Changes in Mitochondrial DNA in Cardiac Hypertrophy in the Rat

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SUMMARY We studied DNA (mtDNA) replication in adult female rat hearts undergoing hypertrophy secondary to constriction of the ascending aorta. MtDNA was measured in isolated mitochondria by a fluorometric method adapted for that purpose. The conditions for removal of contaminating nuclear DNA were developed, and the purity of the mtDNA was assessed from its molecular conformation (open and closed circles) and renaturation-kinetic analysis. The mtDNA concentration in mitochondria, expressed as micrograms of DNA per milligram of mitochondrial protein, increased 2, 4, and 7 days postoperatively by 21, 73, and 98%, respectively. Similar results were obtained when mtDNA was expressed per nanomole of cytochrome c. The population of replicative intermediates of mtDNA was analyzed by electron microscopy. In normal hearts, we observed molecular forms characteristic of animal mtDNA, such as circular monomers and dimers, catenated molecules, D-loops, expanded D-loops, and gapped molecules. D-loop frequency, which was near 50% in the mtDNA of control hearts, was markedly reduced to 5-7% in hypertrophying hearts. This result indicates that the increase in replicative flux of mtDNA is associated with the removal of a block in the conversion of D-loops to other intermediates. Circ. Res. 45:505-515, 1979

IT is now well established that increased tension in the ventricular wall is correlated with increased metabolic activity of the heart. It is also known that the energy requirement of the myocardium is met completely by aerobic metabolism during both rest and strenuous activity (Neely and Morgan, 1974). This dependence of the myocardium on oxidative processes is reflected by the high content of mitochondria, which occupy 35% of the cardiac cell volume (Page et al., 1972).

The increased requirement for ATP during acute work overload is met effectively by the respiratory control mechanism: the accumulating ADP stimulates oxidative phosphorylation. If the hemodynamic overload is sustained, however, the capacity of the existing contractile and energy-producing apparatus is exceeded, and a second adaptive process, cardiac growth, is activated. This adaptive growth is reflected in increased incorporation of labeled amino acids into cardiac subcellular fractions, including mitochondria, as measured both in vivo (Zak and Fischman, 1971) and in vitro (Shahab and Wollenberger, 1970). Quantitative electronmicroscopic analysis (Page et al., 1972) and measurements of mitochondrial cytochromes and respiratory enzymes (Albin et al., 1973) indicate that, in early hypertrophy, mitochondria accumulate in preference to other organelles. Thus, the study of processes leading to mitochondrial proliferation may provide clues about the still elusive feedback mechanism that couples hemodynamic load with biosynthetic pathways. In this respect, the report of Meerson and Pomoinitsky (1972) that the amount of mtDNA, expressed per unit of mitochondrial protein, is increased strikingly after aortic constriction in the rat is of great interest. The mtDNA concentration was found to increase as early as 24 hours after operation and peaked 6 days later at levels 5 times higher than control values. Two months after surgery, the DNA concentration in mitochondria returned to preoperative values.

The present study is aimed at evaluating Meerson's and Pomoinitsky's (1972) observation by means of a more rigorous technique for the isolation and quantification of mtDNA. Accurate determination of the mtDNA present per unit mass of mitochondria is important for an assessment of the coding capacity of mtDNA in relation to induced cardiac growth.

The quantification of mtDNA is difficult, technically, since mtDNA constitutes only 1-2% of the total cellular DNA. Thus, even minor contamination with nuclear DNA can lead to erroneous results. Mitochondria isolated by the rigorous procedures available thus far are grossly contaminated by nuclear fragments, as can be demonstrated by...
the presence of large quantities of linear DNA molecules and by renaturation-kinetics analysis. Nuclear DNA can be removed by treatment of the isolated mitochondrial fraction with DNase. If this procedure is to be adopted as a quantitative assay of mtDNA, however, it must be demonstrated that the mitochondria have intact membranes capable of effective exclusion of DNase, whereas the nuclear DNA, associated with contaminating nuclear fragments, is freely accessible to the action of DNase. We have, therefore, carried out a systematic analysis of DNase treatment of cardiac mitochondria to verify that destruction of nuclear DNA occurs exclusively. For this purpose, we analyzed the molecular forms of the remaining DNA by agarose gel electrophoresis and by renaturation-kinetic analysis. Using the procedure for mtDNA quantification validated in this manner, we showed a 2-fold increase in cardiac mtDNA content following constriction of the ascending aorta.

The increased rate of mtDNA synthesis obtained in hypertrophying hearts allowed us to analyze changes in mtDNA replicative intermediates in a terminally differentiated nondividing cell population. Previous studies correlating molecular forms of mtDNA with growth have used rapidly dividing tissue culture cells (Kasamatsu et al., 1973) or proliferating regenerating liver tissue (Wolstenholme et al., 1973a, 1973b). We observed marked changes in the frequency of mtDNA replicative intermediates isolated from normal and hypertrophied hearts. Of particular note is the reduction in the frequency of D-loop forms from 50% in normal hearts to 5-7% in hearts undergoing hypertrophy. It is concluded that a block in D-loop conversion to expanded D-loop forms is removed when mtDNA replication is stimulated in cardiac hypertrophy.

Methods

Animals

Mature female Sprague-Dawley rats (220–230 g), fed ad libitum, were used for all experiments. Hypertrophy was produced by constriction of the ascending aorta to approximately 30% of its original lumen diameter, as described previously (Albin et al., 1973), by means of a hemostatic clip (Weck Co., Catalog no. 523135) instead of a silver clip.

Isolation of Mitochondria

Rats were killed by a blow on the head, and the hearts were excised and placed in ice-cold relaxing buffer (Zak et al., 1972). Hearts pooled from three rats were minced and homogenized in 0.3 M sucrose containing 2 mM Tris-HCl buffer (pH 7.4), 2 mM EDTA, and 1 mM ethyleneglycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), in a blade-type homogenizer operated at 14,000 rpm for 45 seconds. The resulting homogenate was centrifuged in a Sorvall RC2B refrigerated centrifuge for 10 minutes at 800 g. The supernatant was removed with a propipette and saved. The pellet was resuspended in the same buffer in a Potter-Elvejhem homogenizer and centrifuged as described above. The supernatant was combined with the previous one and centrifuged twice for 10 minutes at 800 g for removal of nuclear contamination. After each centrifugation, the supernatant was syphoned off, leaving a small amount at the bottom of the tube. Mitochondria were isolated from the final supernatant by a 20-minute centrifugation at 8700 g and washed by five cycles of resuspension in 0.3 M sucrose, 2 mM Tris-HCl buffer (pH 7.4), 10 mM EDTA, and 2 mM EGTA and pelleting by 10-minute centrifugation at 8700 g.

Estimation of DNA

A modification of the fluorometric method of Kissane and Robins (1958) was used for DNA determinations. In this procedure, the deoxyribose moiety is reacted with 3,5-diamino benzoic acid (DABA) to yield a fluorescent product. Although the procedure was developed originally for measurements of DNA content in whole-animal tissues, a high baseline level of fluorescence was encountered when the procedure was applied to isolated heart mitochondria. Satisfactory results were obtained, however, when mitochondrial DNA was solubilized by treatment with hot perchloric acid (PCA) prior to the reaction with DABA.

The procedure used was as follows: Mitochondria were suspended in 0.25 M sucrose, and trichloroacetic acid (TCA) was added to a final concentration of 5% (all steps were carried out in ice, unless specified otherwise). After 1 hour, the precipitate was collected by centrifugation for 10 minutes at 12,100 g, washed twice with cold 5% TCA, and suspended in 10 ml of cold absolute alcohol containing 10 mM potassium acetate. Ten minutes later, the precipitate was collected by centrifugation and treated in succession at room temperature for 15 minutes with 5 ml of each of the following: absolute alcohol (once), 2:1 (vol/vol) mixture of alcohol and ether (twice), and ether (once). Finally, the pellet was kept overnight at room temperature in an open test tube. (During this step, ether must evaporate completely, otherwise the reaction with DABA produces material with high spurious fluorescence.) The dry pellet was digested with 0.6 ml of 0.5 N PCA for 20 minutes at 70°C, centrifuged, and the supernatant was removed and saved. The digestion was repeated twice with 0.5- and 0.4-ml aliquots of PCA. The three supernatants were combined and adjusted to 1.5 ml with 0.5 M PCA. An 0.8-ml aliquot of the DNA extract was diluted to 1.1 ml with 0.5 N PCA and neutralized with 10 N KOH, as indicated by K-perchlorate precipitation. After centrifugation for removal of the precipitate, 0.5 ml of the supernatant was removed and acidified with 0.01 ml of 0.5 N PCA, and 0.18 ml of DABA reagent was added. The reaction mixture was incubated at 60°C for 30 minutes in test tubes covered with marbles.
After incubation, the tubes were cooled to room temperature, and 0.3 ml of water, 0.19 ml of 0.5 N PCA, and 0.15 ml of concentrated PCA were added. Any precipitate that formed was removed by centrifugation, and the fluorescence of the supernatant was measured at 420 nm excitation and 520 nm emission wavelength in an Aminco Bowman spectrofluorometer. A blank and the standard DNA were processed at the same time as the unknown samples. We determined empirically that aliquots of the DNA extracts and of the reagents specified above gave the most reproducible results with minimal dilution of DNA; about 2.0 g of pooled hearts were used as a source of mitochondria for two determinations of DNA before and after DNase treatment of mitochondria.

To obtain low blank values of fluorescence, one must take the following precautions: the DABA reagent (1 g of DABA, dissolved in 3.0 ml of water and treated for 30 minutes with 50 mg of activated charcoal, then filtered through Whatman no. 3 filter paper) and the TCA solution must be made freshly before use. We found, also, that any contamination with organic solvents or with sucrose has to be avoided.

A typical calibration curve based on calf thymus DNA is shown in Figure 1. As little as 0.1 μg of DNA can be estimated with this method.

**Treatment of Mitochondria with DNase**

To remove contaminating nuclear DNA, we suspended the washed mitochondria in 0.25 M sucrose containing 5 mM Tris-HCl buffer (pH 7.4), 50 mM NaCl, and 10 mM MgCl₂; they were digested with 100 μg DNase/ml (Worthington) for 30 minutes at 24°C in a final volume of 2.0 ml. After digestion, the mitochondrial suspension was cooled in ice, diluted with 10 ml of 0.25 M sucrose containing 25 mM EDTA and 2 mM EGTA (ice-cold), and centrifuged for 10 minutes at 18,800 g. The resulting pellet was washed once more in the same solution.

**Estimation of Cytochrome a**

Difference spectra of potassium ferricyanide-oxidized and dithionite-reduced mitochondrial preparations, which were solubilized in 2% deoxycholate, were recorded with a Cary model 14 spectrophotometer, as described by Williams (1964). The protein content of the mitochondrial solution was determined by the method of Lowry et al. (1951). Mitochondria that had not been treated with DNase were used for these measurements.

**Isolation of Mitochondrial DNA for Electrophoretic and Renaturation-Kinetic Analysis**

The DNase-treated mitochondria from six pooled hearts were suspended gently in 0.25 M sucrose containing 5 mM Tris-HCl buffer (pH 7.4), 50 mM EDTA, 4 mM EGTA, and 0.15 M NaCl. The mitochondria were lysed by addition of 0.1 volume of 20% sodium dodecyl sulfate (SDS) in a final volume of 2.5 ml and allowed to stand at room temperature for 30 minutes with occasional gentle stirring; Pronase, predigested for 2 hours at 37°C, then was added to a final concentration of 75 μg/ml. After 5 minutes, CsCl was added to a final concentration 1.0 M, and the lysate was frozen immediately. After 1 hour, the lysate was thawed and centrifuged for 10 minutes at 12,100 g. The supernatant was collected and the volume was reduced to 0.6 ml by concentration on a dialysis bag with Sephadex.

**Isolation of mtDNA for Electron Microscopy**

MtDNA was isolated from DNased mitochondria or from non-DNased mitochondria according to the following procedure.

Mitochondrial pellets were lysed, and the lysates were deproteinized in CsCl as described above. After centrifugation, additional CsCl was added to the supernate until the refractive index was adjusted to 1.39. Ethidium bromide then was added to a final concentration of 100 μg/ml. The ethidium bromide-CsCl gradients were centrifuged in a Beckman SW 50.1 rotor at 36,000 rpm for 60–72 hours at 20–24°C. Two bands were observed; the upper band consisted of nuclear DNA and open circular mtDNA, and the lower band contained the mitochondrial covalently closed circular molecules. The interband region containing some replicative forms was pooled with the lower band, and the upper

**Figure 1** Calibration curve for DNA reaction with DABA.
band was analyzed separately. Each fraction was dialyzed against the buffer used for spreading of the DNA for electron-microscopic examination.

Agarose Gel Electrophoresis

The procedure followed was essentially that described by Aaij and Borst (1972), except that ethidium bromide was added to the 2% agarose gels (4 μg/ml) and to the electrophoretic buffer (2 μg/ml). Electrophoresis was carried out at room temperature in glass tubes with a current of 5 mA/gel for 6 hours in buffer. DNA isolated from mitochondria that were treated with DNase at 0°C for 30 minutes was used for analysis.

Renaturation Analysis

Samples of DNA (5 μg/ml) were denatured with 0.1 N NaOH. After 15 minutes at room temperature, these samples were neutralized with 2 M NaH₂PO₄. The DNA was renatured in 0.15 M sodium phosphate buffer, pH 6.8, for 1 hour at 65°C. At this Cot value (6 × 10⁻² mol · sec/liter), all mtDNA is renatured, whereas nuclear DNA is not.

Electron Microscopy of DNA

The mtDNA molecules were spread according to the formamide-urea technique developed by Robberson et al. (1971). The spreading mixture, containing 50% formamide, 2 M urea, 100 mM Tris (pH 8.2), 10 mM EDTA, 50 μg/ml of cytochrome c, and 1 μg/ml of DNA, was delivered slowly onto a water hypophase. The DNA-cytochrome c film was allowed to stand for only a few seconds before being picked up on a parlodion-coated grid.

The grids were stained with uranyl acetate and rotary shadowed with platinum-palladium as described by Davis et al. (1971). The electron micrographs were taken at magnifications of 8,000-12,000 with a Siemens model 101 electron microscope.

Results

Conditions for DNase Digestion of Mitochondria

Isolated mitochondria, which appear to be highly purified as judged by electron-microscopic examination (Zak et al., 1972), are nevertheless contaminated with nuclear fragments, apparently produced during tissue homogenization. Because of the low levels of DNA present in mitochondria, even slight contamination by the nuclear fragments can lead to substantial error in the estimation of mtDNA. Digestion of mitochondria with DNase prior to DNA determination should remove the contaminating nuclear DNA while leaving mtDNA intact, provided that the mitochondrial membrane is not broken during the isolation procedure. Although DNase treatment was used effectively in the past for removal of contaminating nuclear DNA (Gross et al., 1969), the quantitative aspects of the procedure were not examined.

The DNA content of mitochondria digested with DNase at 4°C and at 24°C is compared with that of undigested mitochondria in Table 1. There was no significant decrease in the DNA content of mitochondria when DNase digestion was carried out at 4°C; increasing the temperature to 24°C, however, resulted in loss of 20–30% of the DNA. To determine the conditions under which nuclear DNA is removed without loss of mtDNA, we analyzed the structure and renaturation properties of DNA isolated from DNase-treated mitochondria.

Electrophoretic Analysis of the mtDNA

The purity of mtDNA can be assessed from its molecular configuration. In mammalian cells, the nuclear DNA is linear, whereas mtDNA is present predominantly in open or closed circular configurations. The three configurational species of DNA can be visualized by electrophoresis on 2% agarose gels. Linear molecules migrate more rapidly than closed circular and open circular DNA molecules, irrespective of size; closed circular molecules, in turn, migrate faster than open circular molecules. Densitometric scans of photographic negatives of ethidium bromide fluorescence in DNA electrophoretograms show that a considerable amount of linear DNA is present when mitochondria are not digested with DNase (Fig. 2a). If mitochondria are digested with DNase at 4°C, the DNA has a predominantly open or closed circular configuration with a small quantity of linear molecules (Fig. 2b). The residual linear DNA molecules may represent partially digested mtDNA or contamination with nuclear DNA. When DNA is isolated after DNase digestion at 24°C, however, all of the circular molecules are degraded, and only linear DNA remains (results not shown).

### Table 1 DNA Content, before and after DNase Digestion, and Cytochrome a Content of Isolated Rat Heart Mitochondria

<table>
<thead>
<tr>
<th>DNA content (μg/mg of mitochondrial protein) with DNase treatment</th>
<th>Cytochrome a (nmol/mg mitochondrial protein)</th>
<th>DNA (μg/nmol cytochrome a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4°C</td>
<td>None</td>
</tr>
<tr>
<td>0.650</td>
<td>0.611</td>
<td>0.690</td>
</tr>
<tr>
<td>±0.023</td>
<td>±0.022</td>
<td>±0.056</td>
</tr>
<tr>
<td>(3)</td>
<td>(4)</td>
<td>(5)</td>
</tr>
</tbody>
</table>

The results are given as means ± s.d. The number of determinations is given in parentheses.
CARDIAC HYPERTROPHY AND MITOCHONDRIAL DNA/Rajamanickam et al.

Renaturation-Kinetic Analysis

We further analyzed the purity of mtDNA by examining the renaturation-kinetic behavior of the isolated DNA. The buoyant densities of rat mitochondrial and nuclear DNA are essentially the same and thus cannot be differentiated by CsCl centrifugation (Fig. 3a). Denaturation increases the buoyant density of both mitochondrial and nuclear DNA by about 0.015 g/cm³; the annealing conditions, however, can be selected in such a way that only mtDNA renatures, its density returning to the value found in native DNA. However, nuclear DNA, remains denatured. The result is a clear separation of renatured mitochondrial and denatured nuclear DNA in analysis by CsCl isopyknic centrifugation.

Results of the renaturation analysis of DNA isolated from mitochondrial preparations that were not digested with DNase and preparations that were digested at 4°C and 24°C are shown in Figure 3. MtDNA isolated from undigested mitochondria shows contamination with nuclear DNA that amounts to 30–50% of the total DNA (Fig. 3b). This estimate is similar in magnitude to the decrease in DNA content that was produced by DNase digestion at 24°C (Table 1). DNA extracted from mitochondria subjected to DNase digestion at 4°C (Fig. 3c) or at 24°C (Fig. 3d) contains only a negligible amount of nuclear DNA. These results indicate that DNase digestion at both 4°C and 24°C is adequate to remove contaminating nuclear DNA from mtDNA.

The fluorometrically determined DNA content of mitochondria treated with DNase at 4°C (Table 1) does not decrease despite elimination of nearly all nuclear DNA, as shown above by renaturation analysis. It is likely that oligonucleotides produced by DNase treatment at 4°C remain large enough to

Figure 2 Agarose gel electrophoresis of mtDNA isolated from DNase-treated and untreated mitochondria. Densitometric tracing of negatives of ethidium bromide fluorescence of 2% agarose gels is shown. A: DNA extracted from mitochondria not subjected to DNase treatment. B: Same as A, except that the mitochondria were digested with 100 μg DNase/ml for 30 minutes at 4°C.

Figure 3 CsCl equilibrium centrifugation of mtDNA. Buoyant densities (g/cm³) of marker (M. lysodecticus, indicated by arrow), native, renatured, and denatured DNA are shown on abscissas. The ordinate gives optical densities obtained by scanning of negatives of UV photographs of DNA bands in CsCl gradient. A: Native DNA; mitochondria not treated with DNase. B: Renatured DNA; mitochondria not treated with DNase. C: Same as B, except that mitochondria were treated with 100 μg DNase/ml for 30 minutes at 4°C. D: Same as C, except that DNase treatment was performed at 24°C.
be precipitable with TCA; thus, they would be measured with the DABA reagent, but they would be eliminated by Agarose BioGel chromatography used to purify the DNA prior to gel electrophoresis (Fig. 2b) or renaturation analysis (Fig. 3c).

Time Course of DNase Digestion

If an assay for mtDNA is to be valid, it is necessary to determine whether there was any loss of content reached a minimal value after 30 minutes of digestion and remained unchanged during the subsequent hour of DNase treatment. Therefore, mitochondrial membranes appear to be capable of preventing the DNase from digesting a significant amount of mtDNA. A small quantity of DNase, however, appears to penetrate the mitochondria at 24°C, since there is a conversion of the mtDNA from the circular to the linear form.

From these results, we concluded that digestion of mitochondria with DNase at 24°C is suitable for elimination of contaminating nuclear DNA.

Changes in mtDNA during Cardiac Hypertrophy

Constriction of the ascending aorta in mature rats resulted in cardiac enlargement of 15, 26, and 38% above the size in sham-operated controls 2, 4, and 7 days, respectively, after surgery (Table 2). The mtDNA content, expressed per milligram of mitochondrial protein and per nanomole of cytochrome a, is shown in Table 3. Rats showing similar degrees of cardiac enlargement were pooled for isolation of mitochondria. Hearts of sham-operated rats served as controls. An increase (21%) in mtDNA content was seen in mitochondria isolated from rat hearts 2 days after aortic constriction, whereas the maximal increment (98%) was observed after 7 days. Although 7 days was the longest interval examined, the response in mtDNA content to DNA due to DNase digestion. For this purpose, the DNA content of mitochondrial preparations was measured after varying periods of DNase digestion at 24°C. As shown in Figure 4, the DNA content reached a minimal value after 30 minutes of digestion and remained unchanged during the subsequent hour of DNase treatment. Therefore, mitochondrial membranes appear to be capable of preventing the DNase from digesting a significant amount of mtDNA. A small quantity of DNase, however, appears to penetrate the mitochondria at 24°C, since there is a conversion of the mtDNA from the circular to the linear form.

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### Table 2: Changes in the Ratio of Heart Weight to Body Weight after Aortic Constriction

<table>
<thead>
<tr>
<th>Days after surgery</th>
<th>Normal</th>
<th>Sham-operated</th>
<th>Operated</th>
<th>Sham-operated</th>
<th>Operated</th>
<th>Sham-operated</th>
<th>Operated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart ratio*</td>
<td>0.282</td>
<td>0.292</td>
<td>0.324</td>
<td>0.274</td>
<td>0.351</td>
<td>0.264</td>
<td>0.399</td>
</tr>
<tr>
<td>±0.018</td>
<td>±0.016</td>
<td>±0.006</td>
<td>±0.018</td>
<td>±0.007</td>
<td>±0.04</td>
<td>±0.004</td>
<td>±0.031</td>
</tr>
<tr>
<td>(11)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>Percent hypertrophy</td>
<td>14.9 ± 0.8</td>
<td>26 ± 0.6</td>
<td>38 ± 1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Percent hypertrophy: (Heart ratio in operated rats X 100/heart ratio in sham-operated rats) - 100. The results are given as mean ± SD. The number of experiments is given in parentheses.

* Heart ratio: heart wt in grams x 100/body wt in grams.

### Table 3: Changes in Mitochondrial DNA and Cytochrome a Content after Aortic Constriction

<table>
<thead>
<tr>
<th>Experiment</th>
<th>DNA (µg/mg mitochondrial protein)</th>
<th>DNA (µg/nmol cytochrome a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>0.455</td>
<td>0.463</td>
</tr>
<tr>
<td></td>
<td>±0.05</td>
<td>±0.007</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
</tr>
<tr>
<td>Aortic constriction</td>
<td>0.551*</td>
<td>0.802†</td>
</tr>
<tr>
<td></td>
<td>±0.028</td>
<td>±0.087</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>% Change‡</td>
<td>21</td>
<td>73</td>
</tr>
</tbody>
</table>

Results are given as mean ± SD. The number of determinations is given in parentheses.

* P < 0.02, †P < 0.001. ‡(Aortic constriction – sham operation) – 100.
pressure overload levels off earlier, since the rate of mtDNA accumulation is lower between 4 and 7 days (0.05 μg DNA/day) than between 2 and 4 days (0.125 μg DNA/day). The content of cytochrome a per milligram of mitochondrial proteins does not change in response to pressure overload at any of the times examined. Thus, both cytochrome a and mitochondrial proteins can be used as reference values for calculations of the mtDNA content of mitochondria (Table 3).

Electron-Microscopic Analysis of Molecular Forms of mtDNA

Electron micrographs of various molecular forms of rat cardiac mtDNA are shown in Figures 5 and 6. The mtDNA populations of hearts from normal and sham-operated rats contained a high frequency of D-loops (42-49%; Table 4). The percentage of catenated molecules (3.6-4.9%) was within the range observed by several groups of investigators in a variety of tissues (Clayton et al., 1968; Matsumoto et al., 1976). Intermediates of replication, other than D-loops, were rare. Their low frequency was similar to that normally observed in differentiated organs (Kasamatsu et al., 1973; Paoletti et al., 1972). The percentage of dimer and polymer forms of mtDNA in hearts in which hypertrophy was induced by aortic constriction was similar to that in normal controls.

The analysis of replicative forms of the mtDNA population of hypertrophied hearts, however, showed several differences from normal or sham-operated hearts. The frequency of mtDNA molecules containing D-loops was reduced markedly, from about 45% to 5-7% of the population, in all samples analyzed. In contrast, the frequency of expanded D-loop forms increased substantially in hearts undergoing hypertrophy. It is of note that most of the expanded D-loop forms observed had very large single-stranded regions.

Discussion

Mitochondrial Mass and Cardiac Hypertrophy

One of the earliest changes in the myocardium subjected to acute pressure overload is an increase in the cellular volume occupied by mitochondria. Such a change has been detected by both electron-microscopic (Meerson et al., 1964) and biochemical analysis (Albin et al., 1973). The preferential accumulation of mitochondria is only transient, however; during the later phase of developing hypertrophy, myofibrils accumulate more rapidly than mitochondria. As a consequence, there is a progressive decrease in the fraction of mitochondria per unit of myocardial mass. One measure used to estimate mitochondrial mass has been the cardiac cytochrome c concentration, expressed as nmol/g heart. This concentration increases by about 5% within 24 hours after narrowing of the ascending aorta to 40% of its original diameter; but 3 and 10 days after aortic constriction, it is decreased by 5 and 10%, respectively (Albin et al., 1973; Rabinowitz and Zak, 1975). This sequence of initially increased and subsequently decreased relative mitochondrial mass appears to be the characteristic response of the enlarging myocardium to an acute pressure overload. When the heart enlarges secondary to a gradually imposed pressure overload (Wollenberger and Schultze, 1962; Wollenberger et al., 1966) or to volume overload (Dart and Holloszy, 1969; Oscai et al., 1971), such changes are not detected.

Synthesis and Degradation of Mitochondrial Components in the Hypertrophic Heart

The assembly of mitochondria involves the biosynthesis of components specified by both the nuclear and mitochondrial genomes. In higher organisms, mtDNA transcribes mitochondrial ribosomal RNA, mitochondrial tRNAs, and poly(A)-containing RNAs, which probably are mRNAs coding for some of the peptides of the oligomeric inner mitochondrial membrane complexes, such as cytochrome oxidase, coenzyme Q, cytochrome c reductase, and the oligomycin-sensitive ATPase. Most mitochondrial proteins, however, are synthesized...
outside the mitochondria, on cytoplasmic ribosomes. These include cytochrome c, enzymes of the Krebs cycle, RNA and DNA polymerases, most mitochondrial ribosomal proteins, and the remaining peptides of cytochrome oxidase, coenzyme Q-cytochrome c reductase, and the oligomycin-sensitive ATPase.

In a steady state, mitochondria turn over with an apparent half-life of about 6 days. The consequence of rapid turnover is that changed rates of either synthesis or degradation can contribute effectively to accumulation of mitochondria during growth. In a previous study (Albin et al., 1973), we showed that degradation of cytochrome c is reduced markedly during the first 24 hours of aortic constriction. Other studies suggest, however, that mitochondrial degradation, as estimated by biochemical (Aschenbrenner et al., 1972) or electronmicroscopic (Meerson et al., 1964) methods, may increase immediately following the operation. These different results may be due to the design of the latter experiment, in which no attempt was made to prevent hypoxia during surgery. Oxygen deprivation in vivo has been shown to lead to mitochondrial destruction (Aschenbrenner et al., 1971). It must be kept in mind, however, that this does not have to be the case under all experimental conditions. For example, Kleitke and Wollenberger (1978) have noticed that simulated ischemia in perfused rat heart is associated with increased RNA synthesis measured in isolated mitochondria. An increased rate of synthesis of mitochondrial proteins probably also contributed to mitochondrial accumulation during developing hypertrophy; the data cannot be interpreted quantitatively because rigorous measurement of precursor-specific radioactivity has not been possible yet.

The coordinated accumulation of individual mitochondrial components during cardiac hypertrophy is of considerable interest, since it may shed light on mechanisms regulating mitochondrial biogenesis. We showed previously that cardiac cyto-
TABLE 4 Frequency Distribution of Molecular Forms of DNA from Normal and Hypertrophied Hearts

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>% Hypertrophy</th>
<th>Molecules recorded</th>
<th>Catenated dimers and trimers</th>
<th>D-loop</th>
<th>Expanded D-loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat</td>
<td>—</td>
<td>277</td>
<td>10 (3.6%)</td>
<td>116 (42%)</td>
<td>2 (0.7%)</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>(2 days)</td>
<td>—</td>
<td>473</td>
<td>23 (4.9%)</td>
<td>240 (49%)</td>
</tr>
<tr>
<td>24 hours</td>
<td>24</td>
<td>158</td>
<td>8 (6.0%)</td>
<td>11 (7.0%)</td>
<td>3 (2.0%)</td>
</tr>
<tr>
<td>48 hours</td>
<td>22</td>
<td>541</td>
<td>10 (1.9%)</td>
<td>29 (5.3%)</td>
<td>27 (5.6%)</td>
</tr>
<tr>
<td>96 hours</td>
<td>25</td>
<td>283</td>
<td>8 (2.9%)</td>
<td>15 (5.2%)</td>
<td>4 (1.4%)</td>
</tr>
</tbody>
</table>

chromes c, b, and aa3 change in parallel following aortic constriction (Albin et al., 1973). In contrast, our present results show that mtDNA per unit mass of mitochondria increases considerably during the developing phase of cardiac hypertrophy. The time sequence of the changes in mtDNA agree with the earlier observations by Meerson and Pomoinitsky (1972). They differ quantitatively, however, in that we observed only about 20% of the increment in mtDNA reported by these investigators.

Comparison of the time course of the mtDNA increment with our earlier data on cytochrome c accumulation, for which identical experimental protocols were used, shows that there is no strict correlation between mtDNA content, or its rate of accumulation, and the changes in cytochromes c, b, and aa3. During the first 24 postoperative hours, these inner mitochondrial components accumulate faster than mtDNA, but during the 6 subsequent days, the rate of mtDNA increment far outstrips that of the cytochromes (Fig. 7). The results of Meerson and Pomoinitsky (1972), as well as our unpublished data, show that mtDNA and RNA both increase during developing hypertrophy; but again, there is no precise correlation between the changes in mtDNA and mitochondrial RNA.

It may be postulated that mtDNA must increase during developing cardiac hypertrophy to provide additional template for the transcription of mitochondrial RNA required to support enhanced protein synthesis. Attardi and colleagues (Aloni and Attardi, 1971; Murphy et al., 1975) have shown that HeLa mtDNA is transcribed completely and synchronously, the primary transcripts having a molecular size equivalent to that of the entire mitochondrial genome. Therefore, there is probably only one promoter per DNA strand, and selective transcription of the mitochondrial genome does not occur. The rate of mitochondrial RNA transcription therefore could depend on the amount of available template. However, other factors, such as configuration of the mtDNA, the level of RNA polymerase activity, and possibly control of promotorn function, may also be involved in the regulation of mitochondrial RNA synthesis. Mitochondrial RNA and DNA both increase in cardiac hypertrophy, suggesting a relationship between them. Nevertheless, there are clearly different temporal patterns in the increment of mitochondrial DNA and in the accumulation and rate of synthesis of mitochondrial proteins. Furthermore, there may be translational control of mitochondrial protein synthesis (Poyton and Kavanagh, 1976). We therefore must conclude that the regulation of mitochondrial replication, transcription, and translation is complex, and that the relationship between the amount of mtDNA and the synthesis of RNA and protein is not a simple one.

Frequencies of mtDNA Replicative Intermediates

In animal cells, mtDNA has been shown to replicate by modification of the "Cairns" mode (Cairns, 1963). The overall mechanism has been established by electron-microscopic analysis of the replicative intermediates. Replication is undirectional, and duplex synthesis is asymmetric and specific with respect to strandedness (for review, see Kasamatsu and Vinograd, 1974). A scheme illustrating the replicative cycle of animal mtDNA is shown in Figure 8. Replication is initiated at a specific origin on one DNA strand. The unidirectional synthesis of a 7S

![Figure 7 Relation between the rate of cytochrome c accumulation and mitochondrial DNA content after aortic constriction. Cytochrome c accumulation is expressed as a fractional rate (unit:time^-1) and was calculated as follows: the slope of increasing cardiac cytochrome c content was determined for 12-hour intervals after aortic constriction from a curve based on our previously published data. The slope was divided by the cytochrome c content at the midpoint of each 12-hour interval. The value obtained corresponds to the rate of cytochrome c accumulation expressed as a fraction of the mean cytochrome c content for that particular time interval. The data points for mtDNA/mg mitochondrial proteins are taken from Table 3.](http://circres.ahajournals.org/issue/513)
segment of DNA results in the displacement of the corresponding sequence on the second strand forming the D-loop. In differentiated animal tissues, replication usually pauses at this stage with the consequent accumulation of D-loop forms. After receiving an appropriate signal, DNA synthesis proceeds on the same DNA strand leading to the progressive enlargement of the D-loop, i.e., the expanded D-loop. Initiation of replication of the displaced second strand may occur almost immediately after D-loop expansion begins, producing "Cairns" forms (Wolstenholme et al., 1973a, 1973b), or be delayed until as much as 60-99% of the first strand has been replicated (Robberson et al., 1972; Goddard and Wolstenholme, 1978). The timing of second-strand initiation varies greatly, depending on tissue or cell type. Gapped molecules, which contain both single- and double-stranded segments, arise upon separation of replicating strands before duplex synthesis of the second strand has been completed (see Fig. 8).

One of the most striking observations made in this study is the marked reduction in D-loop forms of mtDNA during developing hypertrophy. The D-loop usually is present with extraordinarily high frequency (up to 50%) in differentiated nongrowing tissues (Piko and Matsumoto, 1977). In rapidly growing tissue culture cells, D-loop frequency has been correlated with the rate of cell division (Kasamatsu et al., 1973). Thus, the number of D-loop forms is highest during exponential growth and decreases as the stationary phase in the culture of mouse L-cells is reached (Kasamatsu et al., 1973).

In fully differentiated tissue stimulated to grow, a different relationship between D-loop frequency and rate of mtDNA replication apparently applies. In this case, the D-loop frequency falls when mitochondrial DNA replication is stimulated. For example, D-loop forms have been observed to be somewhat less numerous in regenerating liver (27%) than in nongrowing rat liver (42%) (Gilbert and Dressler, 1968). Our results show an even more striking decrease in D-loop frequency in the hypertrophied rat heart in which mtDNA synthesis is elevated. Cardiac muscle cells do not divide (Zak, 1974), but a rapid proliferation of mitochondria (Albin et al., 1973), accompanied by a substantial increase of mtDNA, takes place.

The exceptionally high frequency of D-loops indicates that their conversion represents the rate-limiting step in mtDNA replication in normal cardiac muscle. The marked depletion in the D-loop population during hypertrophy and the increase in number of expanded D-loops indicates removal of a block in replication at this site when replicative flux through the cycle is increased.

The frequency of D-loop has been observed to depend on the method of mtDNA isolation. The possibility of selective loss of D-loops, through branch migration, in mtDNA preparations from hypertrophied hearts during the isolation procedure therefore should be considered. Since samples from hypertrophied hearts and controls were treated in an identical manner during the entire procedure, it is unlikely that the observed difference is due to a preferential loss of D-loops in mtDNA preparations from hypertrophied hearts. It also should be emphasized that the low frequency of D-loops in hypertrophied hearts and the high frequency of D-loops in normal rat hearts were observed consistently in several independently prepared samples and were present whether mtDNA was isolated from DNased or from nonDNased mitochondria.

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