

Differences in the Products of Mitochondrial Protein Synthesis *in vivo* in Human and Mouse Cells and their Potential Use as Markers for the Mitochondrial Genome in Human–Mouse Somatic Cell Hybrids

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The proteins synthesized in the mitochondria of mouse and human cells grown in tissue culture were examined by electrophoresis in polyacrylamide gels. The proteins were labelled by incubating the cells in the presence of [³⁵S]methionine and an inhibitor of cytoplasmic protein synthesis (emetine or cycloheximide). A detailed comparison between the labelled products of mouse and human mitochondrial protein synthesis was made possible by developing radioautograms after exposure to slab-electrophoresis gels. Patterns obtained for different cell types of the same species were extremely similar, whereas reproducible differences were observed on comparison of the profiles obtained for mouse and human cells. Four human–mouse somatic cell hybrids were examined, and in each one only components corresponding to mouse mitochondrially synthesized proteins were detected.

Most of the human–mouse somatic cell hybrids that have been examined are characterized by loss of human chromosomes. The number eliminated is variable, but in most cases only a small fraction of the original human parental chromosome complement is retained. Two independent investigations on a series of such hybrids retaining between three and about 20 human chromosomes have failed to detect human mitochondrial DNA (mtDNA) (Clayton *et al.*, 1971; Attardi & Attardi, 1972). Coon *et al.* (1973) obtained human–rodent hybrids in which segregation of either rodent, or human, chromosomes was observed. Some of the hybrids retained a high proportion of both rodent and human nuclear genomes, and in some subcloned derivatives of these both human and rodent mtDNA could be detected.

The results presented in the present paper demonstrate that several of the proteins synthesized *in vivo* by mouse and human mitochondria can be distinguished by electrophoresis in sodium dodecyl sulphate–polyacrylamide gels. These proteins may be of potential use as markers for mitochondrial genes in human–mouse hybrid cells.

The assumption that these proteins are synthesized by the mitochondria is based on studies with selective inhibitors of mitochondrial and/or cytoplasmic protein synthesis.

Experimental

Emetine hydrochloride, cycloheximide, *D*-threo-chloramphenicol and erythromycin were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

The human cell lines studied were D98/AH-2 (Szybalski *et al.*, 1962), fibroblasts (BREN) and a lymphocyte line (BREL), the last two both being derived from the same adult female. The mouse cell lines were GF7, a lymphoid line (Stadler & Adelberg, 1972) and two cell lines grown out from tissue explants in this Department. They are designated 1.4E and 1.1F, being epitheloid and fibroblast-like respectively. They were kindly provided by Mr. David Buck.

The human–mouse hybrids examined (3W4, Z4.12, HOP1.5 and H.24) have been extensively characterized and details of their origins are described elsewhere (Santachiara *et al.*, 1970; van Heyningen *et al.*, 1973; Billardon *et al.*, 1973).

Sample cultures of human and mouse cell lines were checked for mycoplasma contamination by examination of radioautographs obtained after incorporation of [³H]thymidine or [³H]uridine (Schneider *et al.*, 1973).

All cell lines were grown in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (both from Flow Laboratories Ltd., Irvine, Ayrshire, U.K.), 0.1 mg of streptomycin sulphate/ml and 100 i.u. of penicillin G/ml.

Medium for examining [³⁵S]methionine incorporation was prepared from minimum essential medium (lacking methionine) supplemented with Earle's salts (Bio-Cult Laboratories Ltd., Paisley, U.K.). Other additions were 5% (v/v) foetal calf serum and 5% (v/v) foetal calf serum dialysed against Earle's salts. These conditions were found to be the most favourable for efficient incorporation of the label over the time-period tested. Decreasing the

non-dialysed serum contribution to provide a higher specific radioactivity of [^{35}S]methionine did not increase total incorporation.

Cells were detached by trypsin treatment, if necessary, washed once with phosphate-buffered saline [0.85% (w/v) NaCl–2.5 mM-KH₂PO₄–10 mM-Na₂HPO₄], and resuspended at 1×10^6 – 4×10^6 cells/ml in the incorporation medium. After 30 min preincubation at 37°C with inhibitors (see the Results and Discussion section) 10 μCi of [^{35}S]methionine/ml (The Radiochemical Centre, Amersham, Bucks., U.K.; 170–240 Ci/mmol) was added. The incorporation continued for 5 h and was followed by a 'cold chase' for 20 min with 1 mM-L-methionine. The labelled cells were dispersed in portions of between 5×10^5 and 1×10^6 cells/40 μl in 2% (w/v) sodium dodecyl sulphate–5% (w/v) 2-mercaptoethanol–0.0625 M-Tris–HCl buffer (pH 6.8). They were heated to 100°C for 2 min and any faint residual turbidity was removed by centrifugation in a Beckman Microfuge for 4 min. Bromophenol Blue and sucrose were added to the supernatant (0.001% and 10%, w/v, respectively). Electrophoresis was performed in sodium dodecyl sulphate-containing discontinuous polyacrylamide slab gels, by adapting the buffer systems described by Laemmli (1970) and with 12% (w/v) acrylamide in the resolving gel. The gels were fixed in 50% (w/v) trichloroacetic acid, stained in 0.1% (w/v) Coomassie Brilliant Blue dissolved in 50% trichloroacetic acid and then destained in 7% (v/v) acetic acid and dried on to filter paper under suction (Fairbanks *et al.*, 1965). Radioautography was effected by exposure to Kodak Blue Brand X-ray film for 1–4 weeks.

Results and Discussion

The products of mitochondrial protein synthesis may be detected by the incorporation of radioactive amino acids either into isolated mitochondria or into intact cells whose cytoplasmic protein synthesis has been inhibited by cycloheximide or emetine. The two methods are reported to give overall similar patterns of labelled proteins after electrophoresis in sodium dodecyl sulphate–polyacrylamide gels when examined by slicing the gels into sections and determining the distribution of radioactivity (Coote & Work, 1971; Lederman & Attardi, 1973).

In the present study, mammalian cells were labelled with [^{35}S]methionine in the presence of 50 μg of emetine/ml or 200 μg of cycloheximide/ml. At these concentrations of inhibitors, incorporation of radioactivity into trichloroacetic acid-precipitable material is decreased by about 99% when compared with non-inhibited cells. Between 50 and 80% of the residual incorporation is inhibited by 100 μg of D-threo-chloroamphenicol/ml, a specific inhibitor of mitochondrial protein synthesis (see Beattie, 1971).

Electrophoresis of sodium dodecyl sulphate-solubilized whole-cell extracts in polyacrylamide gels resolves the proteins labelled in the presence of emetine or cycloheximide into a small number of bands. These presumably represent discrete polypeptides liberated by treatment with sodium dodecyl sulphate and mercaptoethanol (see Plate 1).

The radioautographic patterns obtained for cells labelled in the presence of either cycloheximide or emetine are extremely similar. Chloramphenicol virtually eliminates the radioactivity incorporated into the most prominent bands, which fall within an apparent molecular-weight range of 15000–50000 (see Plate 1). The residual incorporation is mainly associated with the major cytoplasmically synthesized proteins. Erythromycin, an inhibitor of bacterial and yeast mitochondrial protein synthesis, but apparently not of mammalian mitochondrial protein synthesis (Firkin & Linnane, 1969), has no effect on

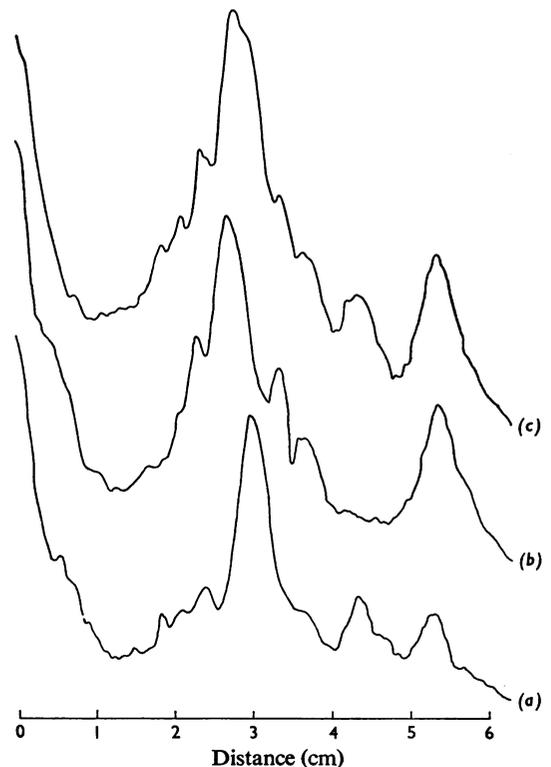
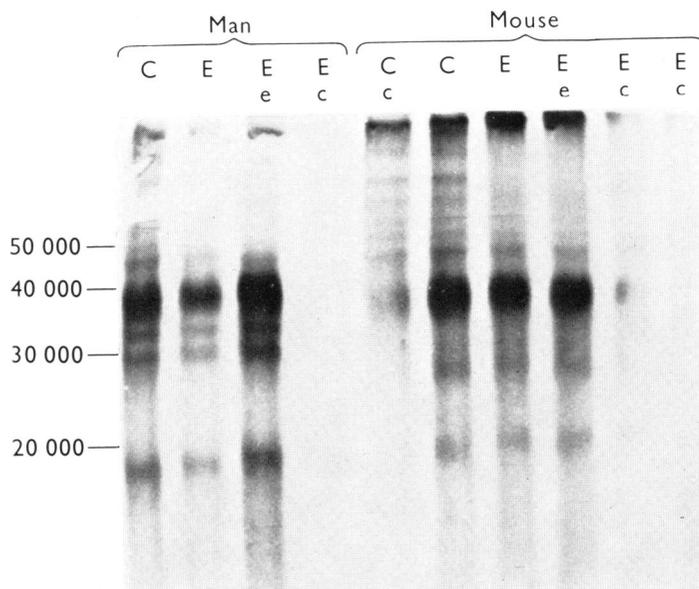


Fig. 1. Microdensitometer trace recordings of the radioautographs obtained after electrophoresis of (a) mouse, (b) human and (c) a mixture of mouse and human cells, solubilized in sodium dodecyl sulphate after [^{35}S]methionine incorporation in the presence of 50 μg of emetine/ml

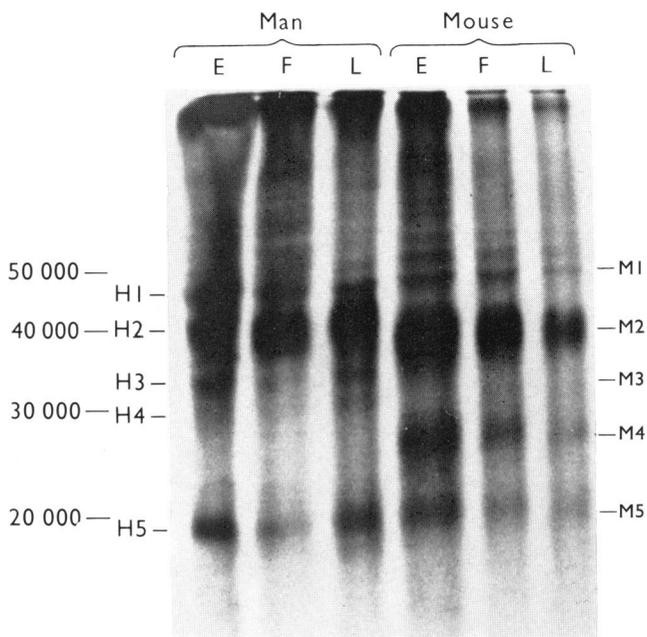
For further details see the text.



EXPLANATION OF PLATE I

Radioautograph of a sodium dodecyl sulphate-polyacrylamide-gel-electrophoretic separation of human (D98) and mouse (1.1F) cell protein labelled with [³⁵S]methionine in the presence of various protein-synthesis inhibitors

C, 200 µg of cycloheximide/ml; E, 50 µg of emetine/ml; c, 100 µg of chloramphenicol/ml; e, 100 µg of erythromycin/ml. Marker proteins (bovine serum albumin, glutamate dehydrogenase, lactate dehydrogenase and cytochrome *c*) run on the same slab gel were used to give the approximate molecular-weight calibrations shown.



EXPLANATION OF PLATE 2

Radioautographic comparison of the emetine-resistant incorporation of [³⁵S]methionine into proteins of various human and mouse cell lines

E, epithelial cell line; F, fibroblasts; L, lymphocytes (see the Experimental section for details of cell lines). Molecular-weight calibrations were obtained as in Plate 1.

the labelling of the putative mitochondrially made proteins.

The most interesting aspect of the present study is the observation of clear differences between the radioautographic patterns of mouse and human mitochondrially synthesized proteins. These differences are maintained when human and mouse cells are mixed before extraction for electrophoresis, the resulting pattern being a summation of the two profiles (see Fig. 1). The patterns obtained for a variety of cell types from the same species were similar and independent of the tissue origin of the cell line (Plate 2).

Samples from at least six independent incorporations into both human and mouse cell lines have been examined. Although the densitometer tracings of radioautograms showed slight variation in the intensity of individual bands, the characteristic differences between human and mouse band positions were reproducible.

The suggestion that the predominant bands in the above patterns are the products of mitochondrial protein synthesis assumes that these organelles are the only site of chloramphenicol-sensitive protein synthesis. Galper & Darnell (1971) have shown that 85% of cycloheximide-insensitive amino acid incorporation into HeLa cells co-purifies with the mitochondria. Under the conditions described here about 90% of the emetine-resistant, chloramphenicol-sensitive [³⁵S]methionine incorporation into whole cells co-fractionated with succinate-cytochrome *c* reductase (a marker for the inner mitochondrial membrane) during the isolation of mitochondria by differential centrifugation.

At least four human mitochondrially made proteins can be distinguished from those of the mouse (see Plate 2). Protein M2 has a small but reproducibly greater migration rate than protein H2. The human proteins H3 and H4 are replaced in mouse by a faint band of protein M3, not visible on these gels. Proteins H5 and M5, which show a small difference in migration rate in this system, can be resolved more clearly on a 14% (w/v) polyacrylamide gel (not shown).

We have also examined four independently isolated human-mouse hybrid cell lines all of which have undergone extensive segregation of human chromosome (see the Experimental section) and two of which have been shown to lack human mtDNA (Clayton *et al.*, 1971). The other two have not been tested, but are presumed to also possess only mouse mtDNA. The radioautographic patterns obtained were identical with those obtained with mouse lines (Plate 2). The inability to detect any of the putative human mitochondrial proteins in the hybrids is consistent with the hypothesis that the genes necessary for the synthesis of these proteins reside in mtDNA and that the differences between the human and

mouse reflects a divergence in the mitochondrial genomes of the two species. The extensive difference in sequence homology between these mtDNA species which were reported by Coon *et al.* (1973) also supports this interpretation. One other explanation of the apparently substantial differences in the molecular weights of some human and mouse mitochondrially made proteins is that they result from post-synthetic modification (by addition of carbohydrate or lipids, or by proteolytic cleavage). If the modification steps are controlled by nuclear genes, the observation of proteins of only the mouse type in the hybrids indicates that the human chromosomes controlling the production of the human specific pattern are absent in the four cases examined.

It is difficult to demonstrate conclusively that low amounts of mycoplasma contamination are not present in cells grown in tissue culture. Nevertheless, although not all cell lines have been screened, samples of human and mouse cells having profiles of chloramphenicol-sensitive proteins identical with those described have been tested for mycoplasma contamination (see the Experimental section) without positive indications.

Finally, it should be borne in mind that the differences observed were obtained by incubating the cells in the presence of an inhibitor of cytoplasmic protein synthesis, whose effect might be to alter the spectrum of mtDNA transcripts and their translation products. The human and mouse profiles may therefore not accurately represent the proteins normally synthesized *in vivo*. Nevertheless, we believe that the observed differences provide potentially valuable markers for the analysis of mitochondrial-nuclear gene interactions in somatic cell hybrids.

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