in DMEM supplemented with 5% FBS.

DNA analyses. To detect the MERRF-8344 mutation, PCR primers were synthesized which spanned the mtDNA (5′→3′) at nt 7955 to 7979 (forward) and 8372 to 8345 (reverse); the reverse primer contained a T→G mismatch at position 8347 that creates an additional recognition site for restriction endonuclease *Ban*II in mutated, but not in normal, mtDNA (46). To detect the MERRF-8356 point mutation, primers were synthesized which spanned the mtDNA at nt 8239 to 8263 and 8380 to 8357; the reverse primer contained a G→T mismatch at nt 8357 which, in combination with the wild-type sequence at position 8356, creates a *Dra*I restriction site in normal, but not in mutated, mtDNA (47). DNA amplifications were performed under standard conditions with the addition of 5 μCi of [α-^{32}P]dATP. An additional fragment amplified with primers spanning the mtDNA at nt 10354 to 10378 and 10542 to 10517 and containing one internal *Dra*I restriction site was included in each MERRF-8356 restriction digest as a control for a complete digestion of the PCR fragments. Ten percent of the PCR product was digested with the appropriate restriction enzyme and electrophoresed through a 12% nondenaturing polyacrylamide gel and exposed to Kodak XAR film. Quantitation of electrophoretically separated restriction digestion products was performed with a Phosphorimager SF (Molecular

Dynamics). All of the cell lines tested negative for the presence of mycoplasma by Southern blot hybridization analysis with pSP64-129, which contains a fragment isolated from M13Mh129 (18). Nuclear DNA markers were examined by PCR analysis of dinucleotide repeat markers (51, 54) of DNA isolated from cybrids, 143B and 143B206 cells, and patients' fibroblasts. Primer pairs D17S950F-D17S950R and D17S931F-D17S931R were used to detect polymorphic DNA sequences on chromosome 17, and primer pair D20S192F-D20S192R was used to detect polymorphic DNA sequences on chromosome 20 (57). In addition, the mtDNA contents of the cybrids and 143B cell line were analyzed by measuring the ratio of mtDNA to nuclear DNA in Southern blot hybridization analyses (2, 38). Southern blots of *Pvu*II-digested DNAs were hybridized with probes corresponding to the ND1 (subunit 1 of NADH dehydrogenase) region of the mtDNA (mp19.BR54 [31]) and to the 18S rRNA gene (pB [59]) and quantitated on a Betascope 603 Blot Analyzer (BetaGen).

Oxygen consumption. Oxygen consumption by intact cells was determined as described previously (32).

Cytochemistry and immunocytochemistry. Cells grown on glass coverslips were stained cytochemically for cytochrome *c* oxidase (COX) and succinate dehydrogenase activities as previously described (32). Immunocytochemical staining to detect COII (subunit II of COX), ND1, and the lipoyl acetyltransferase (E2) portion of the pyruvate dehydrogenase complex was performed as described previously (32, 36, 37). Polyclonal antibodies to COII and ND1 (gifts of J. R. Doolittle) were used at a dilution of 1:250. Polyclonal antibodies to pyruvate dehydrogenase (50) were diluted 1:200. The cells were photographed with a Zeiss II photomicroscope equipped with epi-illumination.

Analysis of mitochondrial translation products. Exponentially growing cybrids, 143B cells, and 143B206 cells were labeled with [^{35}S]Translabel (>1,000 Ci/mmol, 100 to 200 μCi/ml; Dupont NEN) for 10 to 90 min in 2 ml of methionine- and cysteine-free DMEM supplemented with 100 μg of emetine per ml and 5% dialyzed FBS as described previously (12). Generally, protein labelings were performed on cells attached to petri dishes; the cells were then trypsinized, centrifuged, and resuspended in 1% sodium dodecyl sulfate (SDS)–10 mM Tris-HCl (pH 8.0). In addition, some of the protein labelings were performed without prior trypsinization; the labelings were stopped by addition of a solution of 0.5% SDS–5 mM Tris-HCl (pH 8.0) directly to the cells. Labeled proteins were separated on SDS–15 to 20% polyacrylamide exponential gradient gels (10) and subjected to fluorography on Kodak XAR film or analyzed with a Phosphorimager. The assignment of mtDNA-encoded translation products was determined by their similarity to those described by Chomyn and Lai (10) and by their corresponding absence in a parallel experiment performed with 143B206 cells lacking mtDNA. Equal amounts of total cellular protein or a mitochondrial fraction (10 to 25 μg; isolated by differential centrifugation [3]), as determined by the method of Bradford (8), were loaded in each lane in a given experiment. Quantitation of mtDNA-encoded polypeptides from short-term labeling (10 to 30 min) and long-term labeling (80 to 90 min) experiments was performed with a Phosphorimager.

Northern (RNA) blot analyses. Total RNA was isolated from exponentially growing cells (53) and electrophoresed through 1.4% agarose–formaldehyde gels (34), transferred to Hybond-N (Amersham) or Zeta-Probe GT (Bio-Rad) membranes, and hybridized in accordance with the manufacturers' suggested protocols. A plasmid containing the human cytoplasmic β-actin gene, pHFβA-1 (22); the 18S rRNA gene (59); and an mtDNA fragment corresponding to a portion of the COIII gene were labeled by random priming (15). The COIII probe was made by digesting a PCR product (generated with primers spanning nt 8273 to 8295 and 9930 to 9950) with *Stu*I to generate a 507-bp fragment corresponding to nt 9443 to 9950 of the mtDNA. Strand-specific probes for the regions corresponding to COII, A8/6 (ATP synthase subunits 8 and 6), ND1, and the rRNA region (clones mp18.XB66, mp19.XB53, mp19.BR54, and mp9.M4, respectively [31]) were generated by extension of the universal M13 primer as described previously (49). RNA hybridization signals were quantitated with a Phosphorimager or a Betascope and visualized by autoradiography.

High-resolution Northern analyses of mtDNA-encoded tRNAs. Total RNA was electrophoresed through 0.4-mm-thick 20% polyacrylamide–7 M urea gels under highly denaturing conditions (40 V/cm without cooling). The tRNA region of the gel was transferred electrophoretically onto Hybond-N or Zeta-Probe GT membranes with a Trans-Blot SD apparatus (Bio-Rad) in accordance with the manufacturer's protocol.

Mitochondrial tRNAs were detected by hybridization with end-labeled antisense oligonucleotide primers. The probe for $tRNA^{Lys}$ corresponded to nt 8354 to 8320, and that for $tRNA^{Leu(UUR)}$ corresponded to nt 3260 to 3300. The hybridizations were performed at 45°C in accordance with the recommendations of the manufacturer. After hybridization, the filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature and, if necessary, in 0.1× SSC–0.1% SDS at room temperature. The filters were then exposed to X-ray film at −70°C with an intensifying screen.

S1 protection analysis. A 431-nt probe corresponding to nt 8151 to 8582 was made by PCR with primers spanning nt 7955 to 7979 and 8582 to 8560, followed by digestion of the fragment with *Hpa*II to create a 3′ recessed terminus at nt 8151. Following gel purification of this fragment, the recessed 3′ end was labeled on the antisense strand with [α-^{32}P]dCTP in the presence of the Klenow fragment. Five micrograms of total RNA isolated from cell lines was hybridized with approximately 10 ng of the end-labeled oligonucleotide probe in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)-HCl (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, 80% formamide] at 50°C overnight. After hybridization, S1 nuclease (800 U/ml; Gibco BRL) in 300 μl of S1 buffer (280 mM NaCl, 50 mM sodium acetate [pH 4.5], 4.5 mM $ZnSO_4$, 20 μg of denatured salmon sperm DNA per ml) was added and the mixture was incubated at 37°C for 2 h. S1-protected fragments were electrophoresed through an 8% polyacrylamide–7 M urea gel, and the products were visualized by autoradiography.

Primer extension analysis. The 5′ end of A8/6 was mapped by primer extension analysis with a 22-nt oligonucleotide corresponding to nt 8582 to 8561 in the A8/6 gene. This primer was radiolabeled at the 5′ terminus with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase and purified by electrophoresis through a denaturing polyacrylamide gel. The radiolabeled primer was eluted from the polyacrylamide gel and hybridized with 5 μg of total RNA as described previously (33). A matched sequencing ladder was made with the same labeled oligonucleotide as a primer on M13 clone mp19.XB53. Primer extension products were electrophoresed through an 8% polyacrylamide–7 M urea gel alongside the matched sequencing ladder and visualized by autoradiography.

RESULTS

Construction and characterization of MERRF cybrids. To investigate the molecular genetic and phenotypic consequences of the MERRF mutations, mitochondria from patients with the nt 8344 and 8356 mutations were introduced into human cells lacking endogenous mtDNA. Fibroblasts from patients 1 and 2 were enucleated, and the resulting cytoplasts were fused with an excess of cells from human ρ^0 cell line 143B206. After fusion, the cells were replated in medium lacking pyrimidines and containing bromodeoxyuridine. Since 143B206 cells are thymidine kinase deficient and auxotrophic for pyrimidines because of the lack of a functional respiratory chain, only ρ^0 cells which had fused with cytoplasts would be expected to grow in this medium. Independent cell clones were selected and grown for further analysis.

Cybrids were screened by PCR-restriction fragment length polymorphism analysis to determine the levels of mutated mtDNAs they contained. Cybrids possessing either 0 or 100% mutation were chosen for the analyses described in this report; the latter cybrids contained 100% mutated mtDNAs with no detectable amounts of wild-type mtDNAs present (<0.1%; data not shown). The nuclear DNA origin of each of the 16 clones described in this report was confirmed by examining the nuclear DNAs for three dinucleotide repeat markers. The nuclear DNA of each of the cybrids exhibited dinucleotide repeat markers which were the same as those observed in the DNAs of the 143B and 143B206 nuclear parental cell lines and were different from those observed in the nuclear DNAs from patients' fibroblasts (data not shown). In addition, the mtDNA contents of the cybrids and the 143B cell line were analyzed by measuring the ratio of mtDNA to nuclear DNA signals in Southern blot hybridization analyses (2, 38). There were no significant differences in the levels of mtDNA present in cybrids containing mutated mtDNAs compared with the levels in cybrid or control cell lines (data not shown).

Mutant cybrids exhibit a severe respiratory chain defect. The respiratory capacity of the cybrid cell lines was determined by measuring the rates of oxygen consumption of intact cells (Fig. 1). The rates of oxygen consumption were decreased approximately 10-fold in cybrids harboring 100% of either the MERRF-8344 or the MERRF-8356 point mutation (0.58 ± 0.28 fmol per cell per min for MERRF-8344 cybrids and 0.30 ± 0.06 fmol per cell per min for MERRF-8356 cybrids [these values are means ± 2 standard errors]) compared with those containing 100% wild-type mtDNA (4.4 ± 1.79 fmol per cell per min for MERRF-8344 cybrids and 4.29 ± 0.73 fmol per cell per min for MERRF-8356 cybrids). Wild-type cybrids had a rate of oxygen consumption that was similar to that of the 143B cell line (4.01 ± 0.30 fmol per cell per min [32]).

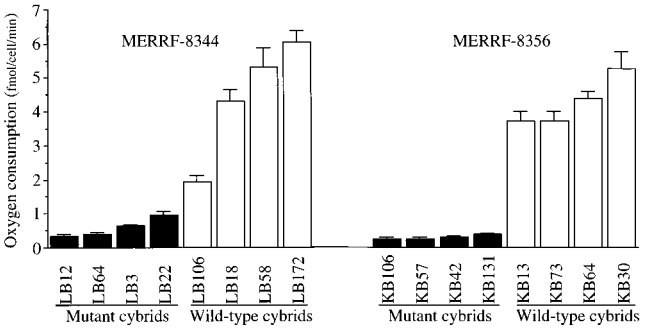

FIG. 1. Oxygen consumption. Rates of oxygen consumption of MERRF cybrids possessing 100% mutated (black bars) or 100% wild-type (white bars) mtDNA are shown, with error bars representing 2 standard errors. The identities of the cell lines are shown below the bars.

Cytochemical staining for succinate dehydrogenase and COX activities was performed on MERRF wild-type (LB18, KB13, KB30, KB64, and KB73) and mutant (LB3, KB42, KB57, KB106, and KB131) cybrids, as well as on the 143B and 143B206 cell lines (data not shown). Cytochemical staining for succinate dehydrogenase activity showed no differences among the cell lines examined, whereas cytochemical staining for COX activity showed a marked decrease in the intensity of the reaction product in mutant cybrids compared with the wild-type cybrids and the 143B cell line. The 143B206 cell line showed no COX activity, as expected for cells lacking mtDNA.

The mutations at nt 8344 and 8356 result in a severe protein synthesis defect. We performed analyses of mitochondrial translation in wild-type (LB18, LB58, KB13, KB30, and KB64) and mutant (LB3, LB12, LB22, LB64, KB42, KB57, KB106, and KB131) cybrids. Mitochondrial translation products were labeled with [^{35}S]methionine and [^{35}S]cysteine in the presence of emetine, which inhibits cytoplasmic protein synthesis. Several independent protein labelings were performed, with both long (80 to 90 min) and short (10 to 30 min) periods of labeling. Two to four independent protein labelings of each cell line, each analyzed in two to five gels, were quantitated. As shown in a representative analysis in Fig. 2A, there was a generalized decrease in the level of labeling of mitochondrial translation products in mutant cybrids compared with those observed in wild-type cybrids and 143B control cells. The degree of protein synthesis inhibition varied depending on which mitochondrial polypeptide was examined; the average was 30% of that observed in wild-type cell lines. For MERRF-8344 cybrids, the values (expressed as a percentage of the level of labeling observed in wild-type cybrids) ranged from 24% for ND2 to 63% for ND3; for MERRF-8356 cybrids, the values ranged from 25% for ND2 to 100% for ND4 (Fig. 2C). Similar decreases were observed in each experiment performed.

No significant correlation was detected between the lysine content of a particular polypeptide and the degree to which its rate of synthesis was decreased in MERRF mutant cybrids compared with wild-type cybrids (Fig. 2C). This absence of a correlation is exemplified by the ND4L polypeptide, which contains no lysine residues yet still had a severe decrease in its level of labeling (36% of normal for MERRF-8344 cybrids and 49% of normal for MERRF-8356 cybrids).

In addition to the quantitative protein synthesis defect, we also observed qualitative differences among mutant and wild-type cybrids: four aberrant translation products, one of which has not been described previously, were observed in all labeling experiments (Fig. 2A and B). All four aberrant translation products were observed only in the mutant cybrids and were apparently identical in both the MERRF-8344 and MERRF-8356 cybrid cell lines. The aberrant translation products migrated at approximately 45 to 50, 34 to 35, 17 to 18, and 10 to 12 kDa. The three aberrant polypeptides migrating at 45 to 50, 34 to 35, and 10 to 12 kDa are likely to be the same as those observed by Chomyn et al. (12).

We investigated whether the protein abnormalities were the result of degradation of the mature mitochondrial translation products. Aberrant translation products were quantitated in various protein labelings. Their amounts were found to be the same relative to the amounts of the normally occurring polypeptides in both the short and long labelings. We also investigated whether the aberrant polypeptides were formed as an artifact of isolation during either trypsinization of the cells or subsequent mitochondrial isolation. To investigate these possibilities, the mitochondrial proteins were labeled and, without prior trypsinization, the labeling was stopped by addition of a solution of 0.5% SDS–5 mM Tris-HCl (pH 8.0) directly to cells. The same decrease in protein synthesis and the appearance of the same four aberrant translation products were observed (data not shown). These results led us to conclude that the protein synthesis defects we observed are most likely due to a primary defect in protein synthesis and in the translation machinery in these mutant cybrid cell lines and are not due to proteolytic degradation of the mature mitochondrial translation products.

To determine the relative steady-state levels of the mtDNA-encoded polypeptides, immunocytochemical detection of two mtDNA-encoded polypeptides (ND1 and COII) and one nuclear DNA-encoded, mitochondrially localized polypeptide (pyruvate dehydrogenase) was also performed (data not shown). Mutant cybrid cell lines (LB64, KB42, KB57, and KB131) showed a significant decrease in their reactions to the anti-ND1 and anti-COII antibodies compared with wild-type cybrids (LB18, KB30, and KB114) or the 143B cell line, whereas the 143B206 cell line showed no reaction to these antibodies. These cell lines exhibited similar qualitative and quantitative reactions to the anti-pyruvate dehydrogenase antibody, suggesting that the number and intracellular distribution of mitochondria were not altered in cells harboring the MERRF mutations.

Northern blot analysis. We performed Northern blot analysis of total RNA isolated from MERRF cybrids to determine if processing of polycistronic precursor transcripts was occurring properly and if the steady-state levels of RNAs derived from this region had been altered by the $tRNA^{Lys}$ point mutations. Total RNA was extracted from exponentially growing cells from representative mutant and wild-type cybrids and examined by Northern blot analysis (Fig. 3). The RNA was hybridized with six probes that identify eight mtDNA-encoded genes (COII, $tRNA^{Lys}$, A8, A6, COIII, 12S rRNA, 16S rRNA, and ND1) and two nuclear DNA-encoded genes (β-actin and 18S rRNA). Eight mutant (LB3, LB12, LB22, LB64, KB42, KB57, KB106, and KB131) and six wild-type (LB18, LB58, KB13, KB30, KB64, and KB73) cybrids were analyzed.

The COII probe detected a band of approximately 1 kb, corresponding to the COII mRNA, in both mutant and wild-type cybrids. This probe also detected a very low-abundance transcript (<0.1% of the COII signal) of approximately 1.9 kb, a size consistent with a transcript corresponding to the COII, $tRNA^{Lys}$, and A8/6 genes (data not shown). This transcript was seen only in MERRF-8356 cybrids containing mutated mtDNA; it was not seen in any of the MERRF-8344 cybrids or control cell lines. We were unable to confirm the identity of this transcript with other probes, as the $tRNA^{Lys}$ probe did not

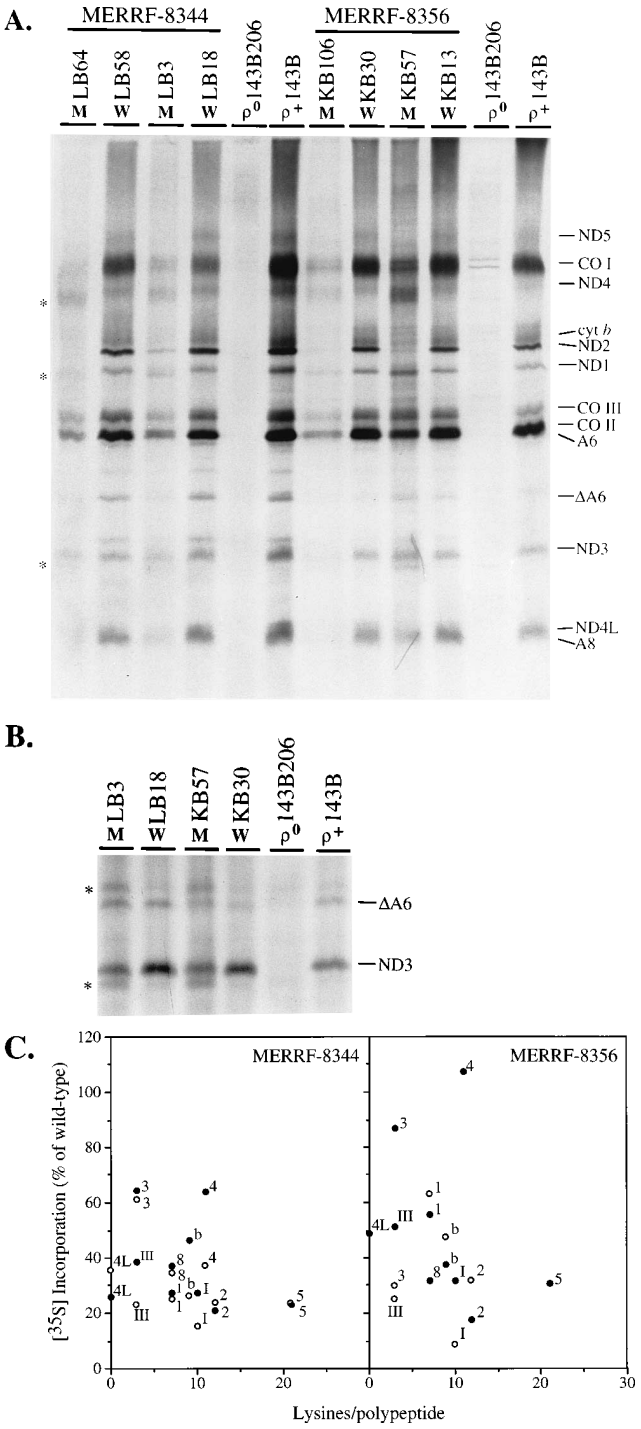

FIG. 2. Analysis of mitochondrial translation in cybrid cell lines. (A) Fluorogram of mitochondrial translation products labeled with [^{35}S]methionine after electrophoresis through 15 to 20% gradient SDS-polyacrylamide gels for the indicated wild-type (W) cybrids and mutant (M) cybrids harboring the nt 8344 or 8356 point mutation. Cells were labeled for 90 min in the presence of 100 μCi of [^{35}S]methionine per ml and 100 μg of emetine per ml. The same amount of the mitochondrial fraction (10 μg of protein) was loaded in each lane of the gel, with the exception of KB57, for which 50% more was loaded to emphasize the presence of the aberrant translation products. The aberrant translation products detected in the mutant cybrids are denoted by asterisks. COI, COII, and COIII are subunits of COX; ND1, ND2, ND3, ND4, ND4L, and ND5 are subunits of NADH dehydrogenase; A8 and A6 are subunits of the ATP synthase; ΔA6 is the carboxy-terminal degradation fragment of A6 (11); and cyt *b* is apocytochrome *b*. (B) Fluorogram of a section of another SDS-polyacrylamide gel shown to highlight the two smallest aberrant translation products located at 17 to 18 and 10 to 12 kDa. The mutant and wild-type cell lines indicated were labeled for 75 min in the presence of 125 μCi of [^{35}S]methionine per ml and 100 μg of emetine per ml. The same amount of the mitochondrial fraction (25 μg of protein) was loaded in each lane of the gel. Abbreviations and symbols are the same as in panel A. (C) Correlation of protein synthesis with lysine content. The average level of labeling of polypeptides in mutant cybrids as a percentage of the average level of the corresponding polypeptide observed in wild-type cybrids is plotted for each of the mitochondrial polypeptides determined. Open circles represent data derived from short-term labeling of cells (20 to 30 min); closed circles represent data from long-term labeling of cells (80 to 90 min). 1, 2, 3, 4, 4L, and 5 are ND1, ND2, ND3, ND4, ND4L, and ND5; I and III are COI and COIII; b is apocytochrome *b*; 8 is A8. COII, A6, and ND6 were not resolved on most gels and were not included in this analysis.

have the sensitivity to detect it and the A8/6 probe identified another transcript, also 1.9 kb long, corresponding to the A8/6-COIII transcript (see below). The presence of this A8/6-COIII transcript precluded the use of the A8/6 probe to confirm the identity of the transcript corresponding to the COII, tRNA^{Lys}, and A8/6 genes detected previously with the COII probe, as the two transcripts had similar electrophoretic mobilities. Additionally, neither the S1 nor the primer extension techniques described below had the sensitivity to detect this low-abundance transcript.

The tRNA^{Lys} probe detected a low-molecular-weight band, presumably corresponding to the tRNA itself, and did not detect any other RNA species in Northern blot analyses. The A8/6 probe detected a band of approximately 840 nt, corresponding to the A8/6 mRNA, and a larger transcript of approximately 1.9 kb, corresponding to the A8/6 and COIII genes (asterisks in Fig. 3B and C). The identity of the 1.9-kb transcript was confirmed by hybridization with a COIII probe, which detected the 1.9-kb transcript (asterisk in Fig. 3B), as well as the authentic COIII mRNA (780 nt). The 1.9-kb transcript was present in the majority of cybrids but was not specific to wild-type or mutant cell lines.

Probes for other regions of the mtDNA were also used for hybridization analyses (data not shown). These included probes to detect the ND1, 12S rRNA, and 16S rRNA genes. The ND1 probe detected the ND1 mRNA (1 kb), as well as another transcript of 2.5 kb, which corresponded to the 16S rRNA, tRNA$^{Leu(UUR)}$, and ND1 genes (RNA 19). RNA 19 was previously observed at low levels in wild-type cell lines but was found to be significantly elevated in cybrid cell lines possessing a pathogenic point mutation at nt 3243 in the tRNA$^{Leu(UUR)}$ gene (32). The identity of the transcript observed here was confirmed by hybridization with a probe which detected 16S rRNA; the probe detected both 16S rRNA and RNA 19. No significant differences in the hybridization signals of RNA 19 compared with those of the ND1 mRNA or 16S rRNA were observed among the mutant, wild-type, and 143B cell lines.

The relative steady-state levels of the mitochondrial transcripts in each cell line, normalized to nuclear DNA-encoded β-actin or 18S rRNA, were determined by quantitation of Northern hybridization signals. These ratios were not found to be significantly different among the MERRF-8344 and MERRF-8356 mutant cybrids, wild-type cybrids, and the 143B cell line, indicating that the steady-state levels of each of the mitochondrial RNA species analyzed were not altered by the presence of the MERRF mutations.

High-resolution Northern blot analysis of tRNA^{Lys}. We examined the effects of the point mutations in tRNA^{Lys} on the size and steady-state levels of the mature tRNA by high-resolution Northern blot analysis with total RNA isolated from mutant (LB3, LB12, LB22, LB64, KB42, KB57, KB106, and

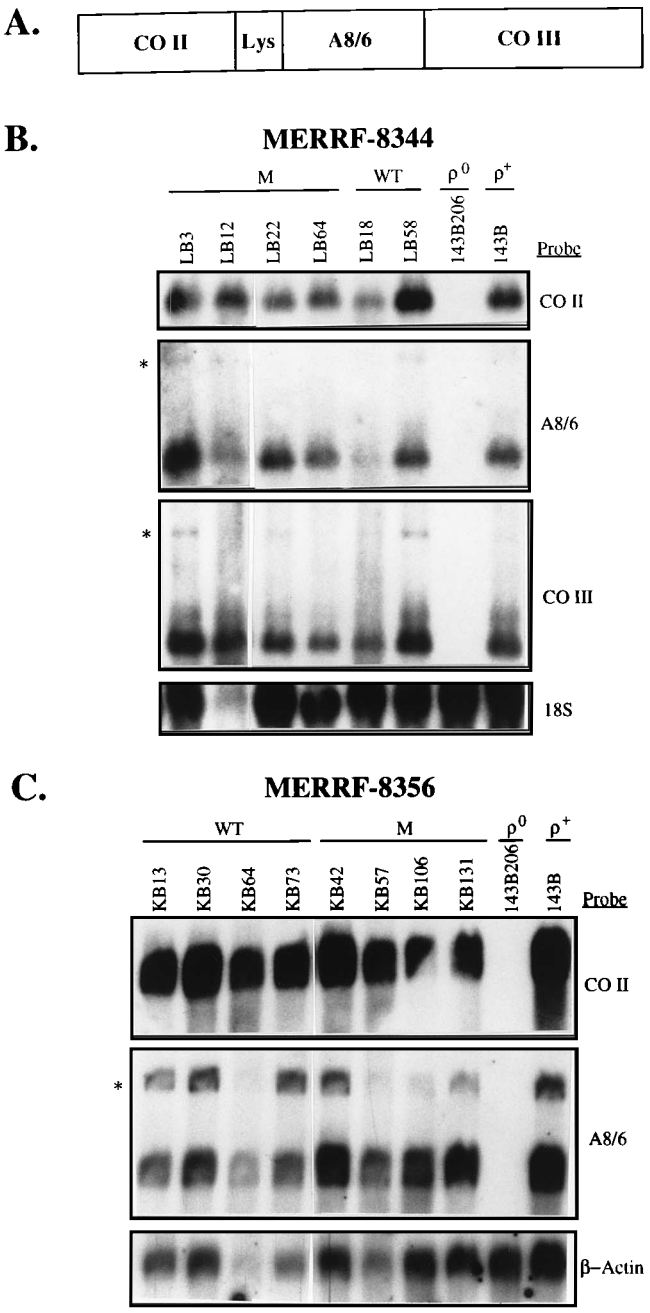

FIG. 3. Northern blot analysis. (A) Map of the region of the mitochondrial genome surrounding $tRNA^{Lys}$. Total RNA was isolated from the indicated wild-type (WT) cybrids and mutant (M) cybrids harboring either the nt 8344 (B) or nt 8356 (C) mutation. The probes used are indicated at the right of each autoradiogram, and the aberrant transcripts detected are indicated by asterisks at the left. Other notation is the same as in Fig. 2.

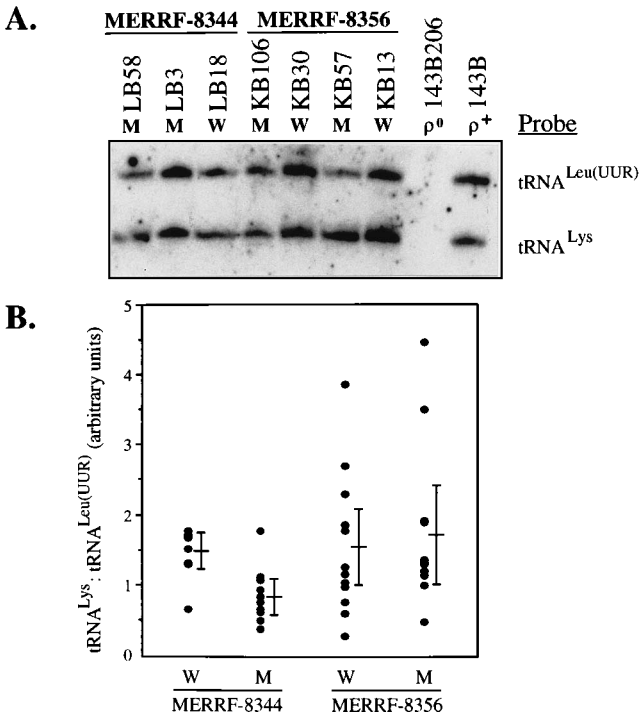

FIG. 4. High-resolution Northern blot analysis. (A) Autoradiogram of hybridization with a probe to detect $tRNA^{Lys}$ and one to detect $tRNA^{Leu(UUR)}$, which was used as a control for equal loading. M, mutant cybrids; W, wild-type cybrids. (B) Graphical representation of $tRNA^{Lys}$-$tRNA^{Leu}$ ratios in wild-type and mutant cybrids. These data were taken from five independent Northern blots. All ratios were normalized to the $tRNA^{Lys}$ signal in the 143B cell line within each blot before comparisons among blots was undertaken. The bars denote means ± 2 standard errors. Other notation is the same as in Fig. 2.

KB131) and wild-type (LB18, LB58, KB13, KB30, KB64, and KB73) cybrids. RNAs were electrophoresed under highly denaturing conditions and, after transfer to membranes, hybridized with an oligonucleotide probe complementary to nt 8354 to 8320 in $tRNA^{Lys}$ (Fig. 4A). A second oligonucleotide probe, from nt 3260 to 3300, was used to detect $tRNA^{Leu(UUR)}$ and to control for differences in RNA loading. Each probe detected a single band corresponding to the size expected for $tRNA^{Lys}$ (72 nt) or $tRNA^{Leu(UUR)}$ (77 nt). The hybridization signals were quantitated (Fig. 4B); there was no significant alteration in the steady-state levels of $tRNA^{Lys}$ present in mutant MERRF-8356 cybrids compared with wild-type cybrids (ratios of $tRNA^{Lys}$ to $tRNA^{Leu(UUR)}$, 1.77 ± 1.38 for mutant cybrids and 1.54 ± 1.04 for wild-type cybrids). There was, however, a significant decrease in this ratio in mutant MERRF-8344 cybrids (0.85 ± 0.15 in mutant compared with 1.45 ± 0.12 in wild-type cybrids, a decrease of 41%; $P < 0.01$).

Fine mapping of the 3′ end of the COII mRNA. To determine whether or not processing of the primary transcript in the region of $tRNA^{Lys}$ was occurring precisely, RNA isolated from cybrids was subjected to fine-mapping analysis. The 3′ end of the COII mRNA was analyzed by S1 nuclease protection (Fig. 5). A 431-nt probe which was complementary to sequences spanning the junctions between the COII (144 nt), $tRNA^{Lys}$ (69 nt), and A8/6 (218 nt) genes (Fig. 5A) was end labeled and hybridized with total RNA isolated from cybrids containing mutated or wild-type mtDNAs and subsequently digested with S1 nuclease. Gel analysis indicated that the probe protected two major regions (Fig. 5B). The smaller protected fragments (143 and 144 nt) corresponded to the 3′ end of COII (144 nt). There was no variation in the size or abundance of these products in the cell lines examined, indicating that misprocessing did not occur at the 3′ end of COII. The larger protected fragments (178 and 179 nt) corresponded to an A-T-rich region within $tRNA^{Lys}$, at nt 8328 to 8329, which was presumably susceptible to digestion by S1 nuclease. ρ^0 cell line 143B206 did not show these S1 protected fragments, as would be expected for cells lacking mtDNA. The protected fragments in the upper portion of the autoradiogram (ranging in size from approxi-

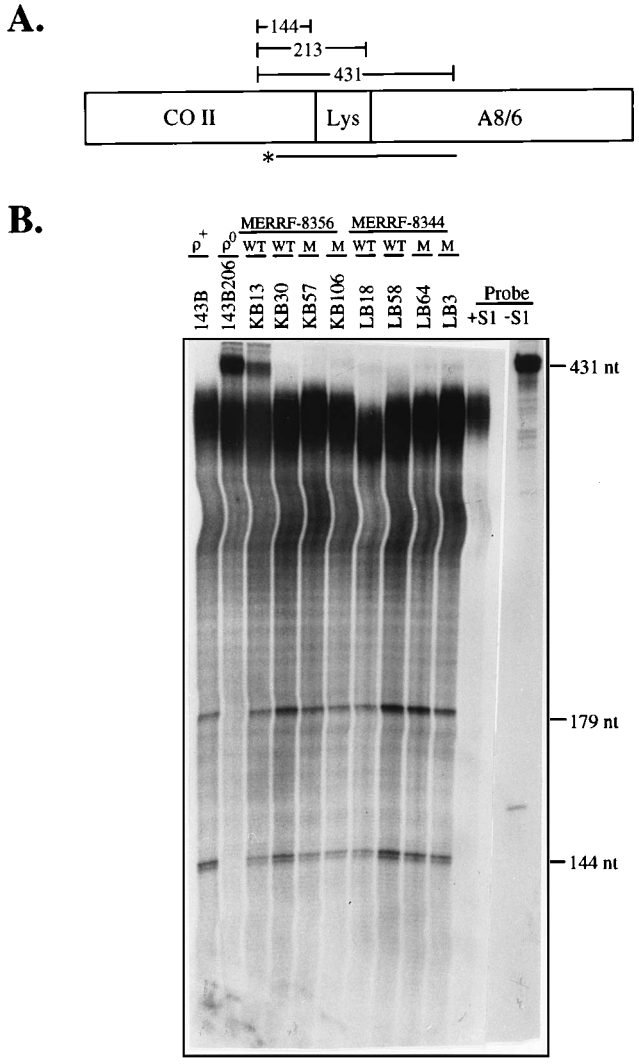

FIG. 5. S1 analysis. (A) An antisense oligonucleotide probe (line below the map) labeled at the 3′ end (asterisk) was hybridized to total RNAs isolated from MERRF cybrids and subsequently digested with S1 nuclease. Expected fragments for processing at the 3′ end of COII (144 nt); for COII and $tRNA^{Lys}$ (213 nt); and for COII, $tRNA^{Lys}$, and A8/6 (431 nt) are shown. (B) Autoradiogram of an 8% denaturing polyacrylamide gel showing S1 digestion products. Two major protected regions corresponding to the 3′ end of COII (143 or 144 nt) and an A-T-rich region in $tRNA^{Lys}$ (178 or 179 nt) were detected. A DNA sequencing ladder (not shown) was run alongside the S1 digestion products for size estimation. WT, wild type; M, mutant.

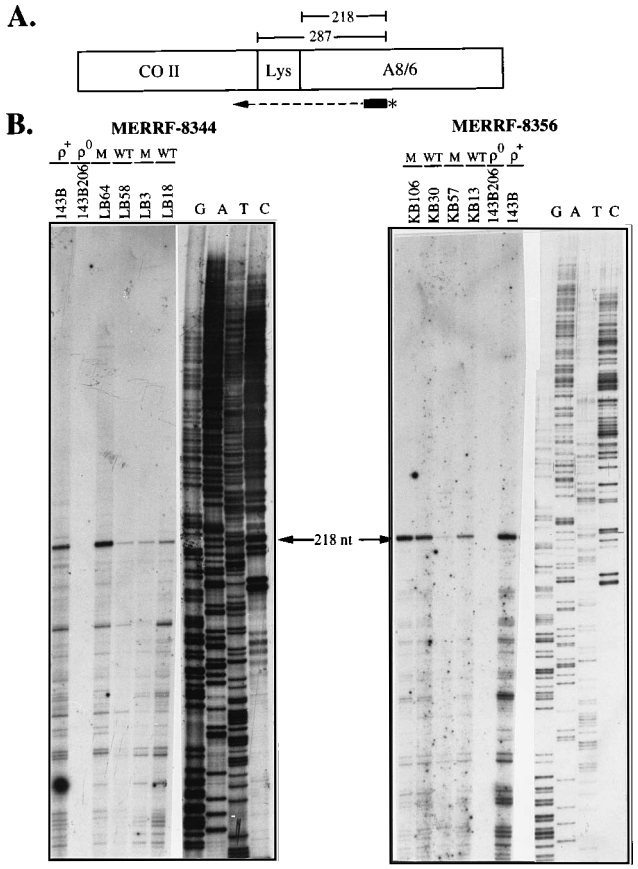

FIG. 6. Primer extension analysis. (A) An oligonucleotide primer (thick bar) labeled at its 5′ end (asterisk) was hybridized to total RNA isolated from MERRF cybrids and extended with reverse transcriptase (dashed arrow). A 218-nt fragment represents processing at the junction between $tRNA^{Lys}$ and A8/6. (B) Autoradiogram of an 8% denaturing polyacrylamide gel showing primer extension products. One major product which corresponds to the 5′ end of the A8/6 mRNA was detected. G, A, T, and C, dideoxy sequencing ladder generated by using the same labeled oligonucleotide primer run in parallel with the primer extension products. Other notation is the same as in Fig. 2. M, mutant; WT, wild type.

mately 250 to 400 nt), were present in all samples, including 143B206 and the S1-digested probe, and probably resulted from incomplete digestion of the probe by S1 nuclease.

High-resolution mapping of the 5′ end of the A8/6 mRNA. A 5′-end-labeled 22-nt oligonucleotide complementary to the region spanning nt 8582 to 8561 in the A8/6 mRNA was used as a primer for extension on total RNA by Moloney murine leukemia virus reverse transcriptase. Primer extension products were electrophoresed alongside a sequencing ladder generated by using the same labeled primer. Figure 6A is a schematic showing the possible fragments expected. The one major extension product observed (Fig. 6B) was 218 nt long and mapped precisely to the 5′ end of the A8/6 mRNA. There were no significant primer extension products longer than this fragment. Primer extension products shorter than 218 nt were present in all samples in similar abundance and most likely resulted from premature termination by the reverse transcriptase.

DISCUSSION

The results presented here show that two different mtDNA genotypes associated with MERRF have essentially the same biochemical and cellular phenotypes. This correlates with previous observations that the MERRF-8344 and MERRF-8356 mutations result in fundamentally similar clinical phenotypes (5, 23, 39, 45–47, 60–62).

The most striking of the defects seen in MERRF cybrids were in the mitochondrial respiratory chain. Mutant cybrids harboring either the MERRF-8344 or the MERRF-8356 point mutation exhibited a severe decrease in their rates of oxygen consumption. Presumably, the reduced rate of synthesis of mitochondrial proteins results in a decrease in the steady-state number of enzyme complexes and the dramatic decrease in mitochondrial respiratory chain activity. These decreases in mitochondrial protein synthesis are not likely to be the result of degradation of unstable translation products, as short-term labeling experiments exhibited the same relative decreases in

protein synthesis as did longer labelings, suggesting that the decreases observed are not the result of increased turnover but more likely were caused by a decrease in the rate of synthesis. Similar decreases in protein synthesis have been described previously for cells possessing the MERRF-8344 mutation and resulted in similar decreases in respiratory chain activity (6, 12).

All mitochondrial translation products seemed to be affected, regardless of the number of lysine residues they contain. There was no direct correlation between the lysine content of a particular polypeptide and the degree of decrease in its rate of synthesis (Fig. 2B). This is exemplified by the rates of translation of the ND4L mRNA (30 to 50% of normal), despite the fact that it contains no lysine codons at all. A correlation of these values was previously reported in cybrids (12) but was not observed in muscle cells (6). A possible explanation is that this phenomenon is analogous to the stringent response which occurs in both bacteria (9, 19) and the mitochondria of *Saccharomyces cerevisiae* (41, 42). Briefly, if high levels of an uncharged tRNA are present in cells or if the cells are starved for an amino acid (because of either depletion of the amino acid or a defect in the interaction of the charged tRNA with the ribosome), cells exhibit a response which includes a decrease in overall protein synthesis, independent of its requirement for that particular amino acid. It is thought that high levels of uncharged tRNA are responsible for inducing the stringent response and also for modulating the rate of translational elongation (9, 19). It is possible that such responses also occur in the mitochondria of mammalian cells.

We also observed the presence of four aberrant translation products, one of which has not been reported previously. All four aberrant translation products were detected specifically in both the MERRF-8344 and MERRF-8356 mutant cell lines and were not seen in wild-type or control cell lines. One possible explanation for the presence of these products would be degradation of the normal mitochondrial translation products. However, we consider this an unlikely possibility. First, aberrant translation products were also present in short protein labelings and were in the same relative proportion to the mature proteins as those seen in longer labelings. Second, these products were also observed when labelings were stopped by addition of an SDS solution directly to the cells (without prior trypsinization). Formally, however, the possibility of very rapid degradation of the mature proteins cannot be excluded.

A second possibility is that the ribosome terminates translation of the mRNAs prematurely, presumably at lysine codons; the aberrant bands we observed would be the stable translation products produced by such a mechanism. However, the absence of a strict correlation between the lysine content of a particular polypeptide and its rate of synthesis in mutant cybrids led us to believe that this is not the primary mechanism operating in MERRF.

A third possibility is ribosome frameshifting, which has been observed in retroviruses, coronaviruses, bacteria, bacteriophages, and yeasts (see references 13, 40, and 56 for reviews): when an amino acid becomes limiting, and in the presence of a favorable sequence on the mRNA, the ribosome shifts either forward or backward and continues translating, usually in a new reading frame. Lindsley and Gallant (35) characterized ribosome frameshifting in bacteria that had been starved for lysine. They identified two heptanucleotide sequences that promoted frameshifting when the bacterial cells were starved for lysine, one (GCCAAGC) for rightward frameshifting and another (CTTCAAG) for leftward frameshifting; the codon for lysine (the "hungry" codon in the original reading frame [underlined above]) is part of the frameshifting consensus sequence. Analysis of the human mtDNA for the presence of these sequences found three perfect in-frame matches for the leftward frameshift consensus sequence (CTTCAAA); remarkably, all three were located in ND5, at Lys-81, Lys-119, and Lys-336. Two sequences (with one mismatch each [in boldface type]) were found for the rightward frameshift consensus sequence, one located in COI at Lys-411 (GCCAAA**A**) and the other located in apocytochrome *b* at Lys-311 (**T**CCAAAC). The sizes of the polypeptides expected from frameshifting at these sites would be 9, 15, and 39 kDa for leftward frameshifting in ND5, 46 kDa for rightward frameshifting in COI, and 35 kDa for rightward frameshifting in apocytochrome *b*. Thus, the aberrant polypeptides we observed in mutant cybrids, with sizes estimated at approximately 45 to 50, 36 to 38, 15 to 18, and 10 to 12 kDa, are consistent with frameshift products in COI, ND5 (or apocytochrome *b*), ND5, and ND5, respectively. We note that many cases of ribosomal frameshifting in retroviruses require the presence of stem-loop or pseudoknot structures which promote ribosome stalling and allow frameshifting to take place at "slippery" sequences (24, 26–28, 52). However, it has been shown that these slippery sequences also require the presence of an in-frame stop codon which also plays a role in promoting the frameshift event (21, 56). In the case of a hungry codon, however, the codon itself serves as a pause site for the ribosome, thus eliminating the need for any structures other than the heptanucleotide consensus sequence itself to promote ribosome frameshifting (4, 16, 35, 55).

The presence of ribosome frameshifting, even though it would occur at lysine codons, could also offer an explanation for the absence of a correlation of lysine content with the levels of decrease in the rates of synthesis of a particular polypeptide. Although ribosome frameshifting occurs at lysine codons, it is not dependent on either the number or location of lysine codons in the mRNA; rather, it depends on the presence of the heptanucleotide consensus sequence surrounding the lysine codon. Pausing at other lysine codons would not necessarily result in decreased translation or premature termination, whereas pausing at lysine codons within a frameshifting consensus sequence allows the ribosome to frameshift without any regard for the number of lysine codons in that particular mRNA. If frameshifting were indeed operative, we would expect the polypeptides involved in frameshifting to be decreased more than those not involved in frameshifting. In fact, this may explain the low levels of ND5 and COI in the mutant MERRF cybrids.

Mechanisms other than frameshifting may also be operative. As stated previously, the ND4L mRNA contains no lysine codons and therefore cannot respond directly to the alterations in $tRNA^{Lys}$; in addition, ND4L resides on the same cistron as does ND4 (with partially overlapping reading frames), and the steady-state levels of the polypeptides produced by these two genes differ greatly in mutant cybrids (they are present in roughly equal amounts in wild-type cell lines). For ND4L, as well as for most of the polypeptides whose synthesis is decreased, a stringent response of the mitochondria (discussed above) may be a more likely mechanism. Alternatively, the pool of free ribosomes could be depleted by ribosomes pausing at lysine codons, resulting in a decreased rate of translation of all polypeptides (irrespective of the number of lysine codons in the mRNA). In addition to explaining the decreased rates of translation, this mechanism is compatible with our observation that the degree of decrease in protein synthesis does not correlate with the number of lysine codons present in a particular mRNA.

Both mutations associated with MERRF are located in the TΨC arm of the tRNA and affect highly conserved nucleotides.

The A at nt 8344 (nt 55 in the standard tRNA numbering system) is highly conserved through evolution (48) and therefore is thought to be functionally important. The mutation at nt 8356 disrupts a highly conserved nucleotide pair (nt 49 and 65 in the standard tRNA numbering system). It is the presence of this base pair, and not the specific sequence at this position, which is conserved, and its absence may have adverse effects on the tRNA structure.

The TΨC arm of the tRNA is known to be important in the interaction of the tRNA with several components of the translation machinery. Thus, both mutations may alter the tRNA's ability to bind to or be recognized by other components and could have several detrimental effects on the function of this tRNA in translation. One such effect would be on the recognition of the tRNA by its cognate aminoacyl tRNA synthetase or by other tRNA synthetases, which may lead to changes in the efficiency of charging or mischarging of the tRNA. Although the mutant cell lines contain 100% mutated $tRNA^{Lys}$, there are still significant, albeit reduced, levels of normal protein synthesis. This suggests that at least some of the mutant tRNAs are being charged, presumably with lysine.

The aminoacyl-tRNAs are delivered to the ribosomes by the elongation factor EF-Tu. EF-Tu interacts specifically with the aminoacyl and TΨC stems of the tRNA (7, 29, 58). The TΨC arm of the tRNA is also important for binding of the tRNA to the ribosome (14, 43, 44). Thus, it is possible that the point mutations in the mtDNA-encoded $tRNA^{Lys}$ interfere with the binding of the $tRNA^{Lys}$ with EF-Tu or the ribosome, decreasing the availability of charged $tRNA^{Lys}$ to the elongating ribosome. It may well be that it is these faulty interactions with the translation machinery, which would appear to the ribosome as a reduction or absence of the charged tRNA itself, that cause the ribosome to pause and which alter normal mitochondrial translation.

The mutations in $tRNA^{Lys}$ may also affect its recognition by enzymes not directly involved in translation, such as the enzymes which process the large polycistronic transcripts of the mtDNA. Because the tRNAs are believed to serve as the signals for the processing enzymes, incorrect interaction may result in no processing, partial processing, or inaccurate processing of the primary transcript. However, we do not think that RNA processing is an important factor in MERRF. In Northern blot hybridization analyses and high-resolution RNA mapping studies, no significant alterations in the accuracy of processing were observed. Specifically, S1 analysis of the 3′ end of the COII mRNA and primer extension analysis of the 5′ end of the A8/6 mRNA revealed that processing of these species occurred precisely in the mutant cybrids. Similarly, high-resolution Northern blot analysis of $tRNA^{Lys}$ suggested that its excision from the primary transcript and posttranscriptional addition of CCA occurred correctly in the mutant cybrids.

One partially processed transcript derived from this region of the mitochondrial genome was detected specifically in cybrids possessing the MERRF-8356 mutation. This transcript, corresponding to the COII, $tRNA^{Lys}$, and A8/6 genes, was present at levels of less than 0.1% of those of the COII mRNA, which would correspond to less than one molecule per cell (17). Therefore, this transcript is unlikely to have functional consequences for these cells. Another partially processed transcript, corresponding to the A8/6 and COIII genes, was also detected. This transcript, not previously observed in human cells, was present in most of the cell lines but was not specific to wild-type or mutant cell lines. The relatively high steady-state levels of this transcript (up to 70% of the levels of the A8/6 mRNA) suggest that processing at the junction of the A8/6 and COIII genes is slow in some cell lines, perhaps because no tRNA is present in the transcript at the point of processing. It is not known why the steady-state levels of this transcript vary so dramatically among these cell lines.

Altered processing could also affect the steady-state levels of the mitochondrial transcripts derived from the $tRNA^{Lys}$ region. By determining the hybridization signal for the mitochondrial transcripts and comparing these with nuclear DNA-encoded β-actin and 18S rRNA, the relative cellular levels of the mitochondrial transcripts were measured. These studies indicated that the steady-state levels of COII and A8/6 mRNAs, as well as transcripts representative of other regions of the mtDNA (including the 12S and 16S rRNAs and the ND1 and COIII mRNAs), were the same in the mutant cybrids as those in wild-type cybrids or 143B cells. Analysis of RNA 19 also indicated that its levels were unchanged in mutant cybrids. An elevation in the levels of this transcript, analogous to that seen in mitochondrial myopathy, encephalopathy lactic acidosis, and strokelike episodes (32), had been reported previously in RNA isolated from muscle tissue of patients possessing the MERRF-8344 mutation (25).

Similarly, we saw no evidence of functionally significant alterations in mitochondrial tRNA levels. We found no significant differences in the steady-state levels of mtDNA-encoded $tRNA^{Lys}$ transcripts in MERRF-8356 cybrids. However, MERRF-8344 cybrids did exhibit a decrease (approximately 40%) in the steady-state levels of $tRNA^{Lys}$. Normally, there is an approximately 10-fold variation in the steady-state levels of mitochondrial tRNAs, and $tRNA^{Lys}$ has been shown to be one of the more abundant tRNAs (31). A 40% reduction in the steady-state levels of $tRNA^{Lys}$ corresponds to approximately 1.7×10^4 molecules per cell. This level is within the normal range of levels of other mitochondrial tRNA species (1.0×10^4 to 8.4×10^4 molecules per cell [31]), and therefore, this level of decrease would not be expected to have any pathogenic consequences. This is supported further by the observation that there were no significant alterations in the steady-state levels of $tRNA^{Lys}$ in MERRF-8356 cybrids, yet they exhibited the same translational defects as did MERRF-8344 cybrids.

On the basis of the results presented here, we feel that qualitative and quantitative protein synthesis defects are the main cause of the respiratory chain deficiencies and, perhaps, the specific clinical features of MERRF. The question of how the mutated tRNA and translation machinery interact to cause these defects is currently being investigated.

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