Mitochondrial DNA was quantitated in total DNA of various normal and mutant strains of human diploid fibroblasts (finite replicative lifespan) and permanent cell lines, using Southern-transfer hybridization to $^{32}$P-labeled pure mtDNA probe and saturation hybridization to $^3$H-labeled cRNA copied from mtDNA. In six normal fibroblast strains, mtDNA copy number increased during serial passage roughly in proportion to cell volume or protein content, whereas normal human mtDNA content per pg of protein depended upon in uiuo donor age but not passage level ("in vitro" age). Copy numbers for mtDNA varied much more widely in individual fibroblast clones than in mass cultures, but were not well correlated with longevity or growth rate. Five mutant fibroblast strains associated with reduced replicative lifespan, and four permanent cell lines, were also examined; in each group, mtDNA values were observed both lower and higher than any obtained for normal fibroblasts. No evidence was found of petite-type deletions from human mtDNA, either at late passage or in individual clones of fibroblasts. Methylation of mtDNA genomes was strikingly nonrandom and apparently decreased with culture age.

Mitochondria, the cellular organelles responsible for oxidative energy production, possess a unique combination of autonomy and interdependence with the cell nucleus. Human mitochondria contain multiple circular copies of their own DNA (mtDNA), which encodes the mitochondrial ribosomal and transfer RNAs, plus about 13 protein chains. Nuclear genes, however, direct the synthesis of another 300-400 mitochondrial proteins (Anderson et al., 1974; Tzagoloff, 1982). It is not known to what extent or in what manner replicative synchrony is maintained between mitochondrial and nuclear genomes, although several levels of interaction are possible (Storrie and Attardi, 1972; Clayton, 1982).

In principle, since a balanced collaboration between nuclear and mitochondrial DNA is required for synthesis of mitochondrial ribosomes and three multimeric proteins, mitochondrial function could be impaired by any gross imbalance in the copy number per cell of mitochondrial DNA molecules or portions thereof. Nuclear DNA replication, on the other hand, may be modulated through mitochondrial synthesis of ATP. Clearly, a deficiency of intact mtDNA genomes can limit the rate of cell proliferation, as in "petite" mutations of yeast (Slonimski and Lazowska, 1977), and it has been suggested that imbalance in either direction could profoundly alter cellular growth parameters (Belcour and Begel, 1978; Shmookler Reis et al., 1980; Hartung, 1982).

We wished to investigate the relationship between mitochondrial DNA and both the growth rate and growth potential of cultured human cells. We have prepared total DNA from a variety of sources, including several normal diploid fibroblast strains at early and late passage. These cells have a limited growth potential or proliferative lifespan which is inversely related to the age of the tissue donor (Hayflick, 1977; Goldstein, 1978). Exhausation of that potential, commonly described as in vitro aging, is accompanied by a progressive slowing of the cell cycle and hence of growth rate (Cristofalo, 1976; Macieira-Coelho, 1977). We have also examined mutant fibroblast strains, from individuals with premature aging and/or chromosomal breakage syndromes, and several "immortal" human cell lines (i.e. with unlimited replicative lifespans). These DNAs were then probed for mtDNA sequences in Southern-blot hybridizations (Southern, 1975) and in saturation hybridizations (Gillespie and Spiegelman, 1965), allowing determination of the number of mtDNA molecules per nuclear equivalent.

Alternative methods of quantitation based on prior isolation of mitochondrial DNA (Bogenhagen and Clayton, 1974) and/or mtDNA (Smith et al., 1971) were excluded as liable to underestimation, due to inevitable losses during preparation or analysis. Such losses could vary systematically with in vitro aging, producing artifactual differences, since fragmented or fragile mitochondria might be preferentially lost during purification, whereas endonuclease nicking of DNA (in uiuo or during mtDNA preparation) would decrease the yield of mtDNA identified as covalently closed circular DNA molecules.

We here report data on copy numbers, methylation, and integrity of mitochondrial DNA in normal and mutant fibroblast strains and in permanent aneuploid cell lines, and we attempt to correlate these data with other cellular parameters including short term growth rate, long term growth potential, and age of donor.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human Fibroblast Cultures and Permanent Cell Lines—Normal and mutant skin fibroblasts were obtained for in vitro culture from healthy male donors as described (Goldstein and Singal, 1974; Harley and Goldstein, 1978), with the exception of Bloom syndrome (GM 1492) obtained from the Genetic Mutant Cell Repository (Camden, NJ). Cells were passaged at 1:8 split ratios, counting three mean population doublings each time cells grew to confluence, in Eagle’s growth medium with 15% fetal calf serum. Permanent cell lines were
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obtained from the American Type Culture Collection (Rockville, MD).

Enzymes—Restriction endonucleases were obtained from Bethesda Research Laboratories and Boehringer Mannheim. Proteinase K from Beckman, pronase from Calbiochem, and RNase A from Sigma.

Isotopes—[α-32P]dATP and [α-35S]dCTP (specific activity, 2000–3000 Ci/mmol) and [8-3H]dCTP (17 Ci/mmol) were purchased from New England Nuclear. [2-3H]Thymidine (specific activity, 56 Ci/mmol) was purchased from ICN.

Methods

DNA Purification—Cells were rinsed 3× with phosphate-buffered saline and lysed in situ on Petri dishes upon addition of SDS to 0.5% plus EDTA and Tris-HCl, pH 8.3. Proteinase K was added at 100 μg/ml and SDS increased to 1% (w/v); samples were then incubated 4 h at 37 °C before and after addition of a further 100 μg/ml of Proteinase K. DNA was extracted twice with ultrapure phenol (Bethesda Research Laboratories), freshly saturated with 0.3 M Tris-HCl, pH 8.3, 20 mM EDTA, 0.18 M NaCl, it was then extracted twice with chloroform:methanol (2:1, v/v). After precipitation and rinsing in 70% ethanol, 0.1 M NaOAc, pH 6, the DNA pellets were redissolved and digested with heat-treated RNase A (100 μg/ml, 1 h at 37 °C) followed by heat-treated pronase (250 μg/ml, 2 h each at 37 °C). Extractions of DNA were repeated as above and DNA was ethanol-precipitated, rinsed twice in 70% ethanol, and redissolved in Tris/EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.3).

Southern-blot Hybridization—DNA samples, either undigested or after complete digestion with restriction enzymes, were loaded on 1.5% vertical agarose slab gels and electrophoresed 16 h at 10 mA. The gels were then denatured and transferred onto nitrocellulose filters (Millipore HAWP, prewashed in 2× SSC at 20 °C) at 0.3 μg of DNA per filter, and hybridized to restriction fragments (HpaII pattern) obtained in most mitochondrial DNA standards is proportional to fragment length, confirming that the probe is essentially uniformly labeled. (Apparent exceptions, at 2.2 kb and 0.5 kb, are due to co-migration of two or more fragments of similar length (Brown and Goodman, 1979).)

Preparation of HeLa mtDNA—Mitochondria were prepared from 5×10⁶ HeLa cells by the "no-gradient" procedure of Bogenhagen and Clayton (1974), utilizing CarSrb for Dounce homogenization as recommended (CarSrb = 10 mM NaCl, 1.6 mM CaCl₂, 10 mM Tris, pH 7.6). Covalently closed circular molecules were then prepared from SDS-lysed mitochondria by two rounds of isopycnic banding in CsCl (1.57 g/ml) containing 300 μg/ml of ethidium bromide, as described by Smith et al. (1971).

Saturation Hybridization with cRNA—Complementary [3H]cRNA was transcribed from HeLa mtDNA by Esherichia coli RNA polymerase in the presence of [3H]GTP and purified as previously described (Shmookler Reis and Biro, 1978). The cRNA product, specific activity 1.8×10⁶ cpm/μg, was hybridized to 16- to 100-fold excess against filter-immobilized DNA.

Total cellular [32P]DNA (or unlabelled cellular DNA brought to 2000 cpm/μg by addition of normal fibroblast [32P]DNA at 48,000 cpm/μg) was denatured by boiling 10 min in 10 mM Tris-HCl, 1 mM EDTA, pH 8.3. It was then diluted rapidly into 6× SSC at 0 °C, bound to nick-endonuclease filters (Millipore HAWF, prewashed in 2× SSC at 95 °C and 6× SSC at 20 °C) at 0.3 μg of DNA per filter, and rinsed, dried, and baked in vacuo as described by Gillespie and Spiegelman (1965). Hybridization was for 18 h at 60 °C, in 3× SSC containing 0.2% SDS and 65 μg/ml of E. coli tRNA. Filters were washed 3×15 min in 4× SSC at 60 °C, then incubated for 30 min at 20 °C with 20 μg/ml of RNAase A in 2× SSC and rinsed 2×10 min in 3× SSC at 20 °C. Filters were air-dried and counted in toluene plus 0.3% (w/v) diphenyloxazole. In some experiments, filters were dried and counted prior to RNase treatment, then rinsed 3× in toluene, dried, and re-wet with water for RNase digestion, etc., as above.

Determination of Cell Volumes—Cell suspensions were obtained at the time of confluence, immediately following harvest with 0.125% trypsin, and were analyzed for median cell volume on a Coulter Counter (Model ZB and Channelizer) calibrated with polystyrene microsphere standards.

Protein Assay—Total cell protein was determined on cell suspensions (obtained and counted as in the preceding section) by the method of Lowry et al. (1951), using bovine serum albumin standards.

RESULTS AND DISCUSSION

mtDNA in Early versus Late Passage Cells from Young and Old Donors—Total DNA was prepared from six normal fibroblast strains at early and late levels of mean population doubling with 97 ± 2% (S.E.) yield (Shmookler Reis and Goldstein, 1980) and digested with HpaII, MspI, or EcoRI restriction endonucleases, electrophoresed in agarose gels, and transferred to nitrocellulose filter sheets. The filters were then hybridized to pure mtDNA probe, prepared as covalently closed circular DNA molecules from isolated HeLa cell mitochondria and labeled with [32P]dCTP by nick translation. The resulting autoradiographs (Fig. 1) were then used to evaluate copy number for the mitochondrial genome and fragments thereof, by comparison to hybridization standards (lanes at left of Fig. 1, containing serial dilutions of pure HeLa mtDNA digested with HpaII). Hybridization to restriction fragments in these mtDNA standards is proportional to fragment length, confirming that the probe is essentially uniformly labeled. (Apparent exceptions, at 2.2 kb and 0.5 kb, are due to co-migration of two or more fragments of similar length (Brown and Goodman, 1979).)

The variant pattern obtained in most samples (Fig. 1) corresponds to the principal form of human mtDNA, which is polymorphic for cleavage sites of numerous restriction enzymes (Brown and Goodman, 1979; Brown, 1980). The variant pattern observed in two of our cell strains (Fig. 1, i and j, and Fig. 2e) is identical with Brown's "Morph 2" seen in 1 out of 21 individuals previously surveyed (Brown, 1980).

The results of several such experiments are summarized in Table I. Values are expressed as "% mtDNA," obtained by dividing the quantity of mtDNA in each channel (determined autoradiographically) by the amount of total cell DNA loaded. Several of these determinations have been confirmed by saturation hybridization (see below). Since the human diploid genome contains ~6.5×10⁶ base pairs, while human mtDNA contains 16,569 bp, each 0.1% mtDNA corresponds to approximately 400 copies of the mitochondrial genome per cell. In each of the "young donor" cell strains, mtDNA increased during serial passage by 30–50%. At the same time, however, the cell volume and protein per cell also increased at late passage (Table I) confirming a well established concomitant of in vitro aging (Simons, 1967; Cristofalo and Kritchevsky, 1969; Haslam and Goldstein, 1974; Mitsu and Schneider, 1976). In strain A2 for example, while mtDNA/cell increased by ~50% at late passage, cell protein content increased by 34% and cell volume increased by 44% (Table I). Thus, although late passage cells generally had gained mtDNA copies per cell, mtDNA expressed per unit volume of cytoplasm or per mass of protein remained relatively constant during serial culture.

The fraction of mtDNA in cell strains derived from three old donors was initially higher than in three young donor strains (1.2% ± 0.06 (S.E.) versus 0.73% ± 0.09, respectively; p < 0.02), but did not increase as much at late passage. Indeed, when mtDNA is considered in relation to cell protein or cell volume serviced by the mitochondria, the difference between old and young donors at early passage is even more pronounced (Table I, right-hand column). In early passage cells, old donors averaged 22.1 ± 2.0 (S.E.) copies of mtDNA per pg of protein, compared to 10.6 ± 0.7 for young donors (p < 0.005). Thus, fibroblasts from old donors, which tend to have shorter replicative lifespan in vitro (Goldstein et al., 1969; Martin et al., 1970; Hayflick, 1977; Goldstein, 1978), may be
distinguished from young donors' fibroblasts even at early passage. This implies that the mtDNA/protein ratio reflects an age-dependent condition of the donors' cells in vivo which persists through in vitro cultivation.

Mitochondrial DNA Copy Number in Permanent Cell Lines and Mutant Cell Strains—In view of the limited variation in mtDNA content observed in normal diploid cell strains, we wished to extend our survey to cells with grossly abnormal growth characteristics. We first examined mtDNA in several permanent human cell lines (Fig. 2, lanes a-d, and Table II). The value obtained for HeLa cells (7200 mtDNA genomes per cell) was in reasonably good agreement with an earlier estimate of 8800 copies per cell, based on [3H]thymidine incorporation into mtDNA of HeLa TK- cells (Bogenhagen and Clayton, 1974). The range of copy number values obtained in these lines, considered either per cell or per mass of protein, was greater than had been found for diploid cell strains throughout their lifespans (Table II; compare to Table I), suggesting that mtDNA levels cannot be important in the escape from mortality of permanent cell lines. It should also be noted that all four permanent lines grew at a similar rate (doubling time ~ 24 h), somewhat faster than even the most vigorous diploid fibroblasts (doubling time ≥ 28 h), implying that mtDNA levels per se do not govern the growth rate of these cells.

Genetically determined syndromes which resemble premature senescence have been found to give rise to cultured fibroblasts with a marked reduction in growth rate and replicative lifespan (Martin et al., 1970; Goldstein, 1978). We have examined mtDNA in fibroblasts from three donors with the Hutchinson-Gilford progeria syndrome and one donor with Werner syndrome (Fig. 2, lanes f-i). Bloom syndrome fibroblasts, characterized by abnormally high levels of DNA breakage and sister-chromatid exchange (Schneider, 1978), were also assessed (Fig. 2, lane e). Both Werner syndrome (Salk et al., 1981) and Bloom syndrome (Schneider, 1978) are associated with an unusually high incidence of malignancy, as well as elevated frequency of chromosomal aberrations. There were no consistent changes in mtDNA correlated with either abbreviated lifespan (Table III, WS2, P5, P11, and P18) or chromosomal instability (Table III, BL and WS2). Three progeria strains and a Werner syndrome strain were low to average in mtDNA copy number, for their doubling level, compared to normal strains (Tables I and III). Cell volumes and protein contents per cell, however, tended to be higher in early passage progeria fibroblasts than in normal strains, perhaps reflecting their slow growth; after adjustment for this their mtDNA concentrations were rather low, in contrast to the high mtDNA concentrations associated with aged donors. Since both immortal cell lines and "short-lived" mutant strains can carry a complement of mtDNA either greater or less than that found in normal diploid fibroblasts at any age, we conclude that the relatively moderate variation in mtDNA copy number seen during the replicative lifespan of diploid fibroblasts is not responsible for the limit on that lifespan.

Clonal Heterogeneity of mtDNA Levels—When seven individual clones isolated from the A2 mass culture were examined for mtDNA sequences (Fig. 3 and Table IV), values were found ranging from 0.4% to 1.3% of the genome (1600–5200 copies/cell). The level of mtDNA/cell in these clones was not correlated with either cell volume or maximal replicative lifespan, but showed a weak inverse correlation with growth rate (r = −0.65) (Table IV). These data support the conclusion that mtDNA number is not important in determining cellular lifespan in vitro. Indeed, if there were any advantage in
Fig. 2. Mitochondrial DNA in permanent human cell lines and in fibroblast strains derived from progeroid and Bloom syndrome donors. DNA samples were digested with Msp1 restriction enzyme (12 units/µg of DNA, 2 h at 37 °C) and assayed as described in the Fig. 1 legend. DNA samples were prepared from permanent cell lines KB (1.7 µg) (a), Chang (1.3 µg) (b), VA13 (WI-38 fetal lung fibroblasts transformed by SV40) (2 µg) (c), and HeLa (1.3 µg) (d); and from human diploid fibroblast strains at MPD levels shown: GM1492 (Bloom syndrome) (2 µg) (e), WS2 (Werner syndrome) (1.7 µg) (f), P18 (Hutchinson-Gilford progeria syndrome) late passage (1.8 pg) (g), P18 (1.7 pg) (h), P11 (progeria syndrome) (1.7 pg) (i), and P5 (progeria syndrome) (1.7 µg) (j).

possessing either high or low mtDNA levels, then given the substantial clonal heterogeneity within a mass culture the population should have shifted toward the mtDNA levels found in "fitter" clones. Since the mass cultures changed little in mtDNA proportion, compared to the heterogeneity they comprise, such selection must not occur significantly. The moderate inverse correlation between mtDNA number and cell growth rate, however, suggests that the replication rate of mtDNA genomes may lag slightly behind nuclear replication in the fastest growing lineages.

Saturation Hybridization—The mtDNA levels in Tables I–IV were obtained from densitometer tracings of Southern transfer autoradiographs (Figs. 1–3) within the linear range of the films. Several such experiments, with appropriate hybridization standards, were used in each determination. However, in addition, many of these results were confirmed by saturation hybridization (Gilbert and Spiegelman, 1965; Shmookler Reis and Goldstein, 1980). 3H-labeled complementary RNA, copied from pure mtDNA template, was hybridized in increasing excess to total cell [14C]DNA immobilized on small nitrocellulose filters. The plateau level of maximal hybridization, approached at high cRNA concentration (ranging from 16–100-fold excess over complementary mtDNA strands), can be most accurately determined by linear extrapolation in a double reciprocal plot of the data (Bishop, 1972).

Representative results from such saturation hybridizations are shown in Fig. 4. DNAs from DS fibroblasts at early and late passage (1.7 pg) (d), P18, (1.7 pg) (e), and P11, (1.7 pg) (f) were obtained from densitometer tracings of Southern blots. Several such experiments, with appropriate hybridization standards, were used in each determination. Howhybridization, approached at high cRNA concentration (ranging from 16–100-fold excess over complementary mtDNA strands), can be most accurately determined by linear extrapolation in a double reciprocal plot of the data (Bishop, 1972).

| Table I |
| --- |
| Effect of in vitro and in vivo age on mitochondrial DNA copy number |
| Lane | Strain (donor, age) | MPD | mtDNA copies/mtDNA | Cell volume | Total protein | mtDNA copies/pg protein | µ<sup>2</sup> pg/cell |
| a | A2 (11, M) | 18 | 0.6 | 2400 | 3000 | 255 | 9.4 |
| b | 58 | 0.9 | 3600 | 4330 | 341 | 10.6 |
| c | DS (36, M) | 17 | 0.7 | 2800 | 3030 | 266 | 10.5 |
| d | 55 | 1.0 | 4000 | 3860 | 395 | 10.1 |
| e | TM (25, M) | 15 | 0.9 | 3600 | 3030 | 303 | 11.9 |
| f | 49 | 1.2 | 4800 | 5130 | 540 | 8.9 |
| g | J089 (67, M) | 16 | 1.2 | 4800 | 1490 | 199 | 24.1 |
| h | 42 | 1.5 | 6000 | 2070 | 365 | 16.4 |
| i | J089 (68, M) | 14 | 1.3 | 5200 | 2500 | 215 | 24.2 |
| j | 31 | 1.2 | 4800 | 2480 | 204 | 23.5 |
| k | J088 (76, F) | 16 | 1.1 | 4400 | 3170 | 243 | 18.1 |
| l | 40 | 1.2 | 4800 | 5380 | 341 | 14.1 |

* Further details, including maximum MPD, and references for these strains can be found in Shmookler Reis and Goldstein, 1982.

* Passage level in mean population doublings.

* Young versus old donor difference is significant at p < 0.02.

* Young versus old donor difference is significant at p < 0.005.

Table II

| Table II |
| mtDNA copy number in permanent cell lines |
| Line | Per cent mtDNA | mtDNA copies/cell | Cell Volume | Total protein | mtDNA copies/pg protein | µ<sup>2</sup> pg/cell |
| KB (CCL17) | 1.8 | 7200 | 1780 | 254 | 28.3 |
| Chang (CCL13) | 0.4 | 1800 | 2700 | 771 | 2.1 |
| HeLa (CCL2) | 1.8 | 7200 | 1480 | 419 | 17.2 |
| VA13 (W138-SV, CCL75) | 0.7 | 2800 | 1600 | 364 | 7.7 |

* American Type Culture Collection, Rockville, MD; with ATCC catalogue code.

Table III

| Table III |
| mtDNA copy number in mutant fibroblast strains associated with premature aging and/or chromosome breakage |
| Strain | MPD | Maximum MPD | Per cent mtDNA | mtDNA copies/cell | Cell Volume | Total protein | mtDNA copies/pg protein | µ<sup>2</sup> pg/cell |
| BL | 46 | 50 | 1.8 | 7200 | 2640 | 409 | 17.6 |
| WS2 | 18 | 37 | 0.9 | 3600 | 2220 | 379 | 9.5 |
| P5 | 14 | 42 | 0.7 | 2800 | ND | ND | ND |
| P11 | 18 | 37 | 0.9 | 3600 | 5160 | 750 | 4.8 |
| P18 | 10 | 53 | 0.4 | 1800 | ND | ND | ND |
| P18 | 49 | 53 | 0.7 | 2800 | ND | ND | ND |

* Bloom syndrome, GM1492 from Genetic Mutant Cell Repository, Camden, NJ.

* Werner syndrome No. 2 (Yatscoff et al., 1978).

* Progeria (Hutchinson-Gilford syndrome) No. 5 (Goldstein and Moerman, 1975).

* ND, not determined.

* Progeria (Hutchinson-Gilford syndrome) No. 11 (Goldstein and Moerman, 1975).

* Progeria (Hutchinson-Gilford syndrome) No. 18 (Goldstein et al., 1982).
Are There Petite-type Deletion Mutations in Human mtDNA?—Senescence of the fungus, *Podospora anserina*, is associated with the random amplification of specific segments of mtDNA at the expense of integral mtDNA genomes (Jamez-Vierny et al., 1986), analogous to ρ° (“petite”) deletion mutations of yeast mtDNA (Slonimski and Lazowska, 1977). In view of this, and the observation by Smith and Vinograd (1972) of small polydisperse circular DNA molecules in HeLa and WI-38 cells, we undertook Southern-transfer hybridizations (Figs. 1–3) in a manner which would favor identification of amplified regions in mtDNA. These experiments revealed no departures from stoichiometry of mtDNA restriction fragments in early or late passage mass cultures, in isolated clones, or in permanent cell lines or mutant cell strains (Figs. 1–3). Apparent departures from molar equivalence of fragments were due to co-migration of two or more restriction fragments (Brown and Goodman, 1979, and see above).

In order to ascertain whether any mtDNA sequences could be identified at sizes smaller than the mitochondrial genome, we electrophoresed undigested total DNA from the DS fibroblast strain at several passage levels and probed the resulting Southern-transfers with [32P]mtDNA under conditions of very high stringency. In addition to intact monomeric and dimeric mtDNA molecules, we observed a number of smaller “extrachromosomal” bands hybridizing to [32P]mtDNA probe (Fig. 5). The most prominent of these was approximately 0.65 kb in length and probably corresponds to the principal species of “D-loop strand” (putative primer for mtDNA H-strand synthesis) released from nicked mtDNA circles (Robberson and Clayton, 1973; Ojala and Attardi, 1978; Clayton, 1982). Hybridization to this band indicated 1000–2000 copies per cell (perhaps more if it is imperfectly homologous to the mtDNA probe or under-represented there), thus comprising a substantial fraction of the mtDNA copy number in DS fibroblasts, 3000–4000 per cell. While there was only a moderate decrease in the amount of 0.65-kb band hybridization during *in vitro* aging, it was considerably diminished in senescent fibroblasts maintained for 5 weeks after the cessation of mitosis (Fig. 5e). In contrast, mtDNA hybridization to a 2.3-kb band from undigested DNA did not change substantially (Fig. 5) and mtDNA genome number per cell remained essentially constant (data not shown) in those postmitotic senescent cells. The preferential loss of the 0.65-kb band, in cells which have essentially ceased dividing and which maintain a stable level of mtDNA, thus suggests that D-loops may play a role not only in regulation of transcription (Clayton, 1982), but also in nuclear control of mtDNA replication.

Although the significance of these small mtDNA molecular species is still speculative, they are clearly not analogous to the mtDNA fragments amplified in senescent *Podospora* cultures. Extrachromosomal circular DNA molecules have been observed recently in nuclei of monkey and human cells (Kro-lewski et al., 1982; Calabretta et al., 1982; Shmookler Reis et al., 1983a) but these are not homologous to mtDNA (Shmookler Reis et al., 1983b). Thus, the observation of small DNA molecules hybridizing to mtDNA is of particular interest. They may form part of the small circular DNA characterized by Smith and Vinograd (1972) which appeared to be cytoplasmic although not localized to mitochondria.

*Methylation of mtDNA*—Previous estimates have indicated very low levels of methylation (not exceeding 0.2–0.6% of cytosines) in mtDNA from mouse and hamster cells (Nass, 1973). We have used restriction cleavage with the enzymes *MspI* and *HpaII* to estimate the extent of methylation in human mtDNA (Waalwijk and Flavell, 1977; Singer et al., 1979; Shmookler Reis and Goldstein, 1982). These endonucleases share a common recognition sequence, -C-C-G-G-, but differ in their responses to 5-methylcytosine (°C): *MspI* (but

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**TABLE IV**

*mtDNA copy number, growth rate, and lifespan for A2 fibroblast clones*

| Cells   | (MPD) | Maximum MPD | Per cent mtDNA | mtDNA copies/cell | Growth rate | Cell volume |
|---------|-------|-------------|----------------|-------------------|-------------|------------|
| A2 M.C.* | ~18   | 60          | 0.7            | 2800              | ++          | ~3000      |
| Clone 1 | ~40   | ND          | 1.2            | 4800              | ++          | ND         |
| Clone 2 | ~40   | ND          | 1.1            | 4400              | +3          | ~4000      |
| Clone 5 | ~40   | ND          | 0.6            | 2400              | ++          | ND         |
| Clone 10| ~40   | 54          | 1.3            | 5200              | +           | ~3600      |
| Clone 14| ~40   | 57          | 0.9            | 3000              | +3          | ~3200      |
| Clone 18| ~40   | 57          | 0.4            | 1600              | +           | ~3000      |
| Clone 30| ~40   | 54          | 1.3            | 5200              | +           | ~3400      |

*Mass culture.

*ND, not determined.

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late passage (17 and 53 MPD) were analyzed in Fig. 4, a and b, confirming a moderate (~50%) increase in mtDNA content per cell at late passage (see Table I, c and d). A heterologous DNA control and a mtDNA standard are also shown. In contrast, comparison of the HeLa and Chang cell lines (Fig. 4, c and d) indicated a 4-fold difference in their mtDNA levels per cell, in close agreement with the results of Southern-transfer quantitations (Table II).

**FIG. 3.** Heterogeneity for mitochondrial DNA in the A2 diploid fibroblast strain: mass culture versus isolated clones. DNA samples were digested with *HpaII* restriction enzyme (12 units/μg of DNA, 2 h at 37 °C) and assayed as described in the Fig. 1 legend. Lanes in the autoradiograph correspond to HeLa mtDNA (5 ng) (a), A2 mass culture total DNA (0.7 μg) (b), clone 1 (1 μg) (c), clone 2 (0.7 μg) (d), clone 5 (0.7 μg) (e), clone 10 (1 μg) (f), clone 14 (0.7 μg) (g), clone 18 (0.3 μg) (h), and clone 30 (1 μg) (i).
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FIG. 4. Saturation hybridizations of mitochondrial [3H]cRNA to total cell DNAs. [3H]cRNA transcribed in vitro from pure HeLa cell mtDNA was hybridized at varying concentrations to DNA samples immobilized on nitrocellulose filters. DNA retention on filters was monitored by simultaneous counting of [3H]DNA (either endogenous by prior long term incorporation of [3H]dTThd (a and b) or exogenous by addition of high specific activity [3H]DNA (c and d) and used to calculate [3H]cRNA hybridized per µg of DNA retained. a: hybridization to E. coli DNA (A), to E. coli DNA plus HeLa mtDNA (A) (2 ng of mtDNA per µg of E. coli DNA), and to DNA of strain DS fibroblasts at 17 (O) and 53 (O) MPD. b: double reciprocal plot of strain DS data from a. c: hybridization to E. coli DNA (A), to E. coli DNA plus HeLa mtDNA (A) (2 ng of mtDNA/µg), to Chang cell line DNA (O), and to HeLa cell line DNA (O). d: double reciprocal plot of Chang and HeLa data from c. Data points shown are the averages of duplicate or triplicate filters, following digestion with RNase A. [3H]RNA counts/min hybridized prior to RNase digestion (but after extensive rinsing) were approximately 4× higher than values after RNase, but were otherwise consistent with these data.

not HpaII) will cut -C-"C-G-G-, while HpaII (but not MspI) will cut -"C-C-G-G- (reviewed by Razin and Riggs, 1980).

In many experiments repeated with the same DNA samples, the patterns and quantities of mtDNA hybridization measured after MspI cleavage were very close to those measured after HpaII cleavage. HpaII digestions, however, generally left 2-5% of hybridizing mtDNA at and near the top of the gel in positions corresponding to undigested mtDNA (see Figs. 1 and 3) indicating molecules with all -C-C-G-G- sites modified to -C-"C-G-G-; such material was not seen after digestion with MspI (Fig. 2) or EcoRI (not shown) and occurred at much lower levels in HeLa mtDNA, isolated as covalently closed circular DNA molecules, following HpaII digestion (Fig. 1). One fibroblast clone (Fig. 3, lane i) had ~10% of its mtDNA at high molecular weight after HpaII digestion and interestingly this was a clone shown previously to have unusually high -CpG- methylation of total nuclear DNA (see Shmookler Reis and Goldstein, 1982, "clone 30').

The uncleaved mtDNA was unaffected by higher ratios of HpaII enzyme to DNA (not shown), but was less abundant in late passage DNA samples than at early passage for each of the fibroblast strains from young donors (Fig. 1, lanes a-f). This relationship was not apparent, however, in fibroblast strains from old donors (Fig. 1, lanes g-l).

If -C-C-G-G- sites are typical of all CpGs in mtDNA, then 2-5% of sites methylated would correspond to 0.06-0.14% of cytosines methylated, far below the fraction observed in total DNA from human fibroblasts (2.2-4.5%; Shmookler Reis and Goldstein, 1982), but in good agreement with earlier estimates for mammalian mtDNA (Nass, 1973). Our calculation of mitochondrial cytosine methylation is based on

$$\frac{\text{C}}{\text{C}} = \frac{\text{CpG}}{\text{CpG}} \times \frac{\text{CpG}}{\text{C}} + \frac{\text{CpG}}{\text{C}}$$

where \(\frac{\text{CpG}}{\text{CpG}} \approx 2-5\%\) (this report), \(\text{CpG}/\text{C} \approx 2.5\%\) (Anderson et al., 1981), and \(\frac{\text{CpG}}{\text{C}} \approx 0.9\) (Ehrlich and Wang, 1981). Since each mtDNA molecule contains 22 potential sites for HpaII/MspI (Brown, 1980), the appearance of a substan-
CONCLUSIONS

We have quantitated mitochondrial DNA in normal human diploid fibroblasts and obtained values ranging from 2400–6000 copies/cell, entirely within the range (1100–8800 copies/cell) spanned by permanent aneuploid cell lines analyzed previously, human HeLa and mouse L cells (Bogenhagen and Clayton, 1974), and concurrently (this report, Table II). The average number of mtDNA genomes per cell varied relatively little during the replicative lifespan of any given diploid cell strain (Table I), increasing in 5 of 6 strains by 10–50% at late passage. When normalized to cell protein content, which also varied, the concentration of mtDNA genomes remained remarkably constant over the period of in vitro culture.

A number of conclusions may be drawn from these results. First, the data begin to answer the question of how closely mtDNA replication is kept in synchrony with nuclear DNA replication: it would appear to be regulated not by direct coupling to the nuclear DNA replication, but rather by the cell mass to be serviced by mitochondria.

Secondly, mitochondrial genome number is unlikely to be a major determinant of cellular senescence in vitro, in view of the moderate alterations observed in mtDNA number during the replicative lifespan, relative to interindividual and interclonal variation, or to values seen in immortal lines. Indeed, when genome numbers per cell are adjusted for changes in cell protein or cell volume, as discussed above, virtually all passage-dependent change in mtDNA copy number is eliminated. Observations by high voltage electron microscopy^2 have indicated a similar constancy during in vitro culture, in the ratio of mitochondria/cytoplasm (estimated from projected mitochondrial area per cell area). There is, however, a late-passage increase in glycolysis (Goldstein et al., 1982) and a decrease in protonmotive capacity (Goldstein and Korczack, 1981). In the light of the present data, these changes are unlikely to result from deficiencies or deletions in mtDNA, but instead may reflect impairments in mitochondrial structure (Lipetz and Cristofalo, 1972; Johnson, 1979), mitochondrial gene expression, or metabolic regulation.

Fibroblasts from old donors displayed significantly higher levels of mtDNA/cell protein or mtDNA/cell volume than cells from young donors, regardless of passage level in vitro. This suggests that a molecular correlate of in vivo senescence may be maintained throughout fibroblast isolation and propagation in vitro. Further studies on additional strains from young and old subjects will be necessary to resolve this question.

The integrity of the mitochondrial genome does not appear to alter during in vitro senescence, since restriction fragments maintain the same relative proportions and mobilities. This is consistent with our earlier report of an essentially invariant pattern of mitochondrial protein synthesis for 24 human cell lines and strains (Yatscoff et al., 1978). In addition to chromosome-size mtDNA (16,569-bp monomeric form), several discrete species of small mtDNA molecules were seen in similar amounts at all passage levels prior to mitotic arrest of the culture. The disappearance of the 0.65-kb DNA species, coincident with prolonged cessation of mitosis (due to saturation arrest plus senescence), suggests its involvement in mtDNA replication. Unlike “petite” mutations in yeast (Slonimski and Lazowska, 1977) and “SEN-DNA” of fungi (Jamet-Vierny et al., 1980), these small mtDNA molecules do not appear to proliferate in lieu of intact mtDNA genomes, nor do they ultimately become the principle form of mtDNA.

Approximately 2–5% of mtDNA molecules are essentially fully methylated at -CCGG- sites, while the remainder are fully unmethylated. The methylated fraction appears to be dependent on fibroblast doubling level in vitro provided that the fibroblasts were derived from young normal donors. It is unlikely that such mtDNA methylation would have any functional significance in the control of mitochondrial gene expression (see, for example, Razin and Riggs, 1980), since entire genomes were methylated uniformly.

In summary, the evidence indicates that mtDNA is not a major determinant of either short term growth rate or long term proliferative potential of human diploid fibroblasts, since neither quantity, integrity, nor methylation pattern of mtDNA is substantially altered during serial passage of mass.

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cultures in vitro. Substantial variation is seen, however, in individual fibroblast clones and in permanent cell lines, ranging from ~1000—8000 mtDNA copies per cell, indicating that mtDNA genome number is not tightly regulated in cultured human cells.

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