High-Resolution Electrophoretic Fractionation and Partial Characterization of the Mitochondrial Translation Products from HeLa Cells†

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ABSTRACT: In the present work, the mitochondrial translation products from HeLa cells have been investigated under greatly improved conditions for both detection and fractionation, as compared to previous work. An electrophoretic analysis of the mitochondrial proteins labeled according to a protocol which produced a high rate of [^{35}S]methionine incorporation into mitochondrially synthesized polypeptides revealed 17 reproducible discrete bands in a sodium dodecyl sulfate ($NaDodSO_4$)-urea-polyacrylamide slab gel system and 19 bands in a $NaDodSO_4$-polyacrylamide linear gradient system; a higher resolution of the mitochondrial translation products was obtained by a bidimensional combination of the two gel systems. This analysis has provided evidence for the existence of as many as 26 reproducible components. The polypeptide nature of the labeled products was indicated by their Pronase sensitivity and by the ribosomal dependence of the [^{35}S]-methionine incorporation, as implied in the chloramphenicol sensitivity. Furthermore, the response of the labeling to antibiotics clearly pointed to an intramitochondrial site of synthesis for these polypeptides. The pattern of polypeptide bands seen in the unidimensional gel electrophoresis analysis of standard preparations was also observed, essentially unchanged, in mitochondrial preparations made in the presence of protease inhibitors or in direct $NaDodSO_4$ lysates of labeled cells: these results strongly argue against the possibility of degradation artifacts. A comparison of the electrophoretic patterns in a $NaDodSO_4$-urea-polyacrylamide gel obtained for a sample pulse labeled with [^{35}S]methionine in the presence of cycloheximide and a sample pulse labeled and then chased for 1 h in complete unlabeled medium in the absence of antibiotics indicated that the majority of the labeled polypeptides do not arise by processing or conversion of others, at least in a short-term experiment. Among the mitochondrial translation products, three have been assigned to cytochrome *c* oxidase subunits I, II, and III. The results are discussed in relationship to the evidence concerning the informational role of mitochondrial DNA (mtDNA) derived from the recent DNA and RNA sequencing analysis.

The mitochondrion contains its own genetic material and a specific transcription and translation machinery (Borst & Grivell, 1978), and it is generally accepted that the mRNAs[1] which are transcribed from the organelle's circular genome are translated on mitochondrial ribosomes. Most of the polypeptides synthesized in mitochondria are hydrophobic proteins of the inner mitochondrial membrane (Schatz & Mason, 1974). In yeast, the mitochondrial translation products detected so far have been identified with components of enzyme complexes of the inner membrane, in particular, three subunits of cytochrome *c* oxidase, one subunit of the cytochrome bc_1 complex (cytochrome *b*), and two subunits of the oligomycin-sensitive ATPase (Borst & Grivell, 1978), and with the var-1 protein, a polypeptide associated with the small ribosomal subunit (Douglas & Butow, 1976; Perlman et al., 1977; Strausberg et al., 1978). However, recent genetic and biochemical evidence has pointed to the existence of a new class of proteins synthesized in mitochondria and encoded in mtDNA ("maturases"), which play a fundamental role in the processing of mitochondrial mRNAs (Lazowska et al., 1980; Claisse et al., 1980; Jacq et al., 1982; Mahler et al., 1982). Furthermore, an analysis of the yeast mitochondrial translation products by a highly resolving $NaDodSO_4$-polyacrylamide gel system has revealed a significantly larger number of discrete components than previously recognized (Douglas & Butow, 1976).

In contrast to yeast, direct information on the nature of the mitochondrial translation products in animal cells, and in particular mammalian cells, has been very slow in coming forth. Among the above-mentioned mitochondrially synthesized polypeptides identified in yeast, only the three subunits of cytochrome *c* oxidase (Yatscoff et al., 1977; Rascati & Parsons, 1979; Hare et al., 1980; Wilson et al., 1981) and cytochrome *b* (Wilson et al., 1981) have been recognized with any degree of confidence as translation products of mammalian mitochondria. Eight to 13 discrete components have been resolved in various mammalian cell types by using different systems of $NaDodSO_4$-polyacrylamide gel electrophoresis (Coote & Work, 1971; Costantino & Attardi, 1975; Jeffreys & Craig, 1976; Yatscoff & Freeman, 1977; Yatscoff et al., 1978). Recently, the determination of the complete sequence of human mtDNA (Anderson et al., 1981) and mouse mtDNA (Bibb et al., 1981) has revealed the existence in mammalian mtDNA of 13 significant reading frames longer than 120 nucleotides. These are transcribed into presumably functional mRNAs (Ojala et al., 1980, 1981; Montoya et al., 1981) and would code for polypeptides in the molecular weight range between 7900 and 66 600. Eight among these reading frames do not appear to be homologous to any segment of yeast mtDNA, and their genetic function is unknown. The discovery of these unidentified reading frames (URFs) has raised intriguing questions concerning the genetic content and evolution of mtDNA.

In the present work, the mitochondrial translation products from HeLa cells have been analyzed under greatly improved conditions for both detection and fractionation as compared

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[1] Abbreviations: mRNA, messenger ribonucleic acid; mtDNA, mitochondrial deoxyribonucleic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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to previous work (Costantino & Attardi, 1975, 1977; Ching et al., 1977). By use of a cell labeling protocol producing a high rate of incorporation of [^{35}S]methionine into mitochondrially synthesized proteins and both unidimensional and bidimensional highly resolving polyacrylamide gel electrophoresis systems for fractionation, evidence has been obtained for the existence of an appreciably larger number of products than reported so far for mammalian systems. Up to as many as 26 reproducible components have been identified and partially characterized in their size, polypeptide nature, site of synthesis, and mutual relationship.

Materials and Methods

Growth of Cells and Labeling Conditions. Suspension cultures of the S3 clonal strain of HeLa cells were grown as previously described (Amaldi & Attardi, 1968). The cultures were free of detectable mycoplasma contamination. For pulse labeling of the mitochondrial translation products, exponentially growing cells were harvested, washed twice with methionine-free modified Eagle's medium containing 5% dialyzed calf serum, and resuspended in this medium at 5×10^6 cells/mL. During the subsequent incubation for cell labeling and chasing, filtered air with 5% CO_2 was flushed over the medium at 2 L/min in order to help stabilize the pH of the medium at very high cell density. Five minutes after cell resuspension, antibiotics were added at 100 μg/mL. For inhibition of cytoplasmic protein synthesis, emetine was generally utilized (Perlman & Penman, 1970; Costantino & Attardi, 1975); however, whenever reversibility of the inhibition was required, cycloheximide was used (Ennis & Lubin, 1964; Colombo et al., 1966). For inhibition of mitochondrial protein synthesis, chloramphenicol was also added (Kroon, 1965; Lederman & Attardi, 1970). Five minutes after antibiotic addition, [^{35}S]methionine (600–1200 Ci/mmol) was added to 8.3 μCi/mL, and the cells were incubated for 2 h. In some experiments, a 1-h chase was performed either by addition of unlabeled methionine to 0.01 M, in the case of emetine block, or, in the case of cycloheximide block, by washing the cells twice in complete unlabeled medium to remove the drug and resuspending them at 2×10^6 cells/mL. The specific activities obtained under the above-mentioned labeling conditions varied, in different experiments, between 200 and 600 cpm/μg of total mitochondrial proteins (specific activities as high as 1600 cpm/μg of protein have been obtained in more recent experiments).

Subcellular Fractionation. After being labeled, the cells were quickly cooled on ice and washed 3 times with cold 0.13 M NaCl, 0.005 M KCl, and 0.001 M $MgCl_2$ (NKM), and an $11000g_{av}$ crude mitochondrial fraction was prepared as previously described (Lederman & Attardi, 1970). The final mitochondrial pellet was resuspended in 0.25 M sucrose in 0.01 M Tris–acetate (pH 7.0 at 25 °C) (1.0 mL/4×10^7 cell equiv) and sonicated (Costantino & Attardi, 1975). The final protein concentration was 5–10 mg/mL. For inhibition of nonphysiological protease activity, in one experiment, phenylmethanesulfonyl fluoride (PMSF) was added fresh at 0.002 M (James, 1978) and EGTA at 0.005 M to all solutions used for cell washing and homogenization and for resuspension of the mitochondrial fraction.

For the preparation of a whole cell lysate, the cells were washed in NKM and then resuspended in 0.6 cell volume of 0.01 M Tris-HCl, pH 7.0, 0.10 M NaCl, and 0.001 M EDTA; after addition of $NaDodSO_4$ to 0.5%, the samples were sonicated and dialyzed overnight against the same buffer.

Purification of Human Cytochrome c Oxidase. For the identification of the cytochrome *c* oxidase subunits among the HeLa cell mitochondrial translation products, mitochondrial membrane protein (20 mg) derived from HeLa cells labeled for 2 h with [^{35}S]methionine in the presence of cycloheximide and then chased for 1 h in unlabeled medium, as described above, was mixed with 580 mg of human placenta mitochondrial membrane protein, and cytochrome *c* oxidase was purified by the cholate–Triton X-100 procedure, as described earlier (Hare et al., 1980).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis through phosphate buffered $NaDodSO_4$–12.5% polyacrylamide [1:40 bis(acrylamide)] column gels was carried out as described (Weber & Osborn, 1969; Costantino & Attardi, 1975). Slab gels using the same buffer, but with 15% polyacrylamide instead of 12.5%, were also used. Linear polyacrylamide gradient slab gels (15–25%) [1:75 bis(acrylamide)] with a 1-cm stacking gel of 5% polyacrylamide [1:37.5 bis(acrylamide)] in Tris–EDTA–$NaDodSO_4$ buffer were prepared and run as described (Studier, 1973; Attardi & Ching, 1979). Electrophoresis through $NaDodSO_4$–15% polyacrylamide [1:50 bis(acrylamide)]/8 M urea slab gels was carried out essentially as described (Downer et al., 1976). However, a 1-cm stacking gel consisting of 5% acrylamide [1:37.5 bis(acrylamide)], 8 M urea in 0.033 M H_3PO_4–Tris (pH 6.3), and 0.1% $NaDodSO_4$ was added (Attardi & Ching, 1979).

For a two-dimensional fractionation, a $NaDodSO_4$–12.5% polyacrylamide [1:50 bis(acrylamide)]/8 M urea slab gel was used and run for the appropriate time. A gel track was cut out, the urea eluted with several short changes of water, and the track overlaid directly onto a higher percentage gel (either a 15% polyacrylamide gel in $NaDodSO_4$–phosphate buffer or a 15–25% linear gradient gel in Tris–EDTA–$NaDodSO_4$ buffer). Bubbles at the gel interface were carefully removed. The track was overlaid with a solution of 0.1% $NaDodSO_4$, 5% β-mercaptoethanol, and tracking dyes, and electrophoresis was carried out normally. Fluorography and autoradiography of the dried gels were performed as described (Bonner & Laskey, 1974).

Results

Resolution of Mitochondrial Translation Products by Electrophoresis in a Single Dimension. In earlier work from this laboratory, a fractionation of the HeLa cell mitochondrial translation products through phosphate-buffered $NaDodSO_4$–polyacrylamide column gels (Weber & Osborn, 1969) revealed, under optimum conditions, the presence of 10 discrete electrophoretic components in the molecular weight range from 11 500 to 40 000 (Costantino & Attardi, 1975, 1977). The pattern of protein components labeled by [^{35}S]methionine in the presence of emetine was found to be virtually identical with that obtained after labeling with other amino acids (Ching et al., 1977). A profile with the major peaks identified by the earlier designation (using an asterisk to distinguish them from the more recent classification) is shown in Figure 1. Beneath is shown the fluorogram, after electrophoresis through a $NaDodSO_4$–15% polyacrylamide slab gel in phosphate buffer, of similar material (EM), as well as of a sample from cells labeled in the presence of emetine and chloramphenicol (EM+CAP). Thirteen bands of greatly varying intensity are recognizable, six of which appear to correspond to peaks in the cylindrical gel electrophoretic profile. Band 1*, corresponding to peak 1*, is very broad, suggesting the presence in it of more than one component. The fluorogram shows a number of bands not resolved in the cylindrical gel profile. The greater resolution and sensitivity of detection inherent in the slab gel system combined with fluorography is clearly apparent.

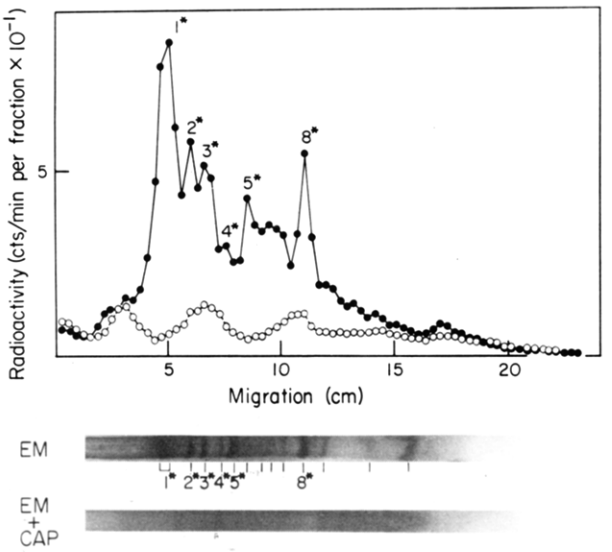

FIGURE 1: Resolution of HeLa cell mitochondrial translation products using the phosphate-buffered $NaDodSO_4$–polyacrylamide gel electrophoresis system. The upper panel shows the profile of the products of mitochondrial protein synthesis labeled with [^{35}S]methionine in the presence of emetine alone (●) or emetine plus chloramphenicol (○), separated on a phosphate-buffered $NaDodSO_4$–12.5% polyacrylamide column gel and assayed after gel smashing by scintillation counting. The peak designations are as in previous work (Costantino & Attardi, 1975). The fluorographs below, indicated EM and EM+CAP, show the fractionation of similarly labeled proteins run on a phosphate-buffered $NaDodSO_4$–15% polyacrylamide slab gel. The bands are labeled as in the column gel, with additional unassigned components also indicated.

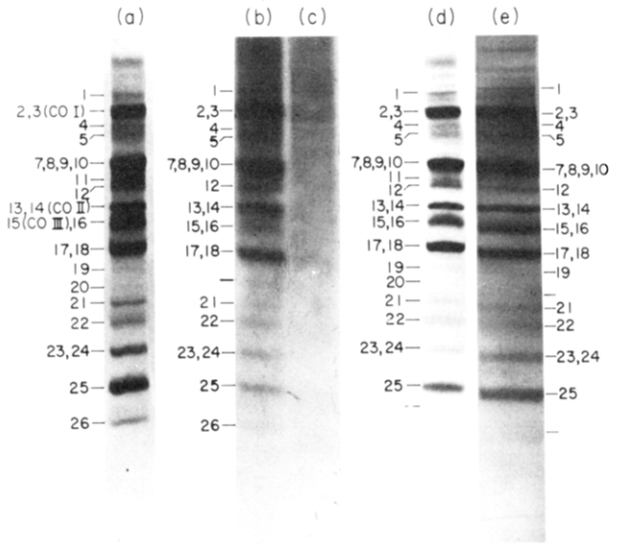

FIGURE 2: Resolution of HeLa cell mitochondrial translation products by electrophoresis through $NaDodSO_4$–urea–polyacrylamide slab gels. (a) Pattern of products of mitochondrial protein synthesis labeled with [^{35}S]methionine in the presence of emetine. (b and c) Patterns of the products labeled in the presence of emetine alone or emetine plus chloramphenicol, respectively, after a 1-h chase and whole cell lysis. (d and e) Patterns of the products labeled in the presence of cycloheximide without or with, respectively, a 1-h chase. Components COI, COII, and COIII, identified as described earlier (Hare et al., 1980), are indicated in lane a. Species of uncertain identification are indicated but not numbered. See text for details.

The labeling of all the bands recognizable in the slab gel electrophoretic pattern appears to be chloramphenicol sensitive.

Gel electrophoresis systems of higher resolution were subsequently applied for separation of the products of mitochondrial protein synthesis. Thus, a phosphate–Tris-buffered $NaDodSO_4$–urea–polyacrylamide slab gel system resolved 17 discrete bands, as shown in Figure 2 (track a). The bands are designated by progressive arabic numerals corresponding to increasing electrophoretic mobilities, with more than one number being assigned to the same band in cases of comigration of different components, as determined by bidimensional fractionation (see below). Component 6, described below, was not recognizable as a discrete band in this type of electrophoretic fractionation, possibly because it comigrated with the heavily radioactive band designated 7,8,9,10. Shown in Figure 2a is the band assignment of the cytochrome *c* oxidase subunits. On the basis of the pattern of labeling of the subunits of highly purified human cytochrome *c* oxidase, subunit I (COI) had been previously shown to correspond to the unresolved components 2 and 3, subunit II (COII) to the unresolved components 13 and 14, and subunit III (COIII) to the unresolved components 15 and 16 (Hare et al., 1980). A more precise identification of these subunits, as indicated in Figure 2a, was made in the present work on the basis of their behavior in a $NaDodSO_4$–polyacrylamide gradient gel and in a bidimensional fractionation (see below).

Electrophoresis of the mitochondrial translation products through a Tris–glycine-buffered $NaDodSO_4$–polyacrylamide linear gradient slab gel could resolve 19 discrete bands, as shown in Figure 3 (track a). Here, too, the bands are designated by arabic numerals, in this case, however, on the basis of their correspondence to the polypeptides resolved in the $NaDodSO_4$–urea–polyacrylamide gel system, as determined by bidimensional fractionation (see below). In the gradient gels, comigration of different polypeptides also occurs, although the polypeptides involved are clearly different from the polypeptides comigrating in the $NaDodSO_4$–urea gels (see below). Other electrophoretic runs, carried out under the same conditions used in the experiments of Figures 2a and 3a, gave very similar patterns; although there was some variation in resolution and some of the minor bands were occasionally not detected due to inadequate fluorographic exposure, the characteristic relative migrations and intensities of the bands were found to be very reproducible.

Shown in Figure 3g,h are the results of a mixing experiment in which cytochrome *c* oxidase, highly purified according to a previously described procedure (Hare et al., 1980) from a mixture of ^{35}S-labeled HeLa cell mitochondria and an excess of human placenta mitochondria, was electrophoresed through a $NaDodSO_4$–polyacrylamide gradient slab gel, which was then subjected to autoradiography to visualize the labeled components. Lane g in Figure 3 shows the electrophoretic pattern of the labeled polypeptides in the purified cytochrome *c* oxidase. Three bands are visible; these presumably correspond to the only three bands which were observed in the same preparation run on a $NaDodSO_4$–urea gel and which coincided perfectly in migration with the stained COI, COII, and COIII subunits (Hare et al., 1980). In lane h, where a sample of HeLa cell total mitochondrial translation products, labeled under the same conditions described above, was run, one can recognize the typical pattern of the main mitochondrially synthesized polypeptides; however, there appear to be a certain number of high molecular weight cytoplasmically synthesized polypeptides which escaped the cycloheximide block. The three labeled components of the purified cytochrome *c* oxidase (Figure 3g) correspond to three main bands of the pattern of mitochondrial translation products. The assignment of COI to band 3 was made on the basis of its alignment relative to the large band representing the partially resolved components 2 and 3 (see also below). The assignment of COIII to band 15 and COII to band 14 was made on the basis of the typical appearance of COII as a sharp band and COIII as a broad

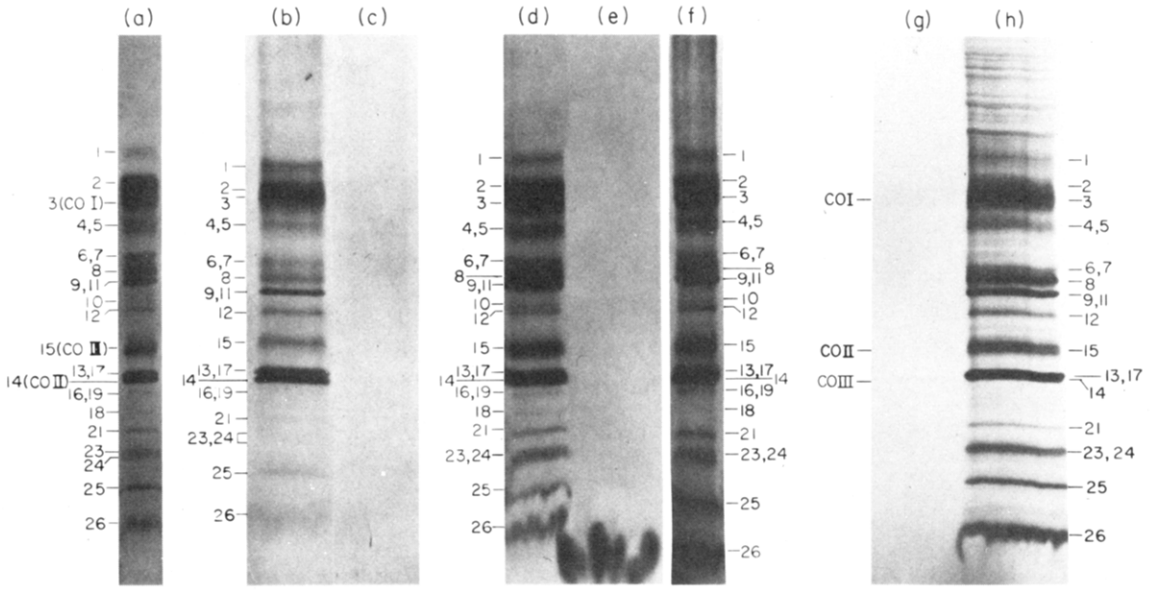

FIGURE 3: Resolution of HeLa cell mitochondrial translation products by electrophoresis through $NaDodSO_4$–polyacrylamide linear gradient slab gels. (a) Pattern of products of mitochondrial protein synthesis labeled with [^{35}S]methionine in the presence of emetine. (b and c) Patterns of the products labeled in the presence of emetine alone or emetine plus chloramphenicol, respectively. (d and e) Patterns of the products labeled in the presence of emetine and electrophoresed directly or after incubation with 25 μg of Pronase at 37 °C for 50 min, respectively. (f) Pattern of the products labeled in the presence of emetine, with mitochondria purification carried out in the presence of PMSF and EGTA. (g and h) Autoradiograph of cytochrome *c* oxidase isolated from a mixture of [^{35}S]methionine-labeled HeLa cell mitochondria and an excess of unlabeled human placenta mitochondria (g; see text for details), run in parallel with a sample of total [^{35}S]methionine-labeled mitochondrial fraction from cells exposed to the radioactive precursor for 2 h in the presence of cycloheximide and chased for 1 h in unlabeled medium (h). Components COI, COII, and COIII, identified as explained in the text, are indicated in lane a.

band [Downer et al., 1976; Hare et al., 1980; the appearance of band 15 as a doublet in lane h of Figure 3, a phenomenon which has been frequently observed for COIII by other authors (Downer et al., 1976; Hare et al., 1980), possibly reflects the occurrence of heme-free and heme-containing polypeptides with slightly different electrophoretic mobility]. The apparent inversion of migration of COII and COIII in the $NaDodSO_4$–polyacrylamide gradient gel, as compared to their behavior in the $NaDodSO_4$–urea–polyacrylamide gel (Figure 2a), has been confirmed by the bidimensional fractionation experiments (see below).

Characterization of the Labeled Components as Polypeptides. The emetine resistance of the labeling of the components resolved in the different gel systems described above suggested that most or all of these components represented polypeptides synthesized on mitochondrial ribosomes. This interpretation was supported by the results of experiments which showed the sensitivity of the labeling to chloramphenicol (Figure 2b,c for the whole cell lysate; Figure 3b,c for purified mitochondria) and therefore the dependence on mitochondrial ribosome function for the [^{35}S] methionine incorporation into the above described components. Furthermore, treatment of the samples with Pronase prior to electrophoresis degraded all the labeled components to small fragments (Figure 3d,e), indicating the polypeptide nature of these components.

Evidence for in Vivo Presence of the Detected Polypeptides. The large number of polypeptides identified here raised the possibility of artifactual generation of protein fragments by site-specific degradative enzymes. Although the possibility of degradation during the preparation was minimized by keeping the samples in the cold and working as rapidly as possible, it could not be eliminated completely. For investigation of the possible contribution of these degradative phenomena to the observed multiplicity of polypeptide bands detected on gel, one experiment was carried out in which PMSF and EGTA were added fresh to all solutions to inhibit protease activity during the mitochondria purification procedure. Figure 3f shows that the resulting profile, after electrophoresis through a $NaDodSO_4$–polyacrylamide gradient gel, is virtually identical with that obtained in the normal preparations made in the absence of the protease inhibitors. Finally, for complete elimination of the preparative manipulations prior to electrophoresis, cells were labeled under appropriate conditions, chased by addition of unlabeled methionine, and directly lysed with $NaDodSO_4$. This sample, plus a chloramphenicol control sample was run on a $NaDodSO_4$–urea–polyacrylamide gel, and the patterns obtained are shown in Figure 2b,c. It is clear that, although the background is higher, most of the bands previously detected in the electrophoretic profile of the mitochondrial lysate are present in similar relative proportions in the pattern of total cell lysate; bands 11, 19, and/or 20 are not recognizable, either because they are really absent, possibly as a result of the chase to which the cells were submitted in this experiment (see, in fact, Figure 2d,e discussed below), or because they do not stand out against the background due to their normally low labeling intensity. The lower relative intensity of band 15, 16 in the cell lysate sample as compared to the mitochondrial sample is probably due to the overexposure of the latter fluorogram (see, in fact, Figure 2d). The above described controls, therefore, would tend to argue against any significant role of nonphysiological degradative phenomena during mitochondria purification in the results observed here.

Bidimensional Fractionation of the Mitochondrial Translation Products. The most highly resolving system proved to be a bidimensional combination of the $NaDodSO_4$–urea and polyacrylamide gradient gel systems. The results of one such experiment are shown in Figure 4. It should be noticed that, because of the width of the overlaid urea gel track and the lack of a stacking gel over the $NaDodSO_4$–polyacrylamide gradient, the spots, especially in the lower molecular weight range, have a tendency to show a streak of faster moving material. It should also be mentioned that, because of the difficulty of obtaining a standard of mitochondrial translation products

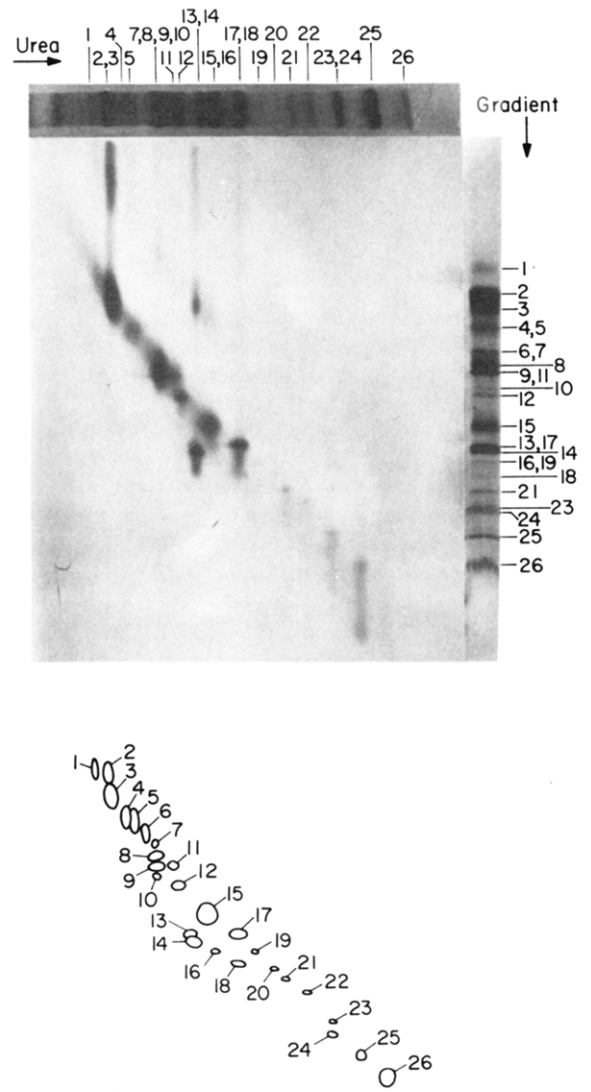

FIGURE 4: Bidimensional fractionation of HeLa cell mitochondrial translation products through a $NaDodSO_4$–urea–polyacrylamide gel and a $NaDodSO_4$–polyacrylamide linear gradient gel. After fractionation of the products through a $NaDodSO_4$–urea–12.5% polyacrylamide slab gel, a track was overlaid onto a $NaDodSO_4$–polyacrylamide linear gradient gel and electrophoresed. A urea gel pattern and a gradient gel pattern are shown above and alongside, respectively, to aid in the recognition of the components. The interpretation of the gel is shown in the line drawing below. The band designations in the single-dimension electrophoretic fractionations reflect their composition, as assigned here.

migrating in the polyacrylamide gradient dimension in a way comparable to the migration of the components already separated in the urea gel, the fluorogram of a $NaDodSO_4$–polyacrylamide gradient gel track run under standard conditions was used as a reference; in this standard, the faster moving components (23, 24, 25, and 26) run somewhat behind the corresponding spots in the bidimensional fractionation. The lower panel in Figure 4 shows the interpretation of the fluorogram. Spot identification was made by using the combined criteria of relative and absolute migrations and intensities; marginally resolved components could be identified by following the edges of the composite spots. Only spots which, according to the above mentioned criteria, were reproducibly present in other electrophoretic runs as that shown in Figure 4 were positively identified. Discrete spots for which there was reproducible evidence in the bidimensional fractionation were numbered successively from the left (i.e., according to increasing electrophoretic mobility in the $NaDodSO_4$–urea gel system), and comigrating species in that dimension were numbered successively from the top (i.e., according to increasing electrophoretic mobility in the gradient gel system). Bands which were resolved in either of the two dimensions were numerically designated by the components contained within them.

The material in the thick band designated 2,3, not resolved in the $NaDodSO_4$–urea gel dimension, gave, in the run through the polyacrylamide gradient, a big oval spot; this has been interpreted to correspond to components 2 and 3, which are clearly separated in a direct run on a gradient gel. Components 4 and 5, which were resolved in the $NaDodSO_4$–urea dimension (see also the less exposed fluorogram of Figure 2d) and gave two spots in the second dimension, were not separated in a direct run on a gradient gel. In the bidimensional pattern one can see, on the right and slightly below the 4–5 doublet, a weakly labeled spot, which is probably another component (6) not recognizable in the $NaDodSO_4$–urea gel. The thick band designated 7,8,9,10 in the $NaDodSO_4$–urea gel was resolved in the second dimension into two main partially overlapping spots designated 8 and 9, a faster moving spot designated as 10, and a slower moving spot designated as 7 (the partial separation of the four components in the second direction is more clear in less exposed autoradiograms, where, however, the weakest spots of the pattern are not visible). Component 7 seemed to comigrate with the putative component 6 in the gradient gel direction, while spot 9 appeared to comigrate with spot 11. Spot 10 may correspond to the band moving slower than band 12 in the $NaDodSO_4$–polyacrylamide gradient reference pattern. Components 13 and 14 (COII), which were not resolved in the $NaDodSO_4$–urea pattern, produced clearly two partially overlapping spots which migrated ahead of the spot corresponding to component 15 (COIII). Similarly, the thick bands 15,16 and 17,18 in the $NaDodSO_4$ urea gel pattern each produced, when run in the second dimension, a main heavily labeled spot (15 and 17, respectively) and a fainter, faster moving spot (16 and 18, respectively). Spot 17 appeared to be aligned with spot 13 in the polyacrylamide gradient direction, and the two spots correspond presumably to the single band designated 13,17 of the gradient gel. The weakly labeled component 19 in the $NaDodSO_4$–urea gel pattern migrated in the gradient gel direction to the same position as band 16, while components 20 and 22 each gave a spot not corresponding to any obvious band in the gradient gel reference pattern. Spots 16, 18, 19, 20, and 22 were faint but reproducibly present. Components 23 and 24, which were not resolved in the $NaDodSO_4$–urea gel, were separated in the polyacrylamide gradient gel dimension. In conclusion, the bidimensional fractionation discussed here, combined with the unidimensional analysis, has provided evidence for the existence of as many as 26 discrete reproducible components.

It can be seen in Figure 4 that subunits II and III of the cytochrome *c* oxidase complex (i.e., species 14 and 15) are inverted relative to one another in their order of migration in the $NaDodSO_4$–urea and gradient gel systems, confirming the conclusion stated earlier. There are other cases of inversion of order of migration in the two gel systems, for example, component 14 vs. component 17.

Table I lists the estimated molecular weights of the components identified in the above described unidimensional and bidimensional fractionation of the mitochondrial translation products. These molecular weights were estimated from the mobility of the various components in $NaDodSO_4$–urea gels relative to that of standard proteins (Hare et al., 1980).

Table I: Estimated Molecular Weights of the Products of HeLa Cell Mitochondrial Protein Synthesis Fractionated on a $NaDodSO_4$-Urea-Polyacrylamide Gel

band designation	M_r[a] ($\times 10^{-3}$)	M_r[b] ($\times 10^{-3}$)
1	51	51
2,3 (COI)	45	90
4	39	39
5	36	36
7,8,9,10	29	116
11	25	25
12	24	24
13,14 (COII)	20	40
15 (COIII),16	18	36
17,18	16	32
19	12	12
20	10	10
21	9	9
22	8	8
23,24	6	12
25	4.5	4.5
26	3.5	3.5
		548[c]

[a] Molecular weight of individual components in the band. [b] Total molecular weight of components in the band. [c] Total.

The correspondence between the components previously resolved in a phosphate-buffered polyacrylamide cylindrical gel and those identified in a $NaDodSO_4$–urea–polyacrylamide slab gel was investigated in a way similar to that described above, by running a two-dimensional gel. The first dimension was run in the urea system and a track overlaid onto a phosphate-buffered polyacrylamide slab gel. In the bidimensional pattern (not shown), bands 1–5 of the $NaDodSO_4$–urea gel pattern were found to correspond to the broad band 1* in the phosphate-buffered gel direction; one component of the thick band in the $NaDodSO_4$–urea gel pattern designated 7,8,9,10 appeared to be resolved from the others and to comigrate with band 11 in the phosphate-buffered gel system. The assignment of the mitochondrial translation products in its present notation to the main components identified in the phosphate-buffered gel system is summarized in Table II. Here, too, there is an inversion in the order of migration of some components in the two gel systems.

Interrelationship between the Identified Mitochondrial Translation Products. The multiplicity of polypeptides identified as mitochondrial translation products in the analysis described above posed the question of whether any precursor to product relationship reflecting physiological conversion processes exists between some of these polypeptides. In Figure 2d,e, the electrophoretic profile in a $NaDodSO_4$–urea gel of the mitochondrial lysate from cells labeled for 2 h with [^{35}S]methionine in the presence of cycloheximide [an inhibitor of cytoplasmic protein synthesis whose action is quickly reversible upon removal of the drug (Ennis & Lubin, 1964; Colombo et al., 1966)] (Figure 2d) is compared to that of a sample from cells labeled with [^{35}S]methionine in the presence of cycloheximide, then washed free of drug, and incubated in complete unlabeled medium for an additional hour (Figure 2e). The two patterns are very similar in number and relative intensity of the bands; band 11 and, possibly, also bands 19 and 20 are apparently absent or strongly decreased in intensity. Also after separation of the mitochondrial translation products in a $NaDodSO_4$–polyacrylamide gradient gel, the pattern obtained for a pulse–chased preparation (Figure 3h) is very similar to that observed for a nonchased preparation, except for the absence of components 10, 16, 19, and, possibly, 18 and for the apparent decrease in amount of component 2 (it is not clear whether the extra faint bands appearing between 13 and 15 and between 14 and 21 in Figure 3h represent conversion products of other mitochondrially synthesized polypeptides or reflect a residual labeling of cytoplasmically synthesized polypeptides). It should be mentioned that, due to the comigration of some of the mitochondrial translation products in the two types of gel systems used here, more components than those observed may have disappeared or decreased in amount after the chase. However, it seems safe to conclude that the majority of the mitochondrial translation products identified in the present work appear to be fairly stable, arguing against their being precursors or processing intermediates destined to be rapidly converted to mature species.

Table II: Comparison of Fractionation of the HeLa Cell Mitochondrial Translation Products in a Phosphate-Buffered $NaDodSO_4$-Polyacrylamide Gel and in a $NaDodSO_4$-Urea-Polyacrylamide Gel[a]

component no. of phosphate gel system	component no. of urea gel system
1*	1, 2, 3, 4, 5
2*	3 of 7,8,9,10
3*	11, 1 of 7,8,9,10
4*	12
5*	17,18
8*	14

[a] The table shows the correspondence between the discrete components resolved in a phosphate-buffered $NaDodSO_4$-polyacrylamide column gel (asterisked numbers in Figure 1) and the components resolved in a $NaDodSO_4$-urea-polyacrylamide gel (Figure 2).

Discussion

Nature of the [^{35}S]Methionine-Labeled Products. The size distribution and relative labeling of the various labeled polypeptides identified in the present work were very reproducible in different preparations, although with some slight variation in the degree of resolution in different electrophoretic runs. The control experiments which were performed strongly argue against the possibility of degradation artifacts as being responsible for the large number of components detected here. Furthermore, the fact that most of these components were observed under quite different conditions of gel electrophoresis, including electrophoresis in the presence of 8 M urea, would tend to exclude the role of aggregation phenomena in generating the patterns observed.

Great differences were found in the degree of [^{35}S]-methionine labeling of the various polypeptides, some of which produced very intense bands or spots in the autoradiogram, while others gave barely visible traces. This variability may be due in part to differences in the rate of synthesis and/or metabolic stability of different polypeptides, but in part certainly reflects differences in their methionine content. It should be noted that the cytochrome *c* oxidase subunits I, II, and III appear to be among the main mitochondrial translation products.

Electrophoretic Resolution of the [^{35}S]Methionine-Labeled Mitochondrial Translation Products. The conditions chosen in the present work for the labeling and analysis of the mitochondrially synthesized polypeptides come close to the optimal detection capabilities presently available. The three unidimensional gel electrophoretic systems utilized here differed considerably in the type of fractionation they produced. Both the Tris–glycine-buffered $NaDodSO_4$–polyacrylamide

linear gradient system and the phosphate–Tris-buffered NaDodSO$_4$–urea–polyacrylamide system exhibited a higher resolving power than the phosphate-buffered NaDodSO$_4$–polyacrylamide gel system but differed from each other in the separation of components in different molecular weight ranges and in the sharpness of the bands they produced. In particular, the gradient gel system gave sharper bands and reasonably good separation, especially in the high molecular weight range; the urea gel system gave a wider separation of components in the low molecular weight range, although it produced bands which were somewhat less sharp than in the gradient system. Furthermore, the urea gel system allowed a much better molecular weight estimation than the gradient system. An exponential polyacrylamide gel system has been previously utilized by Douglas & Butow (1976) for high-resolution electrophoretic analysis of the products of mitochondrial protein synthesis in yeast.

The relative rate of migration and, in some cases, also the relative order of migration of the various mitochondrially synthesized polypeptides differed in the gradient system and in the phosphate-buffered gel system as compared to the urea system. A change in the order of migration of mitochondrial proteins under different conditions of NaDodSO$_4$–polyacrylamide gel electrophoresis has been previously reported (Capaldi et al., 1977; Groot et al., 1978). In the present work, the difference in electrophoretic behavior of the mitochondrially synthesized polypeptides in the urea and in the gradient gel systems was exploited in order to obtain a higher resolution of these polypeptides by a bidimensional combination of the two gel systems. In this bidimensional fractionation, the resolution of some of the components comigrating in the NaDodSO$_4$–urea–polyacrylamide gel or in the polyacrylamide gradient gel was not always satisfactory when analyzed in a given autoradiogram, and one had to rely on a comparison of different autradiograms or different exposures of the same autoradiogram in order to identify with confidence the individual components. Furthermore, some of the spots, although reproducibly present, exhibited a very low labeling intensity. In spite of these limitations, the bidimensional fractionation carried out here, combined with the unidimensional analysis, has provided evidence for the existence of an appreciably larger number of components than in either of the unidimensional fractionation systems employed here, up to 26 discrete components.

A two-dimensional electrophoretic method taking advantage of the migration anomalies exhibited by some polypeptides during electrophoresis in gels of different porosities has been previously used to resolve the subunits of the cytochrome *c* oxidase from two different sources (Poyton et al., 1978).

The analysis carried out in the present work has allowed us to further refine the identification, among the HeLa cell mitochondrial translation products, of the mitochondrially synthesized cytochrome *c* oxidase subunits. The band assignment of COI, COII, and COIII in the electrophoretic pattern of the mitochondrial translation products in a NaDodSO$_4$–urea–polyacrylamide gel has been previously made for HeLa cell material (Hare et al., 1980). Furthermore, the identity of the polypeptides designated as COI and COII has been verified by an alignment of their NH_2-terminal amino acid sequence with the 5′-end proximal sequence of their mRNAs (Chomyn et al., 1981). In the present work, the bands corresponding to COI, COII, and COIII in the electrophoretic pattern of the mitochondrial translation products in a NaDodSO$_4$–polyacrylamide gradient gel have been identified by comparison of their migration with that of the mitochondrially synthesized subunits of highly purified human cytochrome *c* oxidase. This alignment has revealed an inversion in the order of migration of COII and COIII in the gradient gel system as compared to the urea gel system, which has been confirmed by the bidimensional electrophoretic analysis. Such a behavior of COII and COIII had been previously described by Capaldi et al. (1977) for the beef heart enzyme subunits.

Significance of the Large Number of HeLa Cell Mitochondrial Translation Products. In spite of some uncertaintly concerning the precise number of discrete mitochondrial translation products in HeLa cells, the important conclusion of the present work is that the number of mitochondrially synthesized polypeptides detected here is appreciably higher than that reported so far for mammalian cells from this and other laboratories. The higher resolution of the gel systems used here and the increased sensitivity of detection of minor protein components probably account for these differences. Thus, although the pattern of the main bands obtained here in the electrophoretic runs through NaDodSO$_4$–polyacrylamide gradient gels corresponds fairly closely to those reported by other authors (Yatscoff & Freeman, 1977; Yatscoff et al., 1978) under similar electrophoretic conditions, in the latter cases, the comigrating bands were not resolved by runs in a different electrophoretic system, and several of the minor components were not detectable.

The number of mitochondrial translation products detected in the present work in HeLa cells also exceeds the number of significant mtDNA reading frames (Anderson et al., 1981) and mitochondrial mRNAs recognized in human cells (Ojala et al., 1980, 1981; Montoya et al., 1981). In view of the structural features of human mitochondrial mRNAs (Montoya et al., 1981; Ojala et al., 1981) and of the unusual mechanism of translation termination in human mitochondria (Ojala et al., 1981), it seems possible that some of the longer reading frames may be decomposed into shorter ones during the process of translation. In any case, the sum of the molecular sizes of the sequences coding for the mitochondrial translation products identified here, together with the two rRNAs (total = 2513 nucleotides) and the tRNAs (total = 1512 nucleotides), exceeds by 5–10% the single-strand informational content of human mtDNA (16 569 base pairs; Anderson et al., 1981). Furthermore, it seems likely that the difference is appreciably greater; in fact, in the cases where verification has been made, i.e., for COI, COII, and COIII, the molecular weight estimates of the mitochondrial gene products derived from their electrophoretic mobility in NaDodSO$_4$–urea–polyacrylamide gels (Hare et al., 1980) are considerably lower than the values expected from the mtDNA sequence and protein sequence data (Chomyn et al., 1981). From the above considerations it follows that, if all the mitochondrially synthesized polypeptides observed here are encoded in mtDNA, there must be partial overlapping in their coding sequences. Although from the analysis of the mtDNA sequence (Anderson et al., 1981) it would appear that there is no significant overlapping of information within the same strand or between the heavy and light strand, one cannot exclude the occurrence of small reading frames overlapping, in or out of phase, larger ones, or of as yet unidentified spliced genes overlapping other genes in the same or in the other strand. Multiplicity of polypeptides translated from the same reading frame could also arise at the level of translation due to premature termination, ribosome slippage, or progression of the ribosome beyond the reading frame into the adjacent tRNA [in the case of mRNA processing intermediates still carrying at the 3′ end a tRNA

sequence (Ojala et al., 1981)] or at the level of processing or secondary modification of the primary translation products. The pulse–chase experiments in the present work seem to indicate that the majority of the mitochondrial translation products detected here do not arise by processing or conversion of others, at least in a short-term experiment. However, several components were decreased in their labeling after the chase, pointing to their possible processing to other products; the same may be true of other components not resolved in the unidimensional gel electrophoresis analysis. Although the available evidence indicates that all the polypeptides detected in the present work probably exist in vivo in HeLa cells, it is conceivable that some of them do not have any physiological role but simply reflect intrisinc inefficiencies of the translation or processing machinery; these may be tolerated because of the excess biosynthetic capacity of mammalian mitochondria (Attardi et al., 1982).

Partial proteolysis experiments (Cleveland et al., 1977) performed on individual components more completely resolved by bidimensional electrophoretic fractionation should be very informative concerning the number of unrelated molecular species among the HeLa cell mitochondrial translation products. The use of antibodies against synthetic peptides corresponding to appropriately selected portions of the URFs promises to be another powerful approach for analyzing the relationship between the various mitochondrially synthesized polypeptides and, ultimately, for purifying them. The analysis of the nature of the mitochondrially synthesized polypeptides detected in HeLa cells in the present work and of their interrelationship represents a formidable challenge for the future and may reveal unexpected facts of the expression and regulation of the mitochondrial genome in mammalian cells.

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