after digestion with six different restriction enzymes (Fig. 4). We could detect no rearrangement in the L3T4 gene with the complete insert of pcL3T4-C7 as a probe. On the basis of the data from VL3/1 we conclude that no rearrangement is required for expression in T-lineage cells. Although we found no evidence for rearrangement in total brain DNA, we cannot rule out the possibility that a rearrangement occurs in fewer than 5% of brain cells, and that these are the cells that produce the smaller mRNA.

The expression of L3T4 mRNA in mouse brain is intriguing given the high frequency of central nervous system involvement in human acquired immune deficiency syndrome (AIDS) (*19, 20*); the presence of human T-cell lymphotropic virus type III (HTLV-III), the retrovirus responsible for AIDS, in the brain of affected individuals (*19*); and the demonstrated role of human CD4 as a cellular receptor for HTLV-III (*21*). These observations suggest that CD4 is probably expressed in human brain at the protein level. The large evolutionary divergence that we have found between the external domains of L3T4 and CD4 may explain why HTLV-III does not infect mouse T cells. In any event, the tissue-specific expression of an alternative form of L3T4 mRNA suggests a yet undefined role for this molecule in brain.

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URF6, Last Unidentified Reading Frame of Human mtDNA, Codes for an NADH Dehydrogenase Subunit

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The polypeptide encoded in URF6, the last unassigned reading frame of human mitochondrial DNA, has been identified with antibodies to peptides predicted from the DNA sequence. Antibodies prepared against highly purified respiratory chain NADH dehydrogenase from beef heart or against the cytoplasmically synthesized 49-kilodalton iron-sulfur subunit isolated from this enzyme complex, when added to a deoxycholate or a Triton X-100 mitochondrial lysate of HeLa cells, specifically precipitated the URF6 product together with the six other URF products previously identified as subunits of NADH dehydrogenase. These results strongly point to the URF6 product as being another subunit of this enzyme complex. Thus, almost 60% of the protein coding capacity of mammalian mitochondrial DNA is utilized for the assembly of the first enzyme complex of the respiratory chain. The absence of such information in yeast mitochondrial DNA dramatizes the variability in gene content of different mitochondrial genomes.

SINCE THEIR INITIAL DISCOVERY IN human mitochondrial DNA (mtDNA) (*1*), the eight so-called unidentified reading frames (URF's), which together represent about 60% of the protein coding capacity of the mitochondrial genome of animal cells (*2*), have raised considerable interest. Particularly intriguing has been the observation that these reading frames, with the exception of one, have no homology to any of the identified and unidentified reading frames of mtDNA of *Saccharomyces cerevisiae* (*3*) and *Schizosaccharomyces pombe* (*4*), although several of them occur in mtDNA of other lower eukaryotic cells (*5–14*). Recently, direct evidence obtained by the use of antibodies to synthetic peptides predicted from the DNA sequence and by analysis of protease fingerprints has shown that seven of the eight URF's are expressed in HeLa cells (*15–17*).

The smallest of the URF's (URFA6L), a 207-nucleotide (nt) reading frame overlapping out of phase the NH_2-terminal portion of the adenosinetriphosphatase (ATPase) subunit 6 gene has been identified as the animal equivalent of the recently discovered yeast H^+-ATPase subunit 8 gene (*18, 19*). The functional significance of the other URF's has been, on the contrary, elusive. Recently, however, immunoprecipitation experiments with antibodies to purified, rotenone-sensitive NADH-ubiquinone oxido-

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reductase [hereafter referred to as respiratory chain NADH dehydrogenase or complex I (*20*)] from bovine heart, as well as enzyme fractionation studies, have indicated that six human URF's (that is, URF1, URF2, URF3, URF4, URF4L, and URF5, hereafter referred to as ND1, ND2, ND3, ND4, ND4L, and ND5) encode subunits of complex I (*21*). This is a large complex that also contains many subunits synthesized in the cytoplasm (*19*). Support for such functional identification of the URF products has come from the finding that the purified rotenone-sensitive NADH dehydrogenase from *Neurospora crassa* contains several subunits synthesized within the mitochondria (*22*), and from the observation that the stopper mutant of *Neurospora crassa*, whose mtDNA lacks two genes homologous to URF2 and URF3, has no functional complex I (*7, 8*).

The light-strand–encoded URF6 (*1*) remains as the only human mtDNA URF to which no mitochondrial translation product has been assigned by using antibodies to predicted peptides, although a tentative assignment on the basis of protease fingerprints has been reported (*16*). Because of its hydrophobic character, the URF6 product offers limited opportunities for the production of effective immunogens. In spite of this, we prepared four different types of antibodies to URF6-specific peptides—that is, antibodies to an NH_2-terminal peptide, a COOH-terminal peptide, and two different internal peptides of the putative URF6 product (Table 1). Each antibody was reactive with the corresponding synthetic peptide coupled to a carrier. However, when tested in immunoprecipitation experiments under different conditions, these antibodies gave consistently negative results. We subsequently discovered that a complication in the search for the URF6 product was the fact that the migration of this polypeptide as an identifiable band was sensitive to the source of SDS used in the preparation and running of the SDS-urea-polyacrylamide gel. In fact, we found that one of the HeLa cell mitochondrial translation products [component 22 according to the classification by Ching and Attardi (*23*), which has the electrophoretic mobility expected for the putative product of URF6 (*1*)] was clearly recognizable in a gel run in the presence of Spectrum SDS (S0180, Spectrum Chemical) or Sigma SDS (L5750), but was absent or barely detectable in a gel run in the presence of MCB SDS (DX2490-3, MCB Manufacturing Chemists) (*24*). Another factor that affected the migration of component 22 as a discrete band was exposure of the sample to high temperature. Heating the sample at 100°C for 3 minutes resulted in the complete disappearance of the band for component 22 but had no obvious effect on bands for the other components.

Among the conditions in the immunoprecipitation procedure, the SDS solubilization of the mitochondrial fraction at 100°C and the routine use of SDS-urea-polyacrylamide gels in MCB SDS for the electrophoretic analysis of the immunoprecipitates could have prevented the identification of component 22 as the product of URF6 in the early experiments. Indeed, by solubilizing the mitochondrial membranes at 37°C in Sigma or Spectrum SDS, and by electrophoresing the immunoprecipitated samples in SDS-urea-polyacrylamide gels prepared and run in Sigma or Spectrum SDS, we found that one of the four types of URF6-specific antibodies, in particular one against an internal peptide (anti-URF6-I1: Table

Table 1. Synthetic peptides and modes of attachment. The techniques used for the synthesis and characterization of the peptides and their attachment to bovine serum albumin (BSA) have been previously described (*15, 37*). The two internal peptides were chosen as corresponding to two hydrophilic segments of the putative URF6 product [their positions in the DNA sequence (*1*) are indicated], on the basis of the hydropathy plot of this polypeptide (*26*). Asterisks indicate radioactive residues.

Source	Sequence†	Attachment procedure‡	Degree of BSA substitution§
URF6-N (amino terminus)	Met-Met-Tyr-Ala*-Leu-Phe-(Glu)	EDC	16
URF6-C (carboxyl terminus)	(Lys)-Ile-Val-Ile-Glu-Ile-Ala*--Arg-Gly-Asn	GLUT	33
URF6-I1 (internal, 14, 422–14, 454)	Ala-Ile-Glu-Glu-Tyr-Pro-Glu--Ala-Trp*-Gly-Ser	GLUT	24
URF6-I2 (internal, 14, 593–14, 622)	Acetyl-Gly*-Phe-Ser-Ser-Lys-Pro--Ser-Pro-Ile-Tyr	BDB	25

†Residues in parentheses were incorporated for attachment purposes. ‡EDC, water-soluble carbodiimide; GLUT, glutaraldehyde; BDB, bisdiazobenzidine. §Number of peptide molecules per BSA molecule.

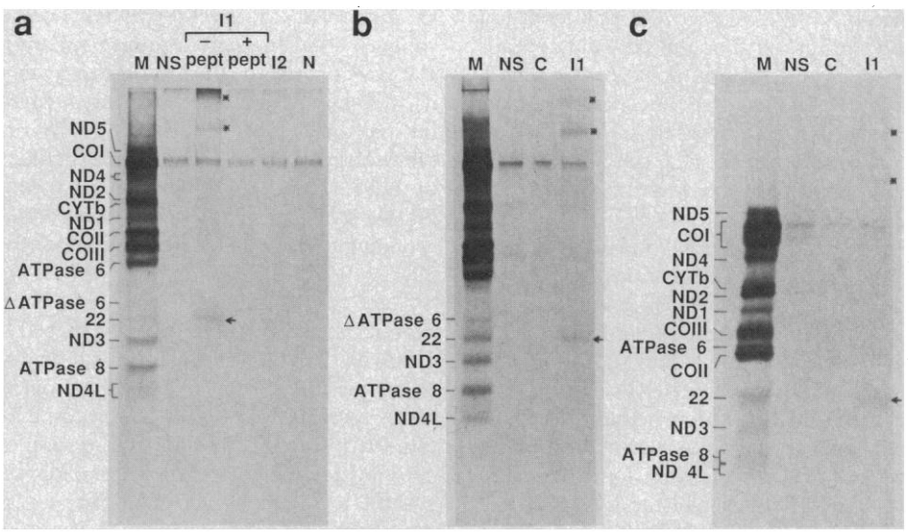

Fig. 1. Identification of the polypeptide encoded in URF6. Samples of an SDS mitochondrial lysate (in Sigma SDS, 75 to 100 μg of protein) from HeLa cells labeled for 2.5 hours with [^{35}S]methionine in the presence of 100 μg/ml of emetine (to inhibit cytoplasmic protein synthesis) were incubated with 125 μg of immunoglobulins from antisera prepared, as previously described (*15*), against the internal undecapeptide I1 or the internal decapeptide I2 or the NH_2-terminal hexapeptide (N) or the COOH-terminal nonapeptide (C) of the URF6 product (Table 1), or from normal serum (NS). Conditions of immunoprecipitations were as detailed elsewhere (*17*) with the modifications described in the text. The immunoprecipitation with antibody to URF6-I1 was carried out in the absence or presence of 10 μg of the corresponding peptide. The immunoprecipitates were run in an SDS–8*M* urea–15% polyacrylamide gel (a and b) or in an SDS–15 to 25% polyacrylamide gradient gel (*15, 17*) (in Sigma SDS) (c). M, pattern of HeLa cell mitochondrial translation products. In the marker pattern observed after electrophoresis in an SDS-polyacrylamide gradient gel (c), COI is resolved into two bands [corresponding to components 2 and 3, according to the classification by Ching and Attardi (*23*)], as shown by immunoprecipitation experiments with antibodies to COII-C on a mitochondrial Triton X-100 lysate (*38*). ND1, ND2, ND3, ND4, ND4L, and ND5, NADH dehydrogenase subunits 1, 2, 3, 4, 4L, and 5 (*21*); COI, COII, and COIII, cytochrome *c* oxidase subunits I, II, and III (*23*); CYT b, apocytochrome b (*39*); ATPase 6 and 8, subunits 6 and 8 of H^+-ATPase (*19*).

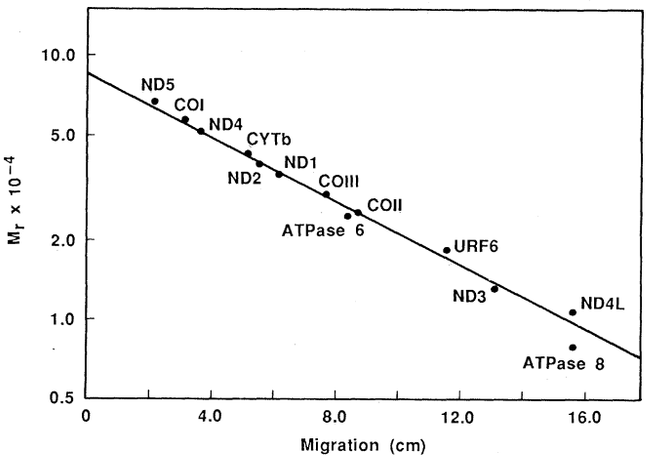

Fig. 2. Electrophoretic mobilities of the human mitochondrial translation products in an SDS–17.5% polyacrylamide gel in MCB SDS plotted against relative molecular mass predicted from the DNA sequence.

1), precipitated specifically component 22, the reaction being inhibited by the corresponding peptide (Fig. 1, a and b). This peptide corresponds to the most hydrophilic segment of the putative URF6 product. In the immunoprecipitate obtained with anti-URF6-I1 there are, in addition to component 22, two other specific bands moving slower than ND5 (indicated by asterisks), with estimated relative molecular masses of 71,000 and 88,000.

Figure 1c shows the pattern obtained when the immunoprecipitate produced by anti-URF6-I1 was run in a different gel system, an SDS–15 to 25% polyacrylamide gradient gel. A pronounced band appeared at the expected position for component 22 (*15, 17, 25*); two specific, slowly moving bands (indicated by asterisks) could also be seen that corresponded closely in size to those observed in SDS-urea-polyacrylamide gels.

The pattern of labeled polypeptides in the SDS mitochondrial lysate showed discrete components (especially after long exposure of the autoradiograms) corresponding in mobility to the slowly moving bands found in the immunoprecipitate. Therefore, it was not possible to decide whether these slowly moving bands represented cytoplasmically synthesized proteins with homology to the I1 internal sequence of the URF6 product (which were labeled during the pulse due to incomplete block of cytoplasmic protein synthesis by emetine), or whether they represented aggregates of component 22 with itself or with other components formed during immunoprecipitation.

As shown in Fig. 2, all the HeLa cell mtDNA-coded polypeptides had mobilities consistent with their expected molecular weights. Only the mobility of ATPase 8 was slightly lower than predicted, presumably because of the lower degree of SDS binding by this protein (*17*). In fact, ATPase 8 is unique among the human mitochondrial translation products for having the amino acid composition of a soluble protein (*26*). The mobility of the URF6 product identified in the present work (Fig. 2) falls on the line best fitting the other data, thus supporting the assignment made here.

To test the possibility that the URF6 product, like the products of six other URF's, is a component of the respiratory chain NADH dehydrogenase, we conducted immunoprecipitation experiments with an antiserum against highly purified native beef heart complex I (*27*), using conditions for mitochondria lysis that leave this complex intact (*21*). This antiserum had been shown to cross-react extensively with the human complex solubilized from kidney submitochrondrial particles (*21*). As shown in Fig. 3a, the immunoprecipitate run in an SDS-urea-polyacrylamide gel (in Sigma SDS) exhibited bands corresponding to the products of ND5, ND4, ND2, ND1, ND3, and ND4L (*21*) and, in addition, a band corresponding to the URF6 product (component 22).

These results suggested that the URF6 product is, like the products of ND5, ND4, ND2, ND1, ND3, and ND4L, a subunit of the respiratory chain NADH dehydrogenase. As previously reported (*21*), it could be estimated from the densitometric tracing of appropriate exposures of the autoradiogram and the methionine content of the translation products mentioned above that the labeled smaller polypeptides (ND3 and ND4L products) were present in the immunoprecipitate in lower than stoichiometric amounts relative to the larger subunits (one-third to one-fifth). This underrepresentation was even more pronounced for the labeled URF6 product, which was present in about one-half the molar amount of the ND3 and ND4L products; the reason for this is not known, although it is probably related to the effects of urea discussed above (*24*).

The electrophoretic pattern in an SDS-polyacrylamide gradient gel of the immunoprecipitate obtained with antibodies to complex I (Fig. 3b) again shows bands corresponding to the six previously identified NADH dehydrogenase subunits and a band corresponding to the URF6 product. This last product appears to be present in a substantially larger amount than in the immunoprecipitate analyzed in an SDS-urea-polyacrylamide gel. We estimated that the relative molar concentrations of the labeled products of ND5, ND4, ND2, ND1, URF6, ND3, and ND4L in the immunoprecipitate were 5, 6, 5, 1, 0.9, 1, and 0.8. The values estimated for the corresponding polypeptides in the marker lane containing a sample of the mitochondrial fraction used for immunoprecipitation (M_I) were ~1.2, 1.6, 5, 1, 1.2, 1, and 0.7, respectively (*28*). It seems likely that, after an 18-hour chase, the mtDNA-coded NADH dehydrogenase subunits labeled during a 2.5-hour [^{35}S]methionine pulse were fully incorporated into the mature complex and that the above values therefore represent their proportions in the complex. It is clear that, whereas there is an underrepresentation of ND2, ND1, and smaller subunits in the immunoprecipitate relative to the mitochondrial fraction, the molar representation of the labeled URF6 product in the precipitate is almost identical to that of the ND1, ND3, and ND4L products, strongly supporting its identification as an NADH dehydrogenase subunit (hereafter referred to as ND6 product). The underrepresentation of the smaller subunits in the immunoprecipitate may be due to a loss during the precipitation procedure, possibly as a result of a destabilizing effect of the antibodies. The probable peripheral position of these subunits in the complex presumably accounts for this behavior. The molar excess of labeled ND2 in the mitochondrial fraction may be due to a higher rate of synthesis or higher stability of this subunit. That the latter interpretation is correct is suggested by the observation that, after a 2-hour [^{35}S]methionine pulse, the molar representation of the newly synthesized ND2 product is similar to that of the other subunits.

Considering the hydrophobic nature of the ND6 product (*26*), it seems likely that this polypeptide, like the other mtDNA-coded subunits of complex I, pertains to the hydrophobic fraction of the enzyme complex: this fraction consists of ~15 polypeptides forming a shell around a core containing the low molecular weight NADH dehydrogenase, or flavoprotein fragment, and the iron-protein fragment (*29*). Indeed, among the polypeptides identified in the hydrophobic fraction, there is one with an estimated M_r of 18,000 (*29*), which could well be the ND6 product. An assignment to specific components of the hydrophobic

fraction has already been made for the products of ND1, ND3, and ND4L by using human URF-specific antibodies in immunoblot assays on SDS-denatured beef heart complex I (*19, 30*).

Recently, antibodies against individual, highly purified iron-sulfur subunits of the beef heart NADH dehydrogenase have become available (*31*). These antibodies offered the opportunity to test the identification of the mtDNA URF products as NADH dehydrogenase subunits. In fact, if one or more of these iron-sulfur subunits were exposed in the complex, it was possible that antibodies to them would precipitate from a mitochondrial lysate the entire complex I, including the mitochondrially synthesized polypeptides of the hydrophobic shell. There is indeed good evidence, from labeling studies with hydrophilic probes, that the 75-kD, 49-kD, and 30-kD iron-sulfur subunits of the iron-protein fragment of NADH dehydrogenase are in part exposed both in the intact enzyme and in the membrane (*30, 32*). Figure 3c shows the results of an immunoprecipitation experiment carried out with antibodies against the 49-kD iron-sulfur protein (*31*), a cytoplasmically synthesized protein (*19*). These antibodies react extensively with the human homologous subunit (*33*). Although the reactivity of these antibodies with non–SDS-dissociated complex I was weak, one can see that all seven URF products were precipitated with absolute specificity from a 0.5% Triton X-100 mitochondrial lysate by antibodies to the 49-kD iron-sulfur protein. It is interesting that the molar proportions of the URF products in this immunoprecipitate (that is, ~1, 1.1, 4, 1, 0.8, 0.9, and 0.5 for ND5, ND4, ND2, ND1, ND6, ND3, and ND4L, respectively) were similar to the proportions of the same polypeptides in the mitochondrial fraction used for immunoprecipitation (M_1 lane). This strongly suggests that complex I is precipitated by the antibodies to the 49-kD subunit in a more intact form than by the antibodies to complex I. These results, besides confirming the accessibility to nonpermeant probes of the 49-kD subunit in the intact complex, provide additional support for the functional assignments of the URF products made in this and previous work. With these assignments, all the genes recognized in the DNA sequence of the mammalian mitochondrial genome have been identified in their function.

Although the respiratory-chain NADH dehydrogenase is a universal mitochondrial enzyme, genes homologous to the mammalian mtDNA URF's have not been found in all mtDNA's analyzed. In fact, there is extraordinary variability among different organisms in the genetic control of the subunits of this enzyme complex. Thus, *Neurospora crassa* mtDNA contains genes that have a convincing amino acid sequence homology to five of the mammalian URF's, that is, URF1 (*6*), URF2 (*7*), URF3 (*8*), URF5 (*9*), and URF6 (*10*). Similarly, *Aspergillus nidulans* mtDNA contains genes with a significant homology to URF1, URF2, URF3, URF4, URF5 (*5*), and URF6 (*10*). Genes with a small but significant amino acid sequence homology to human URF1, URF4, and URF5 also occur in the kinetoplast maxicircle DNA of *Trypanosoma brucei* (*12*) and *Leishmania tarentolae* (*13*); furthermore, a gene with homology to URF1 has been found in *Zea mays* mtDNA (*14*), and two genes related to URF2 and URF5 have been detected in *Chlamydomonas reinhardtii* mtDNA (*11*). However, genes homologous to the mammalian mtDNA URF's are absent in the mitochondrial genomes of *Saccharomyces cerevisiae* (*3*) and *Schizosaccharomyces pombe* (*4*). Compared to the mammalian counterpart, the NADH dehydrogenase region of the respiratory chain exhibits functional and structural differences in *Saccharomyces* strains (*34*), and these differences could be due to, or correlated with, the absence of some or all polypeptides homologous to the mammalian mtDNA URF products from the yeast complex. However, it is also possible that in yeast some or all of these polypeptides are encoded in the nucleus and imported from the cytoplasm.

The differences in genetic control of the respiratory chain NADH dehydrogenase in various organisms represent only one example of the variability in informational content in the mitochondrial genomes from different sources (*2, 3, 10*). Such variability had been previously documented by the lack, in the animal mtDNA's, of genes for the ATPase 9 subunit, for the equivalent of the var 1 protein, and for "mRNA maturases" (*2, 3*). The implications of the functional heterogeneity of mtDNA for any theory on the origin and evolution of mitochondria are likely to be profound. The collection of more extensive data on the gene content of mtDNA in different organisms will allow the construction of a functional genealogy of the mitochondrial genomes, which will complement the phylogenetic trees based on sequence data of mtDNA and mitochondrial proteins. While it has been recognized that rapid primary sequence divergence can obscure the evolutionary ancestry of most mitochondrial ge-

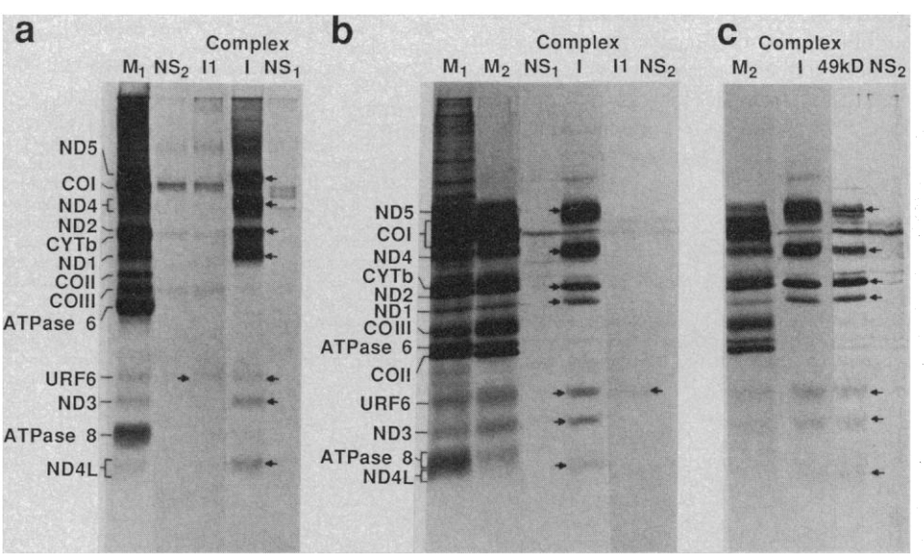

Fig. 3. Assignment to the respiratory chain NADH dehydrogenase of the URF6 product. (a and b) A 0.2% KDOC mitochondrial lysate was prepared from HeLa cells grown for 22 hours in the presence of 40 μg/ml of chloramphenicol (*40*), then labeled for 2.5 hours with [^{35}S]methionine in the presence of 100 μg/ml of cycloheximide (to reversibly inhibit cytoplasmic protein synthesis), and finally chased for 18 hours in unlabeled medium in the absence of inhibitors. Samples of this lysate (100 μg of protein) were incubated with 7 μl of an antiserum to bovine complex I (*21*) or of normal serum (NS_1). The immunoprecipitates were run in an SDS-urea-polyacrylamide gel (a) or in an SDS-polyacrylamide gradient gel (b) (both in Sigma SDS), in parallel with immunoprecipitates obtained by incubating samples of an SDS mitochondrial lysate with immunoglobulins from an antiserum to URF6-I1 or from normal serum (NS_2), as in the experiments of Fig. 1. (c) A sample of a 0.5% Triton X-100 mitochondrial lysate (100 μg of protein) from HeLa cells labeled as in (a) and (b) was precipitated with 70 μg of immunoglobulins from an antiserum to the purified 49-kD iron-sulfur protein of the bovine complex I (49 kD) (*31*) or from normal serum (NS_2). The immunoprecipitates were run in an SDS-polyacrylamide gradient gel. M_1 and M_2, patterns of HeLa cell mitochondrial translation products from cells labeled as described above and, respectively, from cells labeled as in the experiments of Fig. 1. The pattern in lane M_1 shows several large-sized cytoplasmically synthesized components, which were labeled during the 18-hour chase in the absence of inhibitors.

nomes (*35, 36*), a detailed analysis of the functional genealogy of mtDNA may provide the necessary clues to answer such questions as polyphyletic or monophyletic origin of mitochondria and to reconstruct the alternative evolutionary pathways which have led to the present-day variety of mitochondrial genomes.

The URF's of animal mtDNA exhibit a higher divergence rate than the "universal" mitochondrial genes (*2*). Similarly, a comparison between the mitochondrial genes of animal cells and the homologous genes of other eukaryotic cells reveals that the URF's are in general less conserved, in both size and sequence, than the "universal" genes (*5, 7–9, 13*). On the surface this is surprising, since both the products of the URF's and those of the other genes are components of indispensable enzymes. A reasonable explanation for this difference is that the URF products are components of the hydrophobic shell of the NADH dehydrogenase, which has presumably the function of creating an appropriate environment for the catalytic moieties of the enzyme complex within the inner mitochondrial membrane (*29*). This function may not depend strictly on the primary sequence. It is indeed interesting that the amino acid sequences of the URF-related mtDNA genes in filamentous fungi and protozoa often have only a low degree of homology to the sequences of the equivalent mammalian genes, but, on the contrary, an almost perfect correspondence in hydropathy profile over large segments (*4, 6–8, 13*).

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