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Specific cleavage analysis of mammalian mitochondrial DNA

(restriction endonuclease/gel electrophoresis/interspecies comparisons/intraspecies differences)

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ABSTRACT **Mitochondrial DNA from several mammalian species has been digested with a site-specific restriction endonuclease (*Hae*III) from *Haemophilus aegyptius*. A quantitative analysis of the resulting specific fragments indicates that the mtDNA of any individual mammal is predominantly a single molecular clone.**

Gel analysis of specific cleavage products has proven quite sensitive in detecting differences in mtDNA: mtDNAs from the more distantly related mammals studied (e.g., donkey and dog) are found to have few bands in common, and very closely related mammals (e.g., donkey and horse) share only about 50% of their bands. This procedure has detected several intraspecies mtDNA differences. Six distinct human patterns have been found, with one pattern usually differing from another in two or three bands.

mtDNAs from different organs of single individuals have also been analyzed, and no differences have been found.

Our understanding of the organization and regulation of prokaryotic genes is ultimately based on the fine structure analysis of a relatively few specific genetic regions in *Escherichia coli*. Hopefully, fine structure mapping of eukaryotic genes will allow similar insight into the structure and function of eukaryotic genetic material.

Specific cleavage of small DNA virus genomes by restriction enzymes (1, 2) allows the construction of physical maps of DNA molecules which have many of the useful features of recombination maps. The specific fragments produced by enzymatic cleavage are readily isolated, and have been used to map a number of sites of genetic significance. These include origins of DNA replication (3), RNA polymerase binding sites (4), type 1 restriction recognition sites (5), regions coding particular RNA species (early and late mRNA) (6, 7) and also sites of genetic mutations (8). These techniques are potentially extendable to the mapping of ribosomal binding sites and regions that code for specific proteins (in conjunction with *in vitro* protein synthesizing systems).

This strategy for the production of a genetic map is not dependent on the isolation and analysis of mutants and is therefore directly applicable to any simple DNA molecule.

The mammalian mitochondrial genome provides a simple system which is susceptible to specific cleavage analysis. All mammalian mitochondrial DNAs are double-stranded and circular with molecular masses of close to 10^7 daltons (see ref. 9 for a review). In this paper we show the results of specific cleavage of mtDNA from a variety of mammalian sources. Analysis of the characteristic fragment patterns so produced provides an extremely sensitive method for distinguishing closely related mitochondrial genomes.

MATERIALS AND METHODS

Source Materials. Organs from domestic farm animals, namely, cow, pig, sheep, horse, and donkey, were obtained fresh from local slaughterhouses; dog, rabbit, rat, and hamster (Syrian golden) specimens were obtained from conventional suppliers, and sacrificed for fresh tissues in the laboratory; mice were obtained from Dr. G. Haughton; African green monkey livers were purchased from Flow Laboratories; chimpanzee livers were provided by Dr. R. Metzgar of Duke University; buffalo (*Bos bison*) liver was obtained from the Buffalo Ranch, Concord, N.C.; human hearts and livers were obtained as autopsy samples from the N.C. Memorial Hospital, Chapel Hill; leukocytes (removed as part of the treatment) from a patient with chronic myelogenous leukemia (CML) were provided by Dr. A. Huang of Duke University.

Preparation of mtDNA. Mitochondria were prepared at 4° either directly from fresh tissues or cells, or from fresh material stored frozen at −20°. Large livers were first minced with a razor and then an equal volume of S solution (0.25 M sucrose, 1 mM EDTA, pH 7.4) was added. Aliquots of about 15 ml of this material were homogenized quickly (about 3 sec) with a Teckmar Tissumizer, model SDT (48 V). This homogenate was then diluted to 10 tissue volumes with S solution. Small livers (mouse, hamster, and rabbit) were minced, washed in S solution, diluted to 10 tissue volumes with S solution and then homogenized with a Potter-Elvehjem homogenizer fitted with a motor driven teflon pestle. Kidney tissue was treated as were small livers. Heart tissues were disrupted according to procedure 3 of Smith (10). Tissue culture cells were homogenized as described by Kasamatsu *et al.* (11).

Nuclei and unbroken cells were removed from the homogenate by centrifuging twice (500 × *g*, 10 min). Mitochondria were then pelleted (10,000 × *g*, 10 min) and washed once with S solution.

The washed mitochondrial pellet was resuspended in 0.075 M NaCl, 0.05 M EDTA, 0.025 M Tris·HCl at pH 7.4, and made 2% for sodium dodecyl sulfate. This material was then extracted twice with phenol, dialyzed against SSC (0.15 M NaCl–0.015 M sodium citrate, pH 7) and the supercoiled DNA purified by centrifugation in a CsCl ethidium bromide gradient. Ethidium bromide was removed from the mtDNA by three extractions with isopropanol. The mtDNA was dialyzed against SSC and collected by pelleting at 33,000 rpm for 10 hr in a SW50.1 rotor at 4°; the pellet was dissolved in a small volume of TBS (0.15 M NaCl, 0.05 M Tris·HCl at pH 7.4) and stored at −20°.

Digestion. The *Hae*III (endonuclease Z) restriction endonuclease (12) from *H. aegyptius* was prepared as described by Smith (13). Enzyme was used as eluted from the phosphocellulose column or after subsequent ammonium sulfate precipitation; no difference in these two enzyme fractions has been detected. A single batch of enzyme sufficed for all digestions described in this study. The digestion mixture used has been previously described (14). The *Hae*III enzyme

Abbreviations: SSC, 0.15 M sodium chloride–0.015 M sodium citrate, pH 7; SV40, simian virus 40.

(1 μl), as eluted from the phosphocellulose column, completely digested 10 μg of simian virus 40 (SV40) DNA in 10 hr at 37°. mtDNA (10–20 μg) was digested with 5 μl of enzyme for a minimum of 10 hr and then 5 μl more enzyme was added and the digestion continued for 2 hr.

Electrophoresis. Fractionation of DNA fragments by electrophoresis (4 V/cm) in polyacrylamide gels has been described previously (2). Slot gels (30 cm long) were used when the gels were to be dried down on filter paper and were used to expose x-ray film (2). For ethidium bromide staining (1 μg/ml), cylindrical 3.5% acrylamide gels (7 mm by 30 or 60 cm, no agarose) were loaded with about 10–20 μg of mtDNA. Gels were photographed on Polaroid type 55 film. The photographs (Figs. 2–5) displayed in this paper were printed as negatives to enhance the contrast.

RESULTS

The specific limit fragments of small viral DNAs produced by digestion with a site-specific restriction endonuclease are resolved into a characteristic pattern of bands upon electrophoresis in acrylamide gels (1, 2). The bands of DNA fragments can be detected either by staining techniques or by radioisotope label. The characteristic band patterns for mammalian mtDNA obtained in this way were found to be very reproducible. For example, three preparations of hamster liver mtDNA were made, each of which involved pooling the livers from 20 to 30 inbred animals. Separate digestions of these mtDNA preparations were performed and the resulting band patterns were identical. Similar results were obtained in the analysis of large equine livers. Several independent preparations of mtDNA have been made from 300 *g* portions of individual equine livers. The band patterns from any single organ are found to be invariant. Some preparations of mtDNA were contaminated with nuclear DNA. The contaminating nuclear DNA was seen in these restriction analyses as a background smear and never as additional bands of DNA fragments.

To investigate the homogeneity of a mammalian mtDNA preparation, we did a detailed quantitative analysis of the *Hae*III digestion products of HeLa S3 mtDNA. Fig. 1 shows autoradiograms of uniformly ^{32}P-labeled HeLa mtDNA that had been digested and analyzed on 2.5% and 3.5% polyacrylamide gels. The distribution of radioactivity along the gels, as determined by slicing the dried gels and measuring Cerenkov radiation, is also shown in Fig. 1. Some 30 distinct bands are resolved on the autoradiogram of the 3.5% gel. The size of the DNA fragments in each of these bands was estimated by comigration with *Hae*III fragments of SV40 DNA (Table 1); the *Hae*III fragments of SV40 have been well characterized (accuracy ±5%, approximately) and serve as molecular weight standards (15). The relative positions of the SV40 markers as shown in Fig. 1 were determined with three separate gels (not shown). These three gels contained *Hae*III digests of SV40 DNA, S3 HeLa mtDNA, and a mixture of the two DNAs respectively. The fragment sizes for the seven bands of mtDNA which migrate faster than the *Hae*III-H fragment (160 base pairs) of SV40 DNA were determined by extrapolation. The *Hae*III fragments of HeLa S3 mtDNA monitored in Fig. 1 range in size from 70 to 1500 base pairs. Specific fragments smaller than 70 base pairs were not characterized in these experiments although they are probably produced.

The multiplicity of DNA fragments of identical size (and hence unresolved) per band can be determined by an analysis of the radioactivity distributions given in Fig. 1. This type

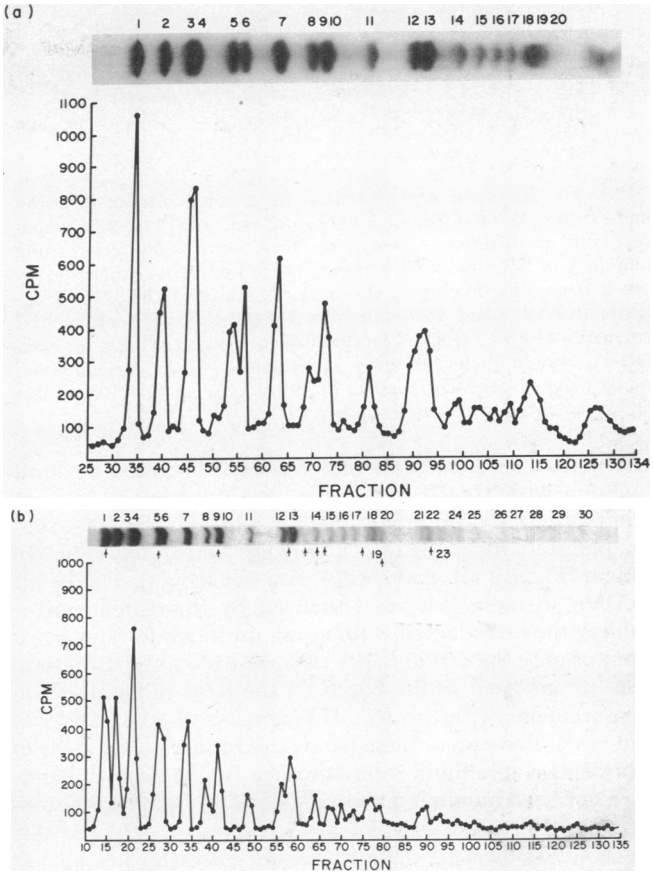

FIG. 1. Autoradiographs and radioactivity distributions of ^{32}P-labeled mtDNA from HeLa S3 digested with *Hae*III and electrophoresed on gels containing (a) 2.5% and (b) 3.5% polyacrylamide. Each band resolved in either (a) or (b) is assigned a number. Cells at 50% confluency were labeled for 48 hr in minimum essential medium containing 10^{-4} M phosphate, 10% fetal calf serum (dialyzed against Hank's balanced salt solution without phosphate), and 5 μCi/ml of ^{32}P. Arrows in (b) point to the relative positions of SV40 size markers (see *Results*). *Hae*III digested Vogt-Dulbecco small plaque strain SV40 DNA was used (13).

of analysis has been well documented in the specific cleavage studies of the homogenous DNAs of animal (14) and bacterial viruses (2). Several of the *Hae*III bands of HeLa mtDNA are shown by this analysis of the data in Fig. 1 to be unresolved multiplets, and are so indicated in Table 1.

The fragments of HeLa S3 mtDNA characterized in Table 1 sum to a total of 15,014 base pairs; we estimate the uncertainty in this sum to be ±1000 base pairs. This total represents a slight underestimate since possible fragments smaller than 70 base pairs were excluded in the study. However, since the size of HeLa mtDNA is estimated to be 16,900 ± 350 base bairs (16), one can conclude that the mtDNA of HeLa S3 represents a population of DNA molecules that is highly homogenous with respect to nucleotide sequence, and probably a pure clone. This conclusion is further supported by the observation that no bands were found to be present in less than a molar yield. Clearly even minor sequence heterogeneity involving *Hae*III cleavage sequences would result in the appearance of such bands and would be recognized as such upon analysis of the radiolabel.

The apparent uniclonal nature of mtDNA, as found in the cloned S3 line of HeLa cells, was investigated further in mitochondria obtained from the livers of several different

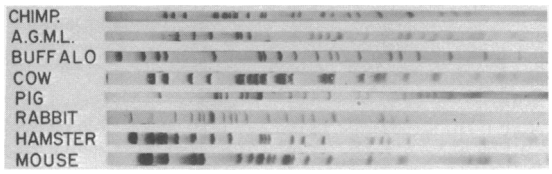

FIG. 2. The data are presented as a composite photograph made from gels of different lengths (30 cm or 60 cm) and which were run at different times. Therefore, conclusions concerning homology of band mobilities among the gel patterns cannot be inferred from this display of the data. The size of the DNA fragments in each band was estimated by comigration with *Hae*III fragments of SV40 DNA. The sums of the fragment sizes for each pattern were in nucleotide pairs: chimpanzee 16,105; African green monkey liver (A.G.M.L.) 15,039; buffalo 15,263; cow 14,105; rabbit 15,602; hamster 14,159; mouse 14,204.

mammalian species. Band patterns were obtained by limit digestion with *Hae*III enzyme for the liver mtDNAs from chimpanzee, African green monkey, buffalo, cow, pig, rabbit, hamster, mouse (Fig. 2), donkey, horse, dog (Fig. 3), human (Fig. 5), rat, and sheep (data not shown). The bands of DNA in these gels are visualized by fluorescence after staining the extruded gel with ethidium bromide. These gels monitor only the 20–25 bands of lowest mobility; as before, sizes are assigned to the bands on the basis of comigration measurements with the *Hae*III fragments of SV40 DNA as standards. The sum of these band sizes for each band pattern represents a minimum estimate since (*i*) the smaller bands have not been monitored and included and (*ii*) a rigorous estimate of the multiplicity of DNA fragments per band was not made. However, these minimum estimates are all less than or equal to (within experimental error) the estimated size of mammalian mtDNA and are therefore compatible with a uniclonal nature for the liver mtDNA of each species. It should be noted that some of the species monitored in Fig. 2 were highly inbred laboratory strains (mouse and hamster).

Possible differences in the band patterns produced by *Hae*III digestion of mtDNAs obtained from different organs of the same animal were looked for by comparing liver and kidney mtDNA from inbred hamsters and heart and liver mtDNA from both human and equine sources (data not shown). No evidence for any organ specific differences could be found.

Several interspecies comparisons were made of mtDNAs digested by the *Hae*III restriction enzyme. Fig. 3 shows two such comparisons: horse with donkey and dog with donkey. Because these long cylindrical gels stretched easily, the interspecies comparison studies required that the *Hae*III digests of the two DNAs be mixed and analyzed on the same gel as shown in Fig. 3. There are at least 12 comigrating bands in the horse–donkey comparison, and at least seven

Table 1. Analysis of HeLa mtDNA fragments

Band	Size	Multiplicity	Total
1	1520	1	1520
2	1295	1	1295
3	1090	1	1090
4	1020	1	1020
5	785	1	785
6	760	1	760
7	645	2	1290
8	565	1	565
9	530	1	530
10	520	1	520
11	440	1	440
12	370	2	740
13	350	3	1050
14	315	1	315
15	292	1	292
16	273	1	273
17	257	1	257
18	239	1	239
19	227	2	454
20	220	1	220
21	175	1	175
22	160	2	320
23	153	1	153
24	138	1	138
25	125	1	125
26	105	N.E.	105
27	100	N.E.	100
28	88	N.E.	88
29	82	N.E.	82
30	73	N.E.	73
		36	15014

N.E., not estimated because of insufficient radioactivity, multiplicity assumed to be unity.

Note: The multiplicity analysis for bands 21–25 was determined from a 4.5% gel (not shown) loaded with more radioactivity than was the gel shown in Fig. 1b.

such bands for the dog–donkey comparison. Donkey bands 1, 2, 6, 8, 9, 10, 11, 12, 15, 17, 18, and 19 comigrate with horse bands 1, 3, 5, 7, 8, 9, 11, 12, 14, 16, 17, and 19, respectively; and donkey bands 1, 2, 11, 13, 15, 18, and 19 comigrate with dog bands 2, 5, 12, 14, 15, 16, and 17, respectively. The other interspecies comparisons examined were buffalo–cow, buffalo–pig, chimpanzee–human, buffalo–horse, chimpanzee–African green monkey, hamster–donkey, and chimpanzee–horse. The general conclusions from these several comparisons are portrayed in the examples depicted in Fig. 3. Species which are closely related in the phylogenetic scheme show a greater proportion of comigrating bands than species which are less related. In comparisons involving two

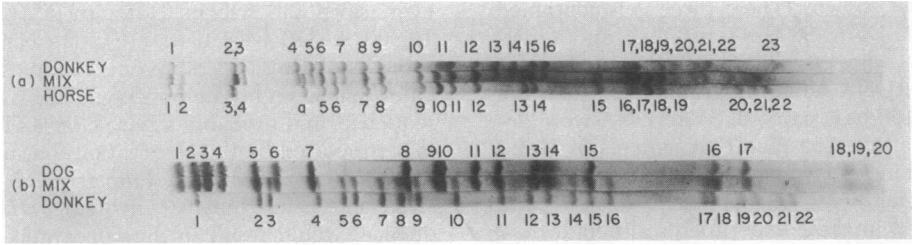

FIG. 3. Interspecies comparisons of *Hae*III digests of mtDNA. (a) Comparison of donkey (*E. asinus*) mtDNA with horse (*E. caballus*) mtDNA; the DNA fragments sum to 14,662 and 15,675 base pairs for the donkey and horse patterns, respectively. (b) Comparison of donkey (*E. asinus*) mtDNA with dog (*Canis familiaris*) mtDNA; the DNA fragments sum to 14,552 and 17,751 base pairs for the donkey and dog patterns, respectively. The donkey patterns depicted in (a) and (b) were derived from the livers of two different animals.

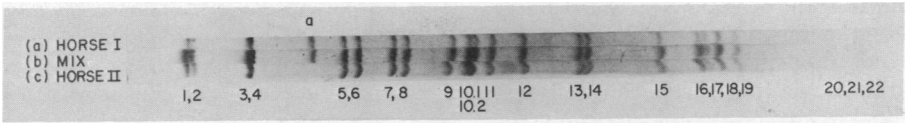

FIG. 4. Intraspecies comparisons of *Hae*III digests of liver mtDNA: differences between two horse patterns. The band pattern for horse I (gel a) differs from that for horse II (gel c) in having the additional band a and a reduced yield of band 16. Note that band 10, which stains as an unresolved multiplet in Fig. 3a, is resolved into two bands (10.1, 10.2) in this figure.

different mammalian orders, a significant proportion (usually greater than one half) of the comigrating bands also comigrate in species comparisons within that order. However, no single *Hae*III fragment monitored is found to be common to all the band patterns. No regions of the mammalian mtDNA molecule can be detected by this analysis to have been completely conserved throughout the evolution of the mammals; all regions of the molecule monitored by this method appear to have undergone mutation.

In the analysis of mtDNA from pooled organs (livers or kidneys) of inbred hamsters and mice the characteristic band patterns were reproducible and invariant for that species. When two quite distinct inbred lines of mice (C57BL and A) were compared in this way the patterns were found to be identical. In monitoring ten horse livers, however, such intraspecies variations were detected. A comparison of the "normal" horse pattern with a variant pattern is shown in Fig. 4. The "normal" band pattern seen in gel (c) in Fig. 4 was found in six animals; the variant pattern in gel (a) is one of four distinct variant patterns found. Gel (b) in Fig. 4 displays the pattern obtained when a mixture of the two mtDNAs is made after digestion with *Hae*III endonuclease. The variant pattern exhibits a band (estimated size 840 base pairs) intermediate between horse bands 4 and 5; additionally horse band 16 (estimated size 310 base pairs) in the variant pattern stains with less intensity than in the normal pattern. The simplest interpretation is that a net insertion of 530 base pairs into one of the components of horse band 16 of the normal pattern has resulted in an increase of its size ... generating the large fragment seen only in this variant pattern. It would thus seem that mtDNA is not immune to forms of mutagenesis resulting in changes in DNA length, as has been suggested (17). The four different variant patterns have been found only once each. All the variant horse patterns have been obtained from pony horses (ponies), whereas the standard pattern has been seen in both pony horses and horses.

The presence of intraspecies variations in the *Hae*III band patterns of mtDNA is seen more strikingly in the analysis of human material (Fig. 5). As for the horse patterns, the intraspecies variations involve only a minor fraction of the bands in the pattern. However, each unambiguously independent human source has yielded a distinct pattern. The band patterns displayed in Fig. 5 derive from mtDNA isolated from HeLa cells, Raji cells [a leukocyte cell line derived from a patient with Burkitt's lymphoma (18)], leukocytes, and two distinct heart preparations derived from adult females at autopsy.

DISCUSSION

The analysis of mammalian mtDNA by the *Hae*III restriction endonuclease has revealed these molecules to be highly homogenous with respect to nucleotide sequence. Similar analyses in which other restriction enzymes (*Eco*RI, *Hin*b, and *Sma*) were used have been conducted in our and other laboratories with similar conclusions (16). It is appropriate to consider exactly what sequence information is revealed in the characteristic band patterns. Clearly two DNA molecules of identical molecular weight could have a high degree of sequence homology, but completely distinct band patterns: in this case the sequence differences would involve only the cleavage sites. Conversely, two DNA molecules could have essentially zero sequence homology but identical band patterns; the number and location of the cleavage sites would be all that was homologous. These are two extreme but possible cases. The band pattern of course reveals directly the number of cleavage sequences per molecule. The nucleotide sequences between cleavage sites are not directly monitored in this analysis; of these sequences the band pattern reveals only the size of the DNA fragment and shows that no new cleavage sequence has been generated within it.

Clearly, the more cleavage sites monitored by a particular enzyme, the more sequence information obtained. The homogeneity of nucleotide sequence as revealed for HeLa mtDNA by analysis of 36 *Hae*III fragments is significantly more revealing than the similar analysis of the three *Eco*RI or the three *Hin*b fragments (16). These observations on the sequence homogeneity of mtDNA are in accord with the results obtained by analysis of heteroduplexes or reassociation kinetics (9, 19). Some band patterns did however reveal the presence of faint bands generated at less than molar yield (see Fig. 3b between dog bands 7 and 8 and donkey bands 8 and 9). In at least those cases the faint bands themselves were quite reproducible and could not be eliminated by exhaustive digestion. This apparent microheterogeneity might represent heterogeneity of primary nucleotide sequence, modification of the DNA, or some physical constraint in the molecule. The limited analysis of mtDNAs derived from different organs, i.e., liver (endodermal in origin), heart and kidney (mesodermal in origin), suggests that indeed the mtDNA of any individual mammal is predominantly a single molecular clone.

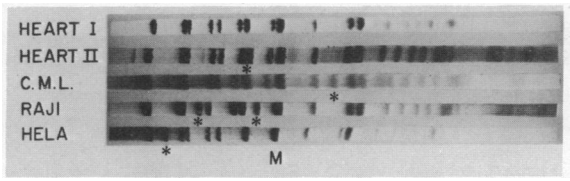

FIG. 5. Intraspecies comparisons of *Hae*III digests of mtDNA: differences between human patterns. The mtDNA samples depicted in the patterns shown above were derived as follows: Heart I, from adult Negro female at autopsy; Heart II, from adult Caucasian female at autopsy; Chronic myelogenous leukemic leukocytes from adult Caucasian male; Raji cells (17) (established transformed leukocyte cell line); HeLa cells, from Flow Laboratories. M denotes the region of difference between this HeLa pattern and that shown in Fig. 1. The lower portion of the Heart II gel was deliberately illuminated for a longer period (with a hand held UV lamp) during photography in order to show that the faint bands near the bottoms of the gels are real. Asterisks denote regions where pattern differences are found.

In this study we compared the specific cleavage patterns of mtDNAs from different mammalian species representing the orders carnivores (dog), rodents (hamster, rat, mouse), lagomorpha (rabbit), artiodactyla (pig, sheep, cow, buffalo), perissodactyla (horse, donkey), and primates (African green monkey, chimpanzee, human). Species capable of interbreeding (horse–donkey, Fig. 3a; cow–buffalo, data not shown) exhibit comigration for about 50% of their mtDNA fragments. In this regard it is both interesting and amusing to report that the greatest degree of apparent homology as revealed in these interspecies analyses was found for the chimpanzee–human comparison; some 62% of the bands monitored (13 of 21 bands) comigrate. As the phylogenetic relationship becomes more distant the number of mtDNA fragments which comigrate decreases. The chimpanzee shares only eight bands with the African green monkey; of those eight bands shared by chimpanzee and African green monkey seven are among those also shared by chimpanzee and human.

The clear demonstration of the minor intraspecies variations found for the horse and human mtDNAs documents the sensitivity of the *Hae*III restriction analysis. The human variations are particularly interesting and of uncertain origin. The two cleavage patterns of mtDNA from autopsy material might reflect individual differences, or possible racial variations (Fig. 5). The differences found in cultured cell lines could reflect further individual variations, or differences due to neoplasia or cultivation in tissue culture. The variant pattern seen in the mtDNA of leukemic leukocytes could simply represent yet another individual difference and not be related to the diseased state of the patient.

Variations have even been detected between two different lines of HeLa cells, namely, clone S3 from Dr. G. Pearson (Fig. 1) and a line from Flow Laboratories (Fig. 5). The patterns differ in that clone S3 mtDNA (Fig. 1) has the resolved bands 8 and the doublet 9,10 whereas the Flow line displays the band labeled M (Fig. 5); the band designated M has been resolved on other gels into a triplet. At least four gels of each HeLa strain have been run and in each case this difference was unambiguous. The mtDNA of KB cells (obtained from Flow Laboratories) was found to be identical to the S3 line of HeLa cells. Another cell line (also from Flow Laboratories) was purchased as "chimpanzee liver cells"; analysis of this mtDNA with *Hae*III digestion showed it to be identical to the Flow line of HeLa cells and quite dissimilar to the mtDNA prepared from the liver of a chimpanzee. In light of the discovery that many cell cultures are contaminated with HeLa cells (20), the potential usefulness of this procedure in testing the authenticity of cell lines is apparent.

The uniclonal nature of mtDNA molecules for each mammal is perhaps not a trivial result. Cells contain many mitochondria, and each mitochondrion may contain two to six DNA molecules (21). In view of the large number of mtDNA molecules per cell, sequence heterogeneity might be expected to occur. It can be speculated that perhaps a mechanism exists to guarantee the homogeneity of mtDNA sequences.

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