

A Simple Method for Measurement of Mitochondrial DNA Content in Total Cell DNA Using Non-Radioactive DNA Probes

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非放射性 DNA 標識を用いた細胞 DNA 中のミトコンドリア DNA の簡便な測定法

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Abstract: Quantitative measurement of mitochondrial DNA (mtDNA) has been very troublesome, because its content in total DNA was very low and quantitative separation of the mtDNA from nuclear DNA (nucDNA) was almost impossible. By using the technique for cloning cells carrying the mtDNA sequences, specific detection of mtDNA became possible in the presence of large excess of nucDNA by DNA-DNA hybridization. For this purpose, we prepared mtDNA probes labeled with a non-radioactive substrate, which made the procedure easier and increased possibility of application. This method revealed that the contents of mtDNA in rat liver strikingly decreased with aging.

Key words: mtDNA, Biotinylated DNA Probe, Aging, Rat Liver

INTRODUCTION

It is well known that mitochondria have own DNA and the system for their replication and expression^{1,2)}. The contents of mtDNA in cells have been reported to vary in wide range depending on the types of cells and the conditions of the cell³⁾. But determination of the amount of mtDNA was difficult, because mtDNA were only very minor fraction of total cell DNA in

almost all cases. For example, in rat liver mtDNA was only 0.5 % of the total DNA³⁾. As *Drosophila* mtDNA have buoyant density of 1.68 g/cm³ as compared to 1.69 g/cm³ of nucDNA in CsCl, Massie *et al.* analyzed mtDNA by buoyant density gradient equilibrium centrifugation of whole lysate of cells to study the age dependent changes in the amount of mtDNA^{4,5)}. But in mammals in which the buoyant density of mtDNA is undistinguishable with that of nucDNA^{3,6)}, it was impossible to use this method for our purpose of detecting the mtDNA. Thus, for determining mtDNA in mammalian cells, we not only have to isolate the mitochondria as quantitative as possible but also have to eliminate the contamination of

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nucDNA completely. We have been studying the age dependent qualitative changes of DNA for several years using rat liver to clarify the possible participation of mtDNA on aging^{7,8)}, however, quantitative results on the mtDNA have not yet been obtained. This defect mainly is due to the difficulty in satisfying two contradictory targets at the same time, *i. e.* to gain the complete recovery of mitochondria with various sizes and densities, and to obtain mtDNA preparation completely separated from nucDNA. In this paper, we will report on experiments aimed to resolve the problem described above using a technique of gene engineering. Namely, by preparing mtDNA probes and determining the amount of hybridized probes to total DNA fraction, it should be possible to quantify the amount of mtDNA. As several investigators have reported the existence of highly homologous sequences in nucDNA with some mtDNA⁹⁾, it was necessary to remove DNA sequences with homology to nucDNA by splitting the specific region using restriction endonucleases and obtain the mtDNA specific sequences as the hybridization probes.

Another attempt for the quantification was to use probes with non-radioactive labels in order to make them much suitable for *in situ* hybridization that we are planning to do. Recently, the sensitivities for detecting the non-radioactive probes extensively increased¹⁰⁻¹²⁾, however, they were still about 1/10 of that of radio-active probes. Although it seemed very difficult to detect a single copy of gene in a cell with a non-radioactive probe, but fortunately, mtDNA existed in several hundred or more per cell (it was assumed that the molecular weight of mtDNA is $1/10^5$ of nucDNA and its

contents are 0.5 % of the total DNA). Consequently as reported below, it was possible to use the non-radioactive probes for mtDNA detection as we planned. By this method, we found that the changes of mtDNA contents is age dependent.

MATERIALS and METHODS

Materials. Livers from Donryu strain male rat were used. Livers from aged rat were kindly supplied from Professors Sato and Tauchi of Aichi Medical University. Restriction endonucleases and DNA molecular size marker were purchased from Nippon Gene (Toyama, Japan). *E. coli* strain HB101 for plasmid preparation and strain MV1184 for single-stranded DNA preparation were used. Plasmid pUC119, helper phage M13KO7 and DNA ligation kit were from Takara Shuzou (Kyoto, Japan). Competent cells for transformation by plasmids were prepared by the method described by Maniatis et al¹⁶⁾. For nucleotides sequence determination, α^{35} -S labeled dATP and the sequencing kit of United States Biochemicals (Cleveland, Ohio) were used. For non-radioactive DNA labeling and its detection, BioNick Labeling System and BluGene from Bethesda Research Laboratories (Gaithersburg, MD) were used. Nitrocellulose sheets (BA85) were obtained from Schleicher & Schuell (Dassel, West-Germany), and Seakem agarose (ME) from FMC Marine Colloids (Rockland, Maine) was used. All other laboratory chemicals were of the highest purity available from major commercial suppliers.

Preparation of mitochondria. The mitochondria were prepared from liver homogenates by using the differential centrifuga-

tion method as described in previous papers^{8,13,14}. To complete the separation from nucDNA, crude mitochondrial suspension was purified by centrifugation in sucrose layers of 1.15 g/cm³ density sucrose layered on 1.2 g/cm³ density sucrose through which only the nuclei may be sedimented. To remove the contaminated nucDNA, resulted mitochondrial preparations were digested by DNase I, and repeatedly washed by sedimentation.

mtDNA purification. mtDNA was extracted from purified mitochondrial suspension by SDS-phenol method as described previously^{7,15} with a slight change, that is 1 M ammonium chloride containing buffer for the Sepharose CL-4B chromatography in place of 0.15 M NaCl to increase the recovery of mtDNA.

Total DNA preparation from tissue homogenates. DNA was extracted from liver homogenate by the modified method of Maniatis et al¹⁶. 1 ml of 10–15 % homogenate (10 mM Tris-HCl buffer, pH 7.6, containing 10 mM EDTA and 0.25 M sucrose) was adjusted to the final concentrations of 0.5 % SDS and 50 mM EDTA, then 40 µg of proteinase K was added and then incubated at 60°C. After every 1 h of incubation, 40 µg proteinase K was freshly added and the incubation was continued. Finally, the incubation was completed after total 3 hours with total of 120 µg/ml proteinase K at the final stage. After digestion by the proteinase, remaining proteins were denatured with phenol by mild shaking for 20 min which procedure was repeated twice and followed by phenol/chloroform (1 : 1 mixture) with 15 min shaking and finally by adding chloro-

form with 15 min shaking, respectively. Then DNA was precipitated with 2 volumes of cold ethanol. To eliminate RNA, the alcohol precipitates were taken into TE buffer (10 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA) incubated with 100 µg/ml RNase A for 1 hour at 37°C. The DNA was purified again by phenol extraction followed by ethanol precipitation.

Bacterial culture and plasmid preparation. *E. coli* HB101 and MV1184 were grown in Luria-Bertani medium and 2x YT medium, respectively. To pick up the cell carrying plasmids which were ligated with mtDNA fragments, agarose plate containing final concentration of 100 µg/ml ampicillin was used. The inserted fragments were checked by digesting plasmid DNA with restriction enzyme. Preparation of plasmids was carried out by alkaline lysis method described elsewhere¹⁶.

Restriction endonuclease digestion. The enzyme was added to the reaction mixture containing DNA and 1/10 volume of the 10x buffer supplied for each corresponding restriction endonuclease by Nippon gene, and incubated at 37°C for over night. The reaction was stopped by adding 1/5 volume of gel-loading buffer containing 25 mM EDTA and 30 % glycerol, and the mixture was heated to 60°C just before loading into agarose gel for electrophoresis. For preparation by electrophoresis, each band was cut out after staining the gel with Ethidium bromide (EtBr), and eluted electrophoretically with Unidirectional Electroelutor. Eluates were then treated with phenol and chloroform to eliminate the dye and agarose debris, and then precipitated the fragments

with 2 volumes of cold ethanol at -20°C .

Cloning the restriction endonuclease fragment of mtDNA. Each fragment obtained by *Hind*III digestion (Fig. 1) was ligated to the corresponding site of plasmid pUC119, and *E. coli* competent cell strain HB101 was transformed by these recombinant plasmids. We digested the purified plasmid DNA with *Hind*III after isolating the plasmid from the cloned bacterial cells, and each clone was tentatively identified from the length of the inserts. Further identification of the inserts was done by partial sequencing as described below. Plasmid was named as pHinB when the *Hind*III fragment B of mtDNA was inserted into the pUC119 and so on. Two kinds of clones that were inserted with the HinB fragment in different directions were obtained. Direction of the inserted fragment was determined by *Eco*RI digestion. After digesting these plasmids with *Eco*RI, followed by electrophoretical separation and ligation, plasmids containing different segment of *Hind*III B were isolated, and named as pHinBEcoC and pHinBEcoD, respectively.

Preparation of DNA fragments for probes. We prepared the fragment for the templates of nick translation as follows: HinE was prepared from pHinE by *Hind*III digestion, the fragments HinBEcoC and HinBEcoD were isolated by splitting the corresponding plasmids with *Hind*III and *Eco*RI. Fragment HinBEcoDSacII was obtained by digesting the pHinBEcoD with *Eco*RI and *Sac*II. These fragments were isolated from the bands of agarose electrophoresis using electrophoretical elution.

Non-radioactive labeling of DNA fragments. We obtained biotinylated DNA fragment by nick translation¹⁷⁾ with biotin-14-dATP from fragments (HinBEcoC, HinBEcoDSacII and HinE) of about 1 kbp in length as templates. The labeling condition was selected so as to obtain rather short polynucleotides that is fitted for *in situ* experiments which we were planning to do. Biotinylated DNA was purified by repeated alcohol precipitation and the size of the labeled probes were checked by electrophoresis after heat denaturation and detection with streptoavidin-conjugated alkaline phosphatase system, BluGene.

Southern blot hybridization. Appropriate amount of DNA digested with *Eco*RI was loaded into 1 % agarose gel and fragments were separated by electrophoresis. After staining with EtBr, the gel was soaked in 0.5 N NaOH containing 1.5 M NaCl, and then neutralized with 0.5 M Tris-HCl, pH 7.5, containing 3 M NaCl. The fragments were transferred to nitrocellulose filter by capillary transferring¹⁶⁾. After baking the filter, prehybridization was carried out in a mixture containing 50 % deionized formamide, 5x Denhardt's solution (1 mg/ml each of Ficoll, polyvinylpyrrolidone and bovine serum albumin, respectively), 5x SSPE (0.9 M NaCl, 0.05 M NaH_2PO_4 and 5 mM EDTA, pH 7.4), 0.1 % SDS and 100 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA, at 42°C for 3 hours. Hybridization was carried out at 42°C over night in mixture consisting of 10-25 ng/ml of probes in prehybridization medium.

Detection of biotinylated probes. Post-hybridization wash was started by washing

twice with 2x SSC (0.015 M sodium citrate and 0.15 M NaCl) containing 0.1 % SDS for 3 min, followed by 0.5x SSC containing 0.1 % SDS for 3 min twice, then the same buffer at 50°C for 15 min, and finally with 0.5x SSC for 3 min. The washed filter was blocked by 3 % bovine serum albumin in 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl at 65°C and then incubated in 1.0 µg/ml streptavidin-alkaline phosphatase conjugate solution for 10 min. After washing the filter twice with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl for 15 min respectively, visualization of the probe was attained by adding nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline buffer (0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl and 0.05 M MgCl₂)¹⁸.

RESULTS AND DISCUSSION

Cloning of mitochondrial DNA fragments. *Hind*III cleaves the mtDNA of Donryu rat into 6 fragments, A to F (6.5, 4.2, 2.5, 2.0, 0.8 and 0.1 kb, respectively) as shown in Fig. 1 (a)¹⁹. However, we recovered only 5 of them, A to E, by electrophoretic preparation, as the shortest fragment cannot be isolated under the condition we employed. Plasmids prepared by inserting these fragments into pUC119 at *Hind*III site were used for transforming *E. coli*. We succeeded in isolating clones from fragment B, C, D and E, but could not obtain clones containing *Hind*III A fragment. As the *Hind*III B fragment has 4 *Eco*RI sites, we specified the direction of fragment in the plasmids by digesting the plasmid DNA with *Eco*RI and measuring the size of produced fragments electrophoretically. Thus, 2 clones were con-

firmed to have *Hind*III B fragment inserted in reverse directions. Plasmid fragments containing either end of *Hind*III B fragments after *Eco*RI digestion were isolated and ligated again. We named these plasmids as pHinBEcoC and pHinBEcoD that include a part of *Eco*RI C fragment and a large part of *Eco*RI D fragment, respectively. The nucleotide sequence of the inserted region of pHinBEcoD was determined as follows: Single-stranded phagimide DNA of the inserted region was sequenced by dideoxynucleotide chain termination technique²⁰ and confirmed to be identical to the sequence located at the expected site of the mtDNA²¹⁻²³.

Homology between mtDNA and genomic DNA. In order to eliminate the regions homologous to nucDNA from the DNA fragment we obtained, we searched by a computer program²⁴ using a DNA data base, GenBank. We searched for the homologous regions to the sequences of three DNA fragments we obtained, *i. e.* HinBEcoC, HinBEcoD and HinE in the rodent genomic DNA library. A part of the HinBEcoD (shaded part of the fragment in Fig. 1 (b)) located in the gene of mitochondrial ribosomal RNA was found to be homologous (more than 60 % in 185 nucleotides) to the nuclear 28S rRNA gene. As ribosomal RNA genes are well known to possess highly repeated sequence, we needed to eliminate that region to avoid the cross-reactivity. Therefore, we searched for a restriction endonuclease site to eliminate the homologous region and to obtain a shorter fragment as a probe. *Avr*II, *Bbv*II, *Eco*52I, *Sac*II and *Stu*I were found to split this. As shown in Fig. 1 (b), *Sac*II was

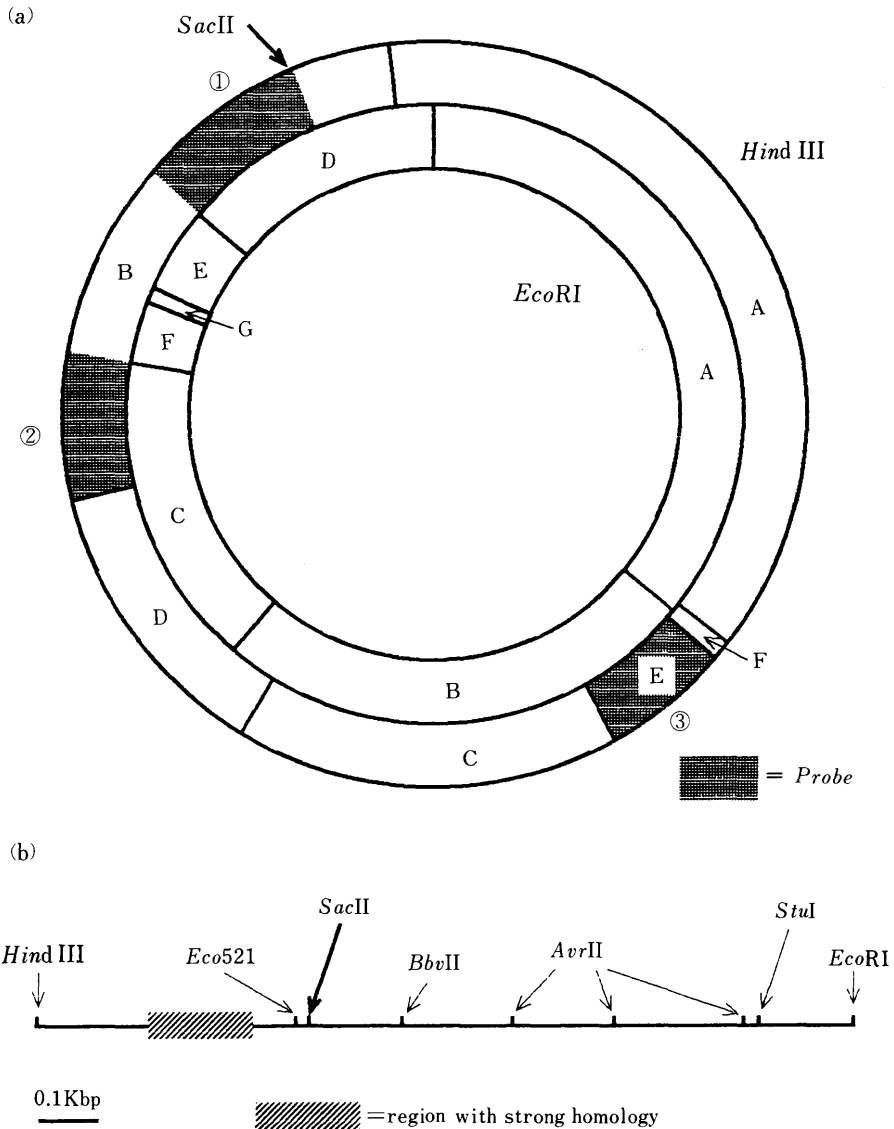


Fig. 1. Restriction map of rat mitochondrial DNA

(a) *Hind*III and *Eco*RI map of mtDNA, 1: HinBEcoDSacII 2: HinBEcoC 3: HinE.

(b) Restriction map of HinBEcoD fragment. Note that top side of the fragment in the circular map (a) is shown at the left side of the linear map shown in (b).

selected for this purpose, to split the HinBEcoD at the position of the arrow as shown in Fig. 1 and produced 1.0 kbp fragment from the *Eco*RI site (HinBEcoDSacII). For our purpose it is preferable to have several fragments that are located on

mtDNA circular genetic map at appropriate distances. Thus, we selected the following fragments: HinBEcoDSacII, HinBEcoC and HinE (cross-hatched regions in Fig. 1 (a)) with sizes of 1.0, 0.8. and 0.8 kbp, respectively. These fragments we obtained as the

mtDNA probe were found to show very low homology with nucDNA, if any, thus, we considered the cross-reactivity as negligible.

Labeling of DNA fragment. We have previously reported that the larger sized mitochondria were observed in aged rat hepatic cells by electron microscopy comparing with those of the young animals, and the number of mitochondria per cell concomitantly decreased^{25, 26}. As the amount of mtDNA measured by the method described in this paper was consequently the sum of these heterogeneously sized mitochondria, it is necessary to know the contents of mtDNA in each heterogeneously sized mitochondrion to clarify the relationship between mtDNA and appearance of these large sized mitochondria upon aging of cells of the liver. We planned to employ the *in situ* hybridization for this purpose. So, we needed to prepare the probes also useful for such experiments, *i.e.* the length of probes limited to about 100 bases. To obtain the best condition for this purpose using a nick translation kit, average length of single-stranded labeled polynucleotides after the reaction was checked by agarose gel electrophoresis as shown in Fig. 2 (a). Three probes thus obtained are expected to hybridize with different fragments of mtDNA digested by *EcoRI*. Namely, HinE is supposed to hybridize with *EcoRI* B, HinBEcoC with *EcoRI* C and HinBEcoDSacII with *EcoRI* D fragment, respectively. These were confirmed by Southern blot hybridization of purified mtDNA digested with *EcoRI*. As shown in Fig. 2 (b) and (c), each probe is hybridized with only the *EcoRI* fragment corresponding to

the probes described above.

Changes of mtDNA contents during aging.

To study the changes in amount of mtDNA during aging, total DNA of rat liver homogenate was extracted from rats of various ages, 8 weeks, 14.5, 20 and 30 months old. The concentration of DNA was determined by measuring UV absorption at 260 nm using absorbance of 1 mg/ml solution as 20.

The DNA preparations were digested with *EcoRI*, and after electrophoretical separation in agarose gel, single-stranded fragments were transferred to nitrocellulose membrane, and hybridization was carried out by using three kinds of probes mixed altogether. Each DNA prepared from various aged rats was loaded to the gel at three different amounts in a ratio of 1, 1/2 and 1/4. In Fig. 3 (a) showed the EtBr stained patterns of the gel and (b) depicts the patterns of BlueGene staining to detect the hybridized bands. It is clear from these results that it is possible to detect only three bands in lanes of (b). DNA prepared from 14.5 and 20 month old rat liver contained less than half of young rat's mtDNA. Content of mtDNA 30 months old rat liver decreased further almost to 1/6 of that in total DNA from young rat, with consideration of the fact that more than twice amount of DNA is loaded on agarose gel of 30 month old DNA as shown in the figure legend.

It is previously reported that some sites for *EcoRI* splitting acquired resistancy at the standard concentration of the enzyme when used against mtDNA from old rat liver⁸. *EcoRI* digestion was complete, however, in every mtDNA in all of total DNA prepara-

tions when enough amount of the enzyme and duration of incubation were employed. In case of incomplete digestion, two or three extra bands consisting of connected adjacent bands should also be hybridized with each probe, but this was not the case in this study.

The results of Southern blot analysis of

total DNA digested by *EcoRI* also answered the other interesting problem. Although we have eliminated the region homologous to nucDNA, the expected sequences were limited to those already registered in the data base, GenBank, and these constitute only a small fraction of the entire genome of rats. Therefore, if un-

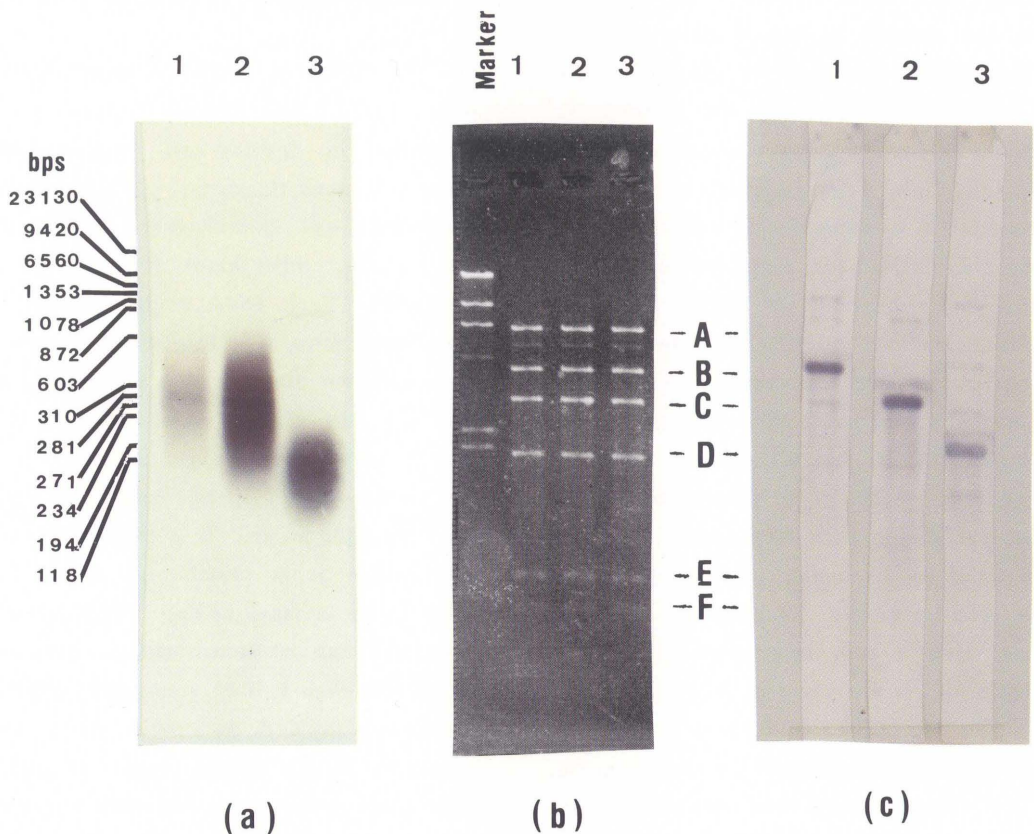


Fig. 2. Biotinylated mtDNA probes

- (a) Size of mtDNA probes labeled with biotin-14-dATP. The solution of purified labeled fragments were heated for 5 min in a boiling-water bath and then chilled quickly in ice, followed by separating electrophoretically in 2 % agarose gel. Lane 1: HinE as template, lane 2: HinBEcoC as template, and lane 3: HinBEcoDSacII as template.
- (b) Patterns of mtDNA digested by *EcoRI*, in each lane: 100 ng of mtDNA which was digested with 12 units of *EcoRI* in a 10 μ l of reaction mixture at 37°C for 2 hours.
- (c) Southern hybridization of *EcoRI* digest of mtDNA shown in (b) with each biotinylate probe followed by detection with BluGene. lane 1: HinE, lane 2: HinBEcoC, and lane 3: HinBEcoDSacII as probes, respectively. The condition of hybridization and detection are described in METHODS.

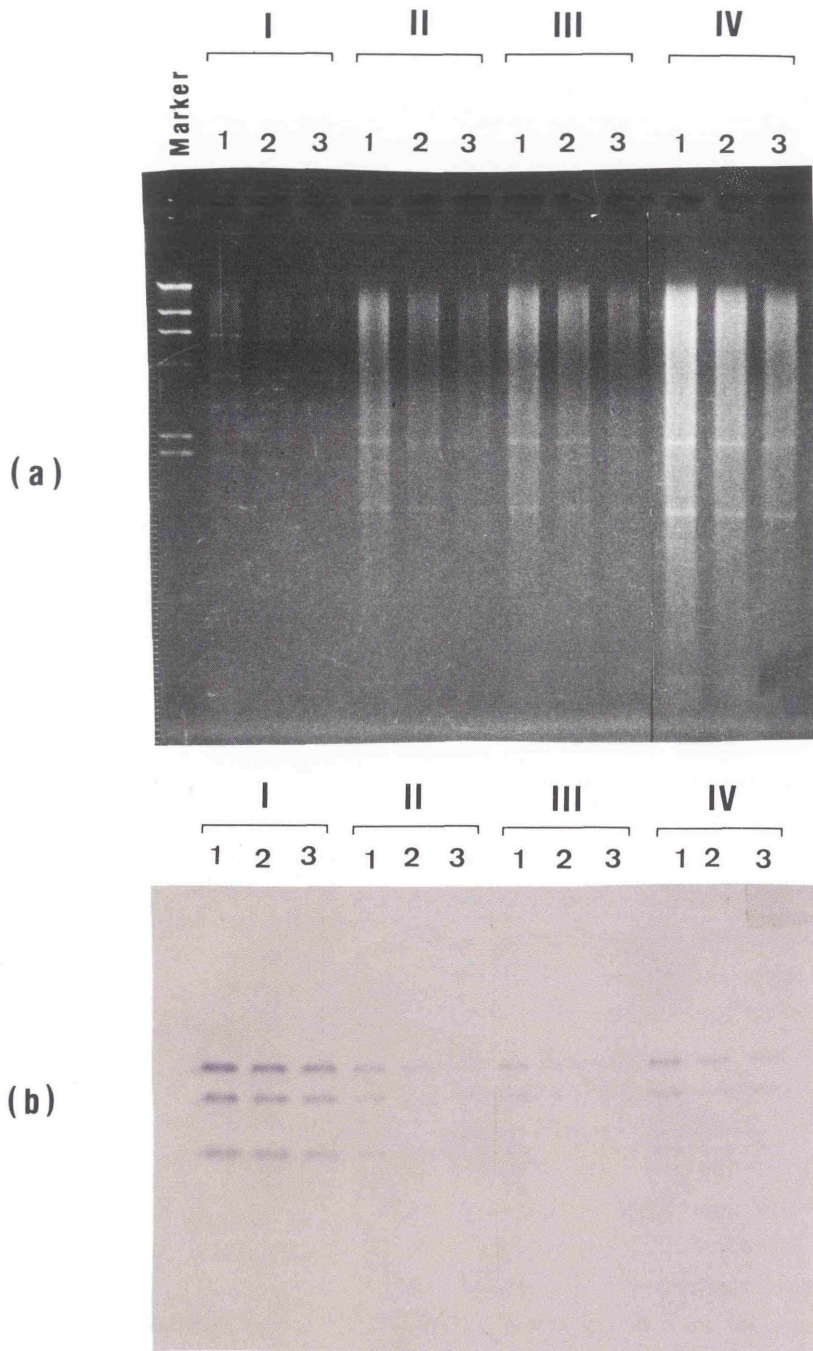


Fig. 3. Change in the amount of mtDNA upon aging
 (a) The electrophoretic patterns of the EtBr stained total DNA digested by *EcoRI*. I: DNA from young rats, 460 ng, II: DNA from 14.5 months old rats, 325 ng, III: DNA from 20 months old rats, 343 ng, IV: DNA from 30 months old rats, 950 ng, were applied on each lane 1. Lanes 2 were loaded with 1/2 amount of lane 1, and lanes 3 were applied with 1/2 amount of lane 2.
 (b) Southern hybridization pattern of the filter transferred from agarose gel shown in (a) with the mixture of three mtDNA probes. Hybridization and detection conditions are described in METHODS.

known homologous region to our mtDNA probes exist in the nucDNA as a repeated sequence, several band(s) other than those from circular mtDNAs (4.2, 2.2 and 2.0 kbp) should inevitably appear. The experiments depicted in the Fig. 3 (b) show that this is not the case, in other words, only three bands were detectable in each lane.

Moreover, it was clear that the amount of probes hybridized with the total DNA decreased during aging, and the intensities of the color of these bands were reasonable as though they are present in the same molar ratios. To check whether the DNA fragments digested with *EcoRI* completed their transfer onto the nitrocellulose membrane, the gels were stained with EtBr after overnight transfer, and examined by UV illumination. Thus, we concluded that in our experiments, *EcoRI* digestion and the transfer of the fragments to the membrane were completed, the detected sequences hybridized with our probes were all from mtDNA, and the ratio of whole circular mtDNA to total DNA decreased during aging. Furthermore, it is suggested from the results that in old rat liver, the decrease of mtDNA contents determined with three probes were almost in parallel with each other. Thus, it is not likely at least to the sites detected with the probes that a part of the mtDNA was deleted, or some part of the mtDNA hybridization was inhibited due to the changes in sequence or to coupling with inhibitory substances during aging.

In aged rat hepatic cells, it was morphologically observed that the numbers of mitochondria decreased with concomitant appearance of larger size mitochondria^{25, 26)}. Our result on the decrease of mtDNA seemed to the reflection of the attenuation

in mitochondria when the number of mtDNA per mitochondrion is considered to be unchanged. For the conclusion on the distribution of mtDNA in mitochondria of aged rat hepatic cells, we must wait for the results of *in situ* hybridization using an electron microscopy now in progress.

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