Volume 4 Number 5 1977 Nucleic Acids Research

Complex forms and replicative intermediates of mitochondrial DNA in tissues from adult and senescent mice

Lajos Pikó and Lloyd Matsumoto

Developmental Biology Laboratory, Veterans Administration Hospital, Sepulveda,CA 91343 and the Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

Received 4 February 1977

ABSTRACT

The occurrence and types of complex forms and replicative intermediates of mitochondrial DNA (mtDNA) were investigated in tissues from C57BL/6J mice aged 10-11 months or 29-30 months. Total mtDNA from brain, heart, kidney and liver was isolated in ethidium bromide-CsCl gradients and examined by electron microscopy after aqueous or formamide spreading. Contour length measurements indicated no difference in the monomer size of mtDNA according to either tissue or donor age. The frequencies of catenated mtDNA, ranging from 4 to 8%, varied significantly according to tissue but changed relatively little as a result of donor age. The main age-related effect observed in this study was a significant increase in the frequency of circular dimers, from about 0.05% in adult tissues to 0.3% in kidney, 0.5% in liver, 0.6% in heart and 1.9% in brain of senescent mice. The frequency of D-loop DNA varied from 30 to 60% and that of larger replicative intermediates from 1 to 10%, suggesting differences in the rate of mtDNA replication according to tissue. The frequencies and types of the various replicative intermediates were unaffected by donor age.

INTRODUCTION

The prevalent form of mitochondrial DNA (mtDNA) in animal cells is a circular duplex molecule of about 5-μm perimeter which constitutes the basic genetic unit [4]. A smaller portion of the mtDNA population occurs in two complex forms: a catenated form consisting of two or more circular duplexes which are topologically interlocked as the links in a chain and a circular dimer form in which two genome-size units are covalently attached in a head-to-tail arrangement [6,8,20]. Catenated mtDNA has been found at frequencies ranging from 2 to 10% in all normal tissues examined. In contrast, circular dimers are seen very rarely in normal tissues, their frequency generally not exceeding 0.1 to 0.2% [6,7,26]. A much higher frequency of complex forms is often found in malignant or otherwise pathological tissues, in some cultured cell lines, and virus-transformed cells [6,29,30]. The mechanisms of accumulation and physiological significance of complex forms of mtDNA are incompletely understood.

Recent studies of mtDNA replication in mouse L cells have described a number of replicative intermediates and have led to a detailed model of mtDNA replication [2,3,22,23,36]. An early replicative intermediate containing a small single-stranded displacement loop (D-loop DNA) is usually present at a high frequency. Replication continues through expansion of the D-loop and duplex synthesis on the displaced strand. Variations within this model have been observed regarding the frequency of D-loop molecules and the synchrony and pattern of duplex synthesis during expansion, for example, in rat liver and rat hepatoma cells [42] and sea urchin oocytes [25]. Although there is evidence indicating mtDNA turnover in adult animal tissues [18], relatively little is known of the pattern of mtDNA replication in various normal tissues.

This paper reports an electron microscope study of structural and replicative forms of mtDNA in four mouse tissues: brain, heart, kidney, and liver. The donors were C57BL/6J male mice belonging to two age groups: adult mice of 10-11 months of age and senescent mice having reached the age of average longevity (29-30 months).

MATERIALS AND METHODS

Animals

C57BL/6J male mice were obtained from the colony of Dr. C. E. Finch, Andrus Gerontology Center, University of Southern California, Los Angeles. These mice were originally received as retired breeders (8 months old) from the Jackson Laboratory, Bar Harbor, ME and were subsequently maintained under the husbandry conditions and health monitoring described previously [11,13,14]. Two age groups were examined: adult mice, 10-11 months old; and senescent mice, 29-30 months old (the age of 50% survival in this colony). Mice at an age of 10-11 months are fully grown with respect to skeletal size and body weight and correspond physiologically to human males aged 20-30 years; the physiological age of the senescent mice used in this study would correspond to that of human males aged 70-80 years [12]. The mice in each group weighed about 30 g and appeared to be in good general health. The internal organs were free of visible pathological signs, except for greatly enlarged but otherwise clear seminal vesicles in three out of the four senescent mice used; the latter condition is prevalent in the majority of C57BL/6J mice 24 months of age or older and is associated with an increased fluid content in the seminal vesicles without detectable malignancy [15].

Isolation of mitochondria

Mitochondria were isolated from brain, heart, kidney and liver; for each

isolation, the organs from two mice were pooled and processed together. Two procedures were used, differing mainly in whether the mitochondrial pellet was treated with DNase before the extraction of the mtDNA or not. Both procedures were carried out in the cold. The tissues were trimmed free of fat, minced, and rinsed in several changes of homogenization buffer. They were then homogenized in about 10X volume of buffer in a Potter-Elvehjem homogenizer with a motor-driven loose-fitting teflon pestle until more than 90% of the cells were disrupted, as monitored by light microscopy of samples stained with methyl green and pyronin Y. In procedure 1, the homogenization buffer was 0.25 M sucrose, 0.01 M Tris-HCl, 0.001 M $CaCl_2$, pH 7.6; in procedure 2, it was 0.25 M sucrose, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6 (STE buffer). The homogenates were centrifuged at 750 g (average) in a Sorvall HB-4 rotor (Dupont Instruments, Newtown, CT) for 7 min. The nuclear pellets were discarded and the supernatants centrifuged at 12,000 g for 12 min in the same rotor. The supernatants were decanted and the mitochondrial pellets were treated as follows. In procedure 1, the mitochondrial pellet was resuspended in STE buffer and repelleted as before. In procedure 2, the mitochondrial pellet was suspended in 2.0 ml of 0.25 M sucrose, 0.01 M Tris-HCl, 0.005 M $MgCl_2$, pH 7.6 containing 100 µg pancreatic DNase (2500 U/mg, electrophoretically pure, Worthington Biochemicals, Freehold, NJ) and incubated at 37°C for 20 min to digest extra-mitochondrial DNA. The action of DNase was quenched with an excess of STE buffer containing 0.005 M EDTA and the mitochondria were repelleted as before. The mitochondria from the organs of two senescent mice were isolated according to procedure 1, and the mitochondria from two senescent mice and two adult mice were isolated according to procedure 2.

Isolation of mtDNA

The procedures used for the isolation of mtDNA were essentially the same as described earlier [26] and were briefly as follows. The mitochondrial pellets were suspended in 1.0 ml of 0.5 M NaCl, 0.1 M Tris-HCl, 0.01 M EDTA, pH 8.0 (NTE buffer) and lysed with an equal volume of warm (37°C) 4% sodium dodecyl sulphate in distilled water. The lysates were adjusted to a final volume of 3.5 ml containing 1.55 g/ml CsCl (optical grade, Harshaw Chemical Co., Solon, OH) and 300 µg/ml ethidium bromide (Boots Pure Drug Co., Ltd., Nottingham, England) and centrifuged for about 30 h at 40 krpm, 20°C, in the Beckman SW50.1 rotor. The tubes were illuminated with near-ultraviolet light and the total DNA from the lower to the upper DNA band was collected. When the isolation of the mitochondria included a DNase treatment of the mitochondrial pellet (procedure 2), most of the DNA (usually at least 90%) that was

recovered banded in the lower band; when the DNase treatment was omitted (procedure 1), the amount of DNA in the upper band generally exceeded that in the lower band due to the presence of contaminating nuclear DNA. The DNA from each tube was dialyzed against Dowex 50 resin (Biorad Laboratories, Richmond, CA) in NTE buffer, concentrated in the dialysis tubing with dry Sephadex G-200 (Pharmacia, Uppsala, Sweden), and further purified by band-velocity sedimentation on a CsCl gradient containing 100 µg/ml ethidium bromide [26]. The contents of the tube except for the upper portion located above the nicked mtDNA band were collected, redialyzed as above, and used for examination by electron microscopy. The velocity sedimentation removed the bulk of the nuclear DNA, and the remaining large linear DNA molecules (in samples obtained by procedure 1) did not interfere seriously with the electron microscopic evaluation of mtDNA.

Electron microscopy

For an analysis of complex forms of mtDNA, the mtDNA was spread by the aqueous basic protein film technique [10] from a solution containing 5 µg/ml of ethidium bromide. Films were transferred onto parlodion-coated 300-mesh copper grids, stained in alcoholic uranyl acetate, rotary shadowed with Pt-Pd, and examined in a Philips EM200 operated at 40 kV. From 1000 to 2000 mtDNA molecules per sample were selected at random and scored for the occurrence of complex forms as described [6,7]. The final score represents the pooled data from at least two spreadings per group. In order to facilitate the identification of circular and catenated dimers and to reduce the chance of accidental overlapping, only those grids containing at least 90% of the mtDNA in the relaxed (open) form and containing fewer than 250 mtDNA molecules per grid square were evaluated. A preliminary study indicated that there was a significant increase in the apparent frequency of catenated forms when the number of mtDNA molecules per grid square reached or exceeded 500. Each complex mtDNA molecule was photographed and final classification was made after analysis of the prints; when necessary, the negatives were traced on a Nikon 6F projection comparator and measured with a map measure. All mtDNA molecules classified as circular dimers were verified by length measurement.

Replicative forms of mtDNA were visualized by formamide spreading [25] and evaluated as follows. Prints (final magnification about X25,000) of randomly taken electron micrographs were scored for the frequencies of the various classes of replicative intermediates. The lengths of the single- and double-stranded regions in the molecules were measured from the enlarged tracings of the negatives. Formamide preparations were also used to measure the contour

length of clean duplex mtDNA circles using duplex ϕX174-RF DNA as an internal standard. A molecular weight of 3.45 X 10^6 was taken for ϕX174-RF DNA [24]. We are indebted to Dr. Paul Johnson for a gift of ϕX174-RF DNA.

The frequency distribution of molecular classes was analyzed in chi-square tests using 2 X 2 and 2 X C contingency tables; a correction for continuity was applied for small class frequencies [39].

RESULTS

Complex forms of mtDNA

Table I summarizes the frequencies of complex forms of mtDNA obtained from the organs of adult mice. In each case, the organs from two mice were pooled and processed together for the isolation of mtDNA; the processing included a DNase treatment of the mitochondrial pellet. The total mtDNA, including lower and upper bands, was used for electron microscopic evaluation after spreading with the aqueous protein film technique.

TABLE I

Frequency of Complex MtDNA in Tissues from Adult Mice*

MtDNA form	Brain	Heart	Kidney	Liver
Monomers	94.6 ± 1.0	94.0 ± 0.9	96.1 ± 0.8	95.4 ± 0.9
Circular dimers	0	0.1 ± 0.1	0.05 ± 0.1	0
Catenanes consisting of:				
2 monomers	5.2 ± 0.9	5.3 ± 0.9	3.5 ± 0.8	4.4 ± 0.9
3 monomers	0.2 ± 0.2	0.5 ± 0.3	0.3 ± 0.2	0.2 ± 0.2
4 monomers	0	0.04 ± 0.1	0	0.05 ± 0.1
Total complex forms (wt%)‡	10.5	11.7	7.8	9.0
Total molecules scored	2174	2435	2162	2193

* Frequencies are given as percent of total number of molecules scored ± the statistical sampling error at the 95% confidence limit.

‡ Total circular dimers and catenanes expressed as a percent of the mtDNA mass.

The frequency of circular dimers was extremely low, from zero to 0.1%, in all the samples examined. Altogether, a total of 4 circular dimers were seen in a population of 8,964 mtDNA molecules from the four organs, or a frequency of about 0.05%. The frequency of catenated dimers and oligomers varied from 4 to 6%, or 8 to 12% of the mtDNA mass. Despite this narrow range, there is a significant heterogeneity in the frequencies of total complex forms (number-%) in the four organs ($P<0.01$).

Table II shows the distribution of complex forms of mtDNA from the organs of senescent mice. In these mice, two procedures were used for the isolation of mtDNA, one involving a DNase treatment of the mitochondrial pellet and the other omitting this step (see Materials and Methods). The pooled organs from two mice were used for each procedure. However, since neither the frequencies of complex forms (including circular dimers) nor those of replicative forms of mtDNA differed significantly between the two groups, the pooled data are presented throughout this paper.

TABLE II

Frequency of Complex MtDNA in Tissues from Senescent Mice*

MtDNA form	Brain	Heart	Kidney	Liver
Monomers	90.7 ± 1.2	95.0 ± 0.7	94.3 ± 1.0	94.8 ± 0.9
Circular dimers	1.6 ± 0.5	0.6 ± 0.3	0.3 ± 0.2	0.5 ± 0.3
Catenanes consisting of:				
2 monomers	6.8 ± 1.0	4.2 ± 0.1	5.0 ± 0.9	4.5 ± 0.9
3 monomers	0.5 ± 0.3	0.1 ± 0.1	0.4 ± 0.2	0.2 ± 0.2
4 monomers	0.1 ± 0.1	0	0.04 ± 0.1	0
5 monomers	0.04 ± 0.1	0.03 ± 0.1	0	0
2 circular dimers	0.04 ± 0.1	0	0	0
1 circular dimer + 1 monomer	0.2 ± 0.2	0	0	0
1 circular dimer + 3 monomers	0.1 ± 0.1	0	0	0
Total complex forms (wt%)†	18.1	9.7	11.1	10.1
Total molecules scored	2396	3600	2243	2201

*† See footnotes in Table I.

The frequency of circular dimers, ranging from 0.3 to 1.6%, is conspicuously elevated in senescent tissues. The frequency is highest in brain, 1.6% (or 1.9% if circular dimers occurring in catenanes are included). As compared with adult tissues, the increase in circular dimer frequency is statistically significant in brain ($P<0.001$), heart ($P<0.01$), and liver ($P<0.01$) but not in kidney. The incidence of circular dimers in senescent brain is significantly higher ($P<0.001$) than in any of the other senescent tissues.

The frequency of catenated forms shows relatively little change in senescent tissues, although it is somewhat elevated in brain ($P<0.01$) and

kidney ($P<0.05$), and somewhat lower in heart ($P<0.05$). The frequency of total complex forms (number-%) is significantly increased in senescent brain ($P<0.001$) and kidney ($P<0.01$), but there is no significant change in heart and liver as compared with the corresponding adult organs.

Size of mtDNA

The contour lengths of the monomer circles of mtDNA from adult and senescent tissues were measured with respect to double-stranded ϕX174-RF DNA marker. Two adjacent ϕX174 circles were measured for each mtDNA molecule. The DNA was spread with the formamide technique so that gapped circles could be recognized; these were not included.

TABLE III

Size of Monomer Circles of MtDNA from Tissues from Adult and Senescent Mice

	Adult mice		Senescent mice	
	No. of molecules	MtDNA size in ϕX174 units*	No. of molecules	MtDNA size in ϕX174 units*
Brain	76	3.07 ± 0.12	122	3.12 ± 0.14
Heart	57	3.13 ± 0.15	109	3.09 ± 0.15
Kidney	50	3.12 ± 0.19	104	3.11 ± 0.20
Liver	88	3.07 ± 0.13	119	3.13 ± 0.17

* The average size ± SD of monomer mtDNA circles in multiples of ϕX174-RF DNA.

There is no significant difference in the average size and distribution of mtDNA in the different groups (Table III). We have also examined the occurrence of "odd-sized" mtDNA molecules, defined arbitrarily as those molecules differing by more than ± 10% from the average size. The frequency of such molecules was 2-3% and did not vary significantly according to either tissue or donor age. The average size of mtDNA for the four tissues is 3.10 ϕX units, corresponding to a molecular weight of about 10.7×10^6. (No correction was made in this calculation for the spontaneous deletion of about 7% of the genome in a small percentage of ϕX174-RF DNA in the *am*3 strain of ϕX174 used in this study; ref.[43]).

Replicative forms of mtDNA

The frequencies of the various types of replicative intermediates of mtDNA [36] were scored from electron micrographs of mtDNA spread with the formamide technique. Molecules containing small displacement loops of up to 5%

genome size were classified as D-loop DNA. Replicative intermediates with 6% or larger expansions were classified as expanded-D molecules. The molecules scored as gapped circles in this work contained single-stranded segments of 5 to 85% genome size.

TABLE IV

Frequency of Replicating Forms of MtDNA in Tissues from Adult Mice*

MtDNA form	Brain	Heart	Kidney	Liver
Clean duplex circle	67.5 ± 5.6	46.9 ± 5.3	45.8 ± 6.3	63.8 ± 4.8
With D loop	31.3 ± 5.6	50.7 ± 5.3	49.6 ± 6.4	27.3 ± 4.5
With expanded D loop‡	0.8 ± 1.0	2.1 ± 1.5	3.4 ± 2.3	7.8 ± 2.7
Gapped circle	0.4 ± 0.7	0.3 ± 0.6	1.3 ± 1.4	1.0 ± 1.0
Total molecules scored	265	341	238	384

* Each monomeric unit of a catenane is scored as a separate molecule. Frequencies are given as percent of total number of monomers scored ± the statistical sampling error at the 95% confidence limit. The terminology of replicative forms follows that of Robberson et al. [36].

‡ Approximately 20% of expanded-D molecules exhibited some degree of branch migration and are included in this class.

Table IV shows the distribution of replicative forms observed in total mtDNA from adult organs. The frequency of D-loop molecules ranged from about 30% (brain, liver) to about 50% (heart, kidney). The frequency of larger replicative forms (expanded-D molecules and gapped circles) varied widely, from about 1% in brain to about 9% in liver. There is a significant heterogeneity in the occurrence of both of these classes of molecules in the four tissues ($P<0.001$).

The frequencies of the different classes of replicative forms in mtDNA from senescent organs (Table V) are similar to those in adult tissues. The only difference amounting to statistical significance ($P<0.05$) is observed in the frequency of D-loop DNA in heart which is somewhat higher, about 60%, in the senescent group. On the other hand, a highly significant tissue-dependent heterogeneity ($P<0.001$) in the frequency of D-loop molecules and that of larger replicative forms also exists in the senescent group. If the data for adult and senescent mice are pooled, one obtains the following frequencies of larger replicative intermediates in the four organs: brain, 0.9%; heart, 2.0%; kidney, 3.9%; and liver, 10.0%. Between any two organs,

the difference in these frequencies is statistically significant.

TABLE V

Frequency of Replicating Forms of MtDNA in Tissues from Senescent Mice*

MtDNA form	Brain	Heart	Kidney	Liver
Clean duplex circle	73.2 ± 2.8	39.2 ± 4.5	53.4 ± 4.6	62.5 ± 3.0
With D loop	26.0 ± 2.7	59.0 ± 4.5	43.1 ± 4.6	27.0 ± 2.7
With expanded D loop‡	0.5 ± 0.4	1.6 ± 1.1	2.2 ± 1.4	7.7 ± 1.6
Gapped circle	0.3 ± 0.3	0.2 ± 0.4	1.3 ± 1.1	2.7 ± 1.0
Total molecules scored	987	449	453	1028

*‡ See footnotes in Table IV.

Pattern of duplex synthesis

Since the mtDNA isolated from liver contained a high percentage of expanded-D forms, these molecules were used for an analysis of the pattern of duplex synthesis on the displaced strand. The size of the duplex segment(s) was measured in 40 expanded-D molecules, selected at random, from control liver and in 50 such molecules from senescent liver. Fig. 1 illustrates the extent of duplex synthesis in these molecules as a function of expansion. The pattern is similar in the two groups. About 50% of the expanded-D molecules have undergone at least some degree of duplex synthesis, and about 3% (in the combined total for the two groups) were totally double-stranded Cairns' forms. Although duplex synthesis can be initiated at any point during expansion, its frequency increases considerably in replicating molecules that have expanded through about 30% of the genome. About 1/5 of the expansions undergoing duplex synthesis had more than one duplex segment, indicating multiple initiations. Although no detailed studies were made, the larger replicative forms observed in the mtDNA from brain, heart, and kidney showed a pattern of duplex synthesis similar to that of liver mtDNA.

DISCUSSION

The experimental animals used in this study appeared to be healthy and the organs used for the isolation of mtDNA were free of malignant growth or other visible pathological signs. Senescent mice at the age of 50% longevity in this colony represent a healthy subpopulation by a number of criteria [13]. Therefore, any changes observed in the properties of mtDNA between the two

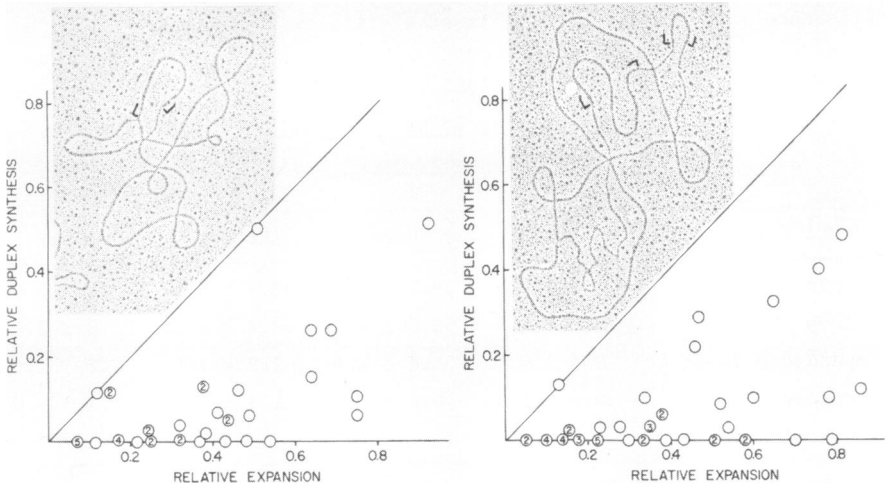

FIGURE 1 Relative duplex synthesis in expanded-D molecules of liver mtDNA from adult mice (left) and from senescent mice (right). The numbers within the circles indicate the number of overlapping data points. The diagonal line gives the position for fully double-stranded expansions. The units are in genome size. Inserts: electron micrographs of liver mtDNA with expansions of 0.15 (left) and 0.7 (right) genome size; duplex regions are indicated by brackets.

age groups can be reasonably attributed to "normal" aging processes rather than to age-related pathological conditions. It is noteworthy that the brain and endocrine glands of senescent C57BL/6J male mice were found free of detectable malignancy in over 300 necropsies [11].

The measurements of contour lengths have failed to reveal any significant variation in the size of mtDNA either between different organs or as a result of donor age. Specific cleavage analysis of mammalian mtDNA with restriction endonucleases also suggests that the mtDNA in different organs of the same individual is highly homogeneous, although a small degree of microheterogeneity may be present [34,35].

The frequencies of catenated mtDNA forms in this study are well within the range generally observed in normal animal tissues [6]. More than 90% of the catenanes observed were dimers and most of the remainder were trimers; no catenane larger than pentamer size was encountered. There is relatively little change in the proportion of catenanes in senescent tissues as compared with control tissues; the biggest change, an increase in the frequency of catenanes from 5.4 to 7.8%, was observed in senescent brain. The factors regulating the frequency of catenated forms of mtDNA are poorly understood,

but studies in HeLa and L cells suggest that both linking and unlinking of molecules occur at a rapid rate resulting in a dynamic equilibrium between monomers and catenanes [3, 16]. The proportion of catenanes tends to be higher in tumors, established cell lines, and transformed cells, but may also depend on the growth conditions of cells [6, 29, 30]. Among normal cells, mature oocytes of sea urchins and mice were found to contain a relatively high percentage (about 25 weight-%) of catenated forms [32, 33]. There is no reason to suspect that the catenated state per se would interfere with the normal genetic function of mtDNA.

The very low frequency (about 0.05%) of circular dimers in the mtDNA from adult mice in this study is in agreement with the reported scarcity and apparent absence of this form in normal tissues of mice and other species [6, 7, 26]. An earlier report of a high content of circular dimers in mtDNA from apparently normal human and beef thyroids [31] was not substantiated by a recent comparative study of thyroid mtDNA [26]. Increased frequencies of circular dimers, ranging from a few percent to up to 50%, have been observed in several human malignancies: granulocytic leukemia [8, 9], oncocytomas of the salivary glands [30], and a few solid tumors [38]. Circular dimers constitute several percent of the mtDNA in mouse L cells, but the frequency of these forms could be increased significantly by exposure of the cultures to adverse growth conditions and metabolic inhibitors [27, 28, 29]. A subline of L cells containing 100% circular dimers of mtDNA has also been obtained [6, 22, 23]. The mechanism by which mtDNA monomers are converted to the circular dimer form has not been established, but a replication error may be involved by analogy to circular dimer formation in bacteriophage ϕX174 DNA [1], λdv plasmid DNA in recA$^-$ host [19], and SV40 viral DNA [17].

In view of the apparent association of circular dimers of mtDNA with pathological conditions and physiologically abnormal cells, the increased frequency of these forms in the organs of senescent mice is of interest and potentially of pathognostic significance. The values of circular dimer frequency for the four tissues may be considered as minimal estimates since structurally abnormal mitochondria, which may conceivably contain an increased incidence of aberrant mtDNA, could have been preferentially lost during cell fractionation [40]; however, DNase treatment of the mitochondrial pellet in the present study did not significantly affect the frequency of circular dimers and other complex forms. These results suggest that the age of the donor needs to be considered as an additional factor which may affect the content of circular dimers in the mtDNA isolated from animal and human tissues. For

example, in previous studies, the elevated content of circular dimers (about 1%) observed in thyroid mtDNA of older cows [26] and mtDNA of cow heart [6] may well have been related to donor age.

The highest incidence of circular dimers, about 2%, was observed in mtDNA from senescent brain; it remains to be determined whether certain cell types or regions of the brain are involved preferentially. Age-related degenerative changes in mitochondrial structure (for example, in the cristae) and defects in the respiratory function of the mitochondria have been noted in a number of tissues, for example, mouse brain [21], flight muscle from aging blowfly [37,41], rat skeletal muscle [5], and mouse liver [40]. The increased frequency of circular dimers of mtDNA, as observed in the present study, may well be related to some of these aberrations in mitochondrial structure and function. The mitochondrial changes themselves may be secondary to age-related disturbances in cellular metabolism due to intrinsic and extrinsic factors [12]. Nevertheless, if defects in the mitochondrial genetic system develop, this may have serious consequences in non-dividing cells such as nerve cells. If the mutant mitochondria have a selective advantage, they can replace the normal mitochondrial population within the cell, causing permanent functional damage or cell death.

The replicative forms of mtDNA observed in the four tissues in the present study can be arranged in a sequence similar to that found in mouse L cells [2,3,22,23,36] and in rat liver and rat hepatoma cells [42]. A minor difference from the L-cell model is that the degree of asymmetry in the replication of the two strands is smaller in mouse liver mtDNA and probably also in the other tissues examined. The frequency of D-loop DNA was 30 to 60% and varied significantly according to tissue. Significant tissue-dependent differences were also found in the frequencies of larger replicative forms, ranging from 1 to 10%.

The presence of larger replicating intermediates in the four organs indicates that the mtDNA is indeed turning over in these tissues, as has been shown also by radioisotope labeling studies of mtDNA in the corresponding organs of the rat [18]. The apparent half-lives of mtDNA in the rat organs were: 6.7 days in heart, 9.4 days in liver, 10.4 days in kidney, and about 31 days in brain [18]. Multiplying these values with the fraction of larger replicative forms observed in the present study, one can estimate the average time needed for the replication of an mtDNA molecule: approximately 3 h in heart, 7 h in brain, 10 h in kidney, and 20 h in liver. These values are subject to change, since the actual turnover rates of mtDNA in mouse tissues may be different. Nevertheless, these results suggest that significant

tissue-dependent differences exist in the rate of replication of mtDNA. In comparison, the time required for the complete replication of an mtDNA molecule in mouse L cells in the logarithmic phase of growth has been estimated at about 2 h [2].

Neither the frequencies and types of the various replicative intermediates of mtDNA, nor the pattern of duplex synthesis appear to have been significantly affected by donor age. These observations suggest that there are no gross disturbances in the replication process of mtDNA as a result of aging and that the rates of synthesis and turnover of mtDNA remain relatively unchanged in senescent tissues. A much larger sample of replicative forms would need to be examined to detect possible minor variations in the pattern of replication of mtDNA.

ACKNOWLEDGMENTS

We thank Phyllis Hotchkin and Roxanne Meyer for excellent technical assistance. We are indebted to Dr. Caleb E. Finch for providing us with the mice used in this study. The interest and advice of the late Professor Jerome Vinograd is gratefully acknowledged. One of us (L.M.) was supported in part by National Institutes of Health postdoctoral fellowship No. CA53287 from the National Cancer Institute and in part by research funds from the Clinical and Research Center on Aging, VA Hospital, Sepulveda, CA.

REFERENCES

1. Benbow, R.M., Eisenberg, M. and Sinsheimer, R.L. (1972) Nature New Biol. 237,141-144
2 Berk, A.J. and Clayton, D.A. (1974) J. Mol. Biol. 86,801-824
3 Berk, A.J. and Clayton, D.A. (1976) J. Mol. Biol. 100,85-102
4 Borst, P. (1972) Annu. Rev. Biochem. 41,333-376
5 Chen, J.C., Warshaw, J.B. and Sanadi, D.R. (1972) J. Cell. Physiol. 80,141-148
6 Clayton, D.A. and Smith, C.A. (1975) Int. Rev. Exp. Pathol. 14,1-67
7 Clayton, D.A., Smith, C.A., Jordan, J.M., Teplitz, M. and Vinograd, J. (1968) Nature (London) 220,976-979
8 Clayton, D.A. and Vinograd, J. (1967) Nature (London) 216,652-657
9 Clayton, D.A. and Vinograd, J. (1969) Proc. Natl. Acad. Sci. U.S.A. 62,1077-1084
10 Davis, R.W., Simon, M. and Davidson, N. (1971) in Methods in Enzymology. Vol. 21, pp. 413-428. Academic Press, New York
11 Finch, C.E. (1973) Brain Res. 52,261-276
12 Finch, C.E. (1976) Quart. Rev. Biol. 51,49-83
13 Finch, C.E. and Foster, J.R. (1973) Lab. Anim. Sci. 23,339-349
14 Finch, C.E., Foster, J.R. and Mirsky, A.E. (1969) J. Gen. Physiol. 54,690-712
15 Finch, C.E. and Girgis, F.G. (1974) J. Gerontol. 29,134-138
16 Flory, P.J., Jr. and Vinograd, J. (1973) J. Mol. Biol. 74,81-94
17 Goff, S.P. and Berg, P. (1975) in Tumor Virus Meeting on SV40, Polyoma and Adenoviruses. Abstracts of Papers, p. 42. Cold Spring Harbor

18 Gross, N.J., Getz, G.S. and Rabinowitz, M. (1969) J. Biol. Chem. 244,1552-1562
19 Hobom, G. and Hogness, D.S. (1974) J. Mol. Biol. 88,65-87
20 Hudson, B. and Vinograd, J. (1967) Nature (London) 216,647-652
21 Johnson, J.E., Jr., Mehler, W.R. and Miquel, J. (1975) J. Gerontol. 30,395-411
22 Kasamatsu, H., Robberson, D.L. and Vinograd, J. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 2252-2257
23 Kasamatsu, H. and Vinograd, J. (1974) Annu. Rev. Biochem. 43,695-719
24 Kim, J.-S., Sharp, P.A. and Davidson, N. (1972) Proc. Natl. Acad. Sci. U.S.A. 69,1948-1952
25 Matsumoto, L., Kasamatsu, H., Pikó, L. and Vinograd, J. (1974) J. Cell Biol. 63, 146-159
26 Matsumoto, L., Pikó, L. and Vinograd, J. (1976) Biochim. Biophys. Acta 432,257-266
27 Nass, M.M.K. (1969) Nature (London) 223,1124-1129
28 Nass, M.M.K. (1970) Proc. Natl. Acad. Sci. U.S.A. 67,1926-1933
29 Nass, M.M.K. (1974) in Hormones and Cancer. K.W. McKerns, ed. pp.261-307. Academic Press, New York
30 Paoletti, C. and Riou, G. (1973) in Progress in Molecular and Subcellular Biology. Vol. 3, pp. 202-248. Springer-Verlag, New York
31 Paoletti, C., Riou, G. and Pairault, J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69,847-850.
32 Pikó, L., Blair, D.G., Tyler, A. and Vinograd, J. (1968) Proc. Natl. Acad. Sci. U.S.A. 59,838-845
33 Pikó, L. and Matsumoto, L. (1976) Develop. Biol. 49,1-10
34 Potter, S.S., Newbold, J.E., Hutchinson, C.A., III and Edgell, M.H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72,4496-4500
35 Robberson, D.L., Clayton, D.A. and Morrow, J.F. (1974) Proc. Natl. Acad. Sci. U.S.A. 71,4447-4451
36 Robberson, D.L., Kasamatsu, H. and Vinograd, J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69,737-741
37 Sacktor, B. and Shimada, Y. (1972) J. Cell Biol. 52,465-477
38 Smith, C.A. and Vinograd, J. (1973) Cancer Res. 33,1065-1070
39 Snedecor, G.W. and Cochran, W.G. (1967) Statistical methods. 6th edn. The Iowa State University Press, Ames, Iowa
40 Wilson, P.D. and Franks, L.M. (1975) Biochem. Soc. Trans. 3,126-128
41 Wohlrab, H. (1976) J. Gerontol. 31,257-263
42 Wolstenholme, D.R., Koike, K. and Cochran-Fouts, P. (1973) Cold Spring Harbor Symp. Quant. Biol. 38,267-280
43 Zuccarelli, A.J., Benbow, R.M. and Sinsheimer, R.L. (1972) Proc. Natl. Acad. Sci. U.S.A. 69,1905-1910