

CATENATED OLIGOMERIC CIRCULAR DNA MOLECULES FROM
MITOCHONDRIA OF MALIGNANT AND NORMAL MOUSE AND RAT TISSUES

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INTRODUCTION

Mitochondrial DNA (mtDNA) of all metazoan species examined, from nematode worms to man, is in the form of circular molecules with species-specific contour lengths of 4.5–6.0 μm (for recent reviews see Swift and Wolstenholme, 1969, Borst and Kroon, 1969; Rabinowitz and Swift, 1970, Wolstenholme et al., 1971). A number of oligomeric forms of circular mtDNA have been described. A circular molecule twice the normal contour length (circular dimer) has been found to distinguish mtDNA of a number of human granulocytic leukemic leukocytes from mtDNA of normal leukocytes and to account for as much as 48% of the molecules observed in such leukemic mtDNAs (Clayton and Vinograd, 1967, 1969). Similar molecules have been observed in mtDNA from mouse L cells (Nass, 1969 *a, b*; 1970, Kasamatsu et al., 1971) in mtDNA from human and beef thyroid (Paoletti et al., 1972) and in low frequency (<0.1% of molecules examined) in normal rat liver mtDNA (Kirschner, R. H., and D. R. Wolstenholme, unpublished).

Molecules consisting of two interlocked 5 μm circles (catenated dimers) have been found to account for up to 10% of the molecules observed in mtDNA of a variety of normal tissues (Clayton et al., 1968, Pikó et al., 1968). In HeLa cell mtDNA, as well as catenated dimers forms comprising as many as seven interlocked 5 μm circles were found (Hudson and Vinograd, 1967). Such catenated trimers and higher oligomers have been

shown to occur with a variety of frequencies in mtDNA from normal tissues (Clayton et al., 1968, Paoletti et al., 1972), cells in culture, and tumors (Nass, 1969 *a, b*; 1970).

Higher frequencies of both circular dimers and catenated dimers and higher oligomers of mtDNA have been found in mtDNA of mouse L cells in the stationary phase of growth, after starvation for methionine or phenylalanine, after treatment with cycloheximide or chloramphenicol (Nass, 1969 *b*) or puromycin (Nass, 1970), and after transformation of hamster kidney cells with polyoma virus (Nass, 1970). Treatment of chick fibroblasts and Ehrlich mouse ascites tumor cells with cycloheximide was also reported to result in mtDNA with higher frequencies of catenated dimers and higher oligomers (Nass, 1970).

We have determined the frequencies of different multiple circular forms in mtDNA from cells of some hepatomas and normal liver of mice and rats. The results are the subject of the present report.

MATERIAL AND METHODS

Tumors

Chang rat solid and ascites hepatomas (Chang et al., 1967) and Novikoff rat ascites hepatoma (Novikoff, 1957) were obtained from Drs. Carl F. Tessmer and Jeffrey P. Chang, M. D. Anderson Hospital and Tumor Institute, Houston, Texas. Ehrlich mouse ascites hepatoma was obtained from Dr. Peter M. M. Rae, the Whitman Laboratory, University of Chicago. All rats used in the present

study were female albinos weighing 150–300 g. All mice used were mature albino females. Both the rats and mice were obtained from Sasco Company, Omaha, Nebraska.

Rat ascites cells were grown in the peritoneal cavity of rats and maintained by syringe passage of 1.0 ml of ascites fluid every 7 days. Cells used for the preparation of mtDNA were harvested on given days after infection. Chang solid hepatomas were maintained in subcutaneous pockets on the backs of rats (Chang et al., 1967). A piece of tumor was excised from a rat, placed in 10 vol of isotonic saline, and broken up in a Waring Blendor (5 s, slow speed, Waring Products Div., Dynamics Corp of America, New Hartford, Conn.). The cell suspension so produced was squeezed through two layers of muslin, and 5 ml was injected into a previously formed subcutaneous air pocket of about 50 ml capacity on the back of the rat. Intraperitoneal solid tumors were produced by injecting 10 ml of this cell suspension into the peritoneal cavity of a rat. Both the back and peritoneal solid tumors grew at varying rates and were therefore harvested when the rats showed given weight gains.

Mouse ascites cells were grown in the peritoneal cavity of mice and maintained by syringe passage of 0.1 ml ascites fluid every 14 days. Cells used for the preparation of mtDNA were taken either on the 20th day after infection or when a batch of simultaneously infected mice showed a given mean weight gain. Solid tumors were produced by injecting 0.1 ml of ascites fluid from a mouse 20 days after infection with tumor cells, subcutaneously on the backs of mice. The tumors were harvested 20 days after infection.

Preparation of Mitochondria

Rats and mice were sacrificed by cranial fracture. Solid Chang tumors were excised, taking care not to include associated normal tissues, and immersed in 10 vol of a buffer (homogenizing medium) containing 0.3 M sucrose, 1 mM disodium ethylenediaminetetraacetate (EDTA) and 0.01 M Tris (tris[hydroxymethyl]-aminomethane)-HCl (pH 7.4) at 4°C and mitochondria were then prepared as described by Kirshner et al. (1968). Mitochondria were similarly prepared from cells of different tumors but with the following modifications. Cells of Novikoff rat ascites hepatomas and of Chang rat ascites hepatomas were broken open by the procedure described in Wolstenholme et al. (1973).

Cells of both solid and ascites Ehrlich mouse hepatomas were broken open by suspending the cells in homogenizing medium and subjecting them to two strokes at top speed in a Potter-Elvehjem homogenizer.

DNA Extraction and Purification

DNA was extracted from mitochondria as described previously (Kirschner et al., 1968). Each mtDNA was finally purified by elution from a column of methylated albumin kieselguhr (Sueoka and Cheng, 1962).

Electron Microscopy

MtDNAs were prepared for electron microscopy by the aqueous protein monolayer technique of Freifelder and Kleinschmidt (1965), and rotary shadowed with platinum-palladium, exactly as described by Wolstenholme and Gross (1968). MtDNA from a mouse ascites hepatoma was also prepared for electron microscopy by the formamide protein monolayer technique following the procedure of Davis et al. (1971), as described by Wolstenholme et al. (1973). This preparation was then shadowed from one direction at an angle of about 8° with 10 mg platinum:palladium (80:20) wire evaporated from a 25 m tungsten wire at a mean distance of 10 cm from the grids in a Kinney model KSE-2 vacuum evaporating unit (Kinney Vacuum Co., Boston, Mass.), and then rotary shadowed under otherwise identical conditions. Finally, carbon was evaporated onto the grids to ensure stability of the membranes in the electron beam.

All grids were examined in a Hitachi HU-11B electron microscope or in a Siemens Elmiskop 101 electron microscope at a magnification of approximately $\times 6000$. Shadowed molecules were photographed (using projector pole piece 2 in the Hitachi, and projector polepiece 1 in the Siemens) at an original magnification of $\times 12,000$. Exact calibrations were made for each microscope using a diffraction grating replica (Ernest F. Fullam, Inc., Schenectady, N. Y., 2160 lines/mm). Measurements of molecules were made on positive prints using a map measure, at a magnification of $\times 150,000$.

For each of two mtDNA preparations, one from Ehrlich mouse ascites hepatoma cells and the other from Novikoff rat ascites hepatoma cells, aqueous protein monolayers were prepared for electron microscopy using two different DNA concentrations. Grids were oriented in the electron microscope so that movement of a stage control could result in a scan of a grid in a direction perpendicular to a grid bar. Relative field densities of molecules in the different protein monolayer preparations of a single mtDNA were estimated from counting the number of molecules which passed under a point on the electron microscope screen when three scans of each of six grid squares were made for each protein monolayer preparation. These scannings were done at a magnification of $\times 6000$.

RESULTS

It was determined by electron microscope examination that more than 90% of at least one preparation of mtDNA from each of the different malignant and normal tissues used in these experiments was circular. Each of the mtDNAs comprised highly twisted or supercoiled circular molecules and open circular molecules in various proportions (Tables II and III) as has been found for mtDNAs of other metazoans (see reviews of Swift and Wolstenholme, 1969, Wolstenholme et al, 1971). The supercoiled form of mtDNA has been shown to be a covalently closed molecule (Kroon et al, 1966; Dawid and Wolstenholme, 1967; Clayton and Vinograd, 1967), that is, phosphodiester bonds are found throughout the lengths of the two nucleotide strands of the molecule (Vinograd and Lebowitz, 1966). As a single phosphodiester bond break converts the supercoiled form of mtDNA to the open circular form (Dawid and Wolstenholme, 1967), the proportion of molecules in the supercoiled form in a mtDNA preparation is an indication of the maximal random hydrolysis by endodeoxyribonuclease (DNase) to which that mtDNA has been subjected.

Most of the circular DNA from each tissue examined had a unimodal contour length of about 5 μm (Fig 1, Table I). Observed differences between contour lengths of circular mtDNA molecules of malignant and normal tissues of the same species did not exceed 5% (Table I)

In mtDNA from all of the tissues examined, forms were found which apparently comprised two attached 5 μm circles (Tables II and III, Figs. 2 and 3). Between 6 and 8% by weight of normal mouse liver mtDNA was in this form (Table II). In contrast, 15–22% of the mtDNA from Ehrlich mouse hepatoma cells was accounted for by dimeric forms. In addition, up to 19% of the circular mtDNA from Ehrlich hepatoma was in the form of molecules apparently comprising between three and seven joined 5 μm circles (Fig 4). Such higher oligomers were either not found or were rare in mtDNA prepared from normal mouse liver

In order to test the accuracy of visually scoring molecules as the various oligomeric forms, a number of dimers, trimers, tetramers, and pentamers from a preparation of Ehrlich hepatoma mtDNA were visually scored and each molecule was photographed and measured. The lengths were compared with the mean length of the mole-

TABLE I
Contour Lengths of Circular DNA Molecules Classified as Monomers, from Mitochondria of Hepatomas and of Normal Liver of Mice and Rats

| | Mean contour length in microns | Number of molecules measured |
|---|--------------------------------|------------------------------|
| Mouse | | |
| Ehrlich ascites hepatoma | 4.86 \pm 0.02 | 50 |
| Normal liver | 4.83 \pm 0.03 | 50 |
| Rat | | |
| Chang solid (intra-peritoneal) hepatoma | 4.94 \pm 0.04 | 50 |
| Novikoff ascites hepatoma | 4.70 \pm 0.04 | 50 |
| Normal liver | 4.95 \pm 0.02 | 50 |

The standard error of each mean contour length is given.

cules scored as monomers. The results which are summarized in Fig 1, indicated the scoring to be accurate and also confirmed that these molecules were multiples of the unit length molecule

The frequencies of the different oligomeric forms in two electron microscope preparations of an Ehrlich hepatoma mtDNA sample which had relative field densities of approximately 1:8 were found to be similar (Table II). Similar frequencies of the different forms were also found when this same mtDNA was prepared for electron microscopy in the presence of formamide. These findings indicate that the observed associations of molecules are due to some kind of bonding rather than to simple overlapping of the molecules when the DNA-protein monolayers were formed.

By shadowing DNA molecules in protein monolayer preparations with a heavy metal, first from one direction and then while rotating the specimen, Hudson and Vinograd (1967) demonstrated that the bonding between the component molecules of dimeric forms of HeLa cell mtDNA was apparently topological; that is, the dimers comprise two monomers interlocked like links in a chain. MtDNA from Ehrlich hepatoma cells was shadowed in a similar way. In suitably oriented oligomers, the component molecules also appeared to be interlocked (Figs 2 and 3) (This interlocking was found to be more easily demonstrated in

TABLE II
Frequencies of Different Forms of Circular DNA Molecules from Mitochondria of Ehrlich Mouse Hepatoma Cells and from Normal Mouse Livers

| | Total circular molecules examined | Percentage of circular molecules as catenated | | | | | | | | | | Percentage weight of circular DNA as | | | |
|-------------------------------|-----------------------------------|---|-----------|-----------|-----------|-----------|-----------|---------------------|------------------|----------------------------|--|--------------------------------------|--|--|--|
| | | Dimers | Trimers | Tetramers | Pentamers | Hexamers | Septamers | Supercoiled circles | Catenated dimers | Catenated higher oligomers | | | | | |
| Ehrlich mouse hepatoma | | | | | | | | | | | | | | | |
| Ascites | | | | | | | | | | | | | | | |
| (1) 20 day | 827 | 13.8 ± 2.4* | 3.4 ± 1.2 | 1.6 ± 0.9 | 0.6 ± 0.5 | 0.1 ± 0.2 | 0 | 74.1 ± 2.6 | 21.5 | 15.7 | | | | | |
| (2) 20 day | 927 | 9.0 ± 1.9 | 1.9 ± 0.9 | 1.0 ± 0.6 | 0.1 ± 0.2 | 0 | 0 | 72.4 ± 2.7 | 15.4 | 9.2 | | | | | |
| (3) 23% weight gain | | | | | | | | | | | | | | | |
| (a) | 857 | 12.6 ± 2.2 | 4.0 ± 1.3 | 1.7 ± 0.9 | 0.6 ± 0.5 | 0.2 ± 0.3 | 0 | 62.1 ± 2.9 | 19.5 | 18.0 | | | | | |
| (b) | 860 | 13.2 ± 2.2 | 4.5 ± 1.4 | 2.1 ± 1.0 | 0.4 ± 0.4 | 0.1 ± 0.2 | 0.1 ± 0.2 | 63.3 ± 2.8 | 20.2 | 19.2 | | | | | |
| (c) | 875 | 11.3 ± 2.1 | 3.7 ± 1.3 | 1.5 ± 0.8 | 0.8 ± 0.6 | 0.2 ± 0.3 | 0.1 ± 0.2 | 61.5 ± 2.9 | 17.7 | 18.0 | | | | | |
| Solid subcutaneous (back) | 828 | 13.8 ± 2.4 | 2.4 ± 1.0 | 1.6 ± 0.9 | 0.7 ± 0.6 | 0.1 ± 0.2 | 0.1 ± 0.2 | 66.3 ± 2.9 | 21.6 | 14.7 | | | | | |
| Normal mouse liver | | | | | | | | | | | | | | | |
| (1) | 1002 | 3.0 ± 1.1 | 0 | 0 | 0 | 0 | 0 | 89.9 ± 1.8 | 5.8 | 0 | | | | | |
| (2) | 1052 | 4.3 ± 1.2 | 0.1 ± 0.2 | 0 | 0 | 0 | 0 | 65.2 ± 2.8 | 8.2 | 0.1 | | | | | |

Entries 1 and 2 for the Ehrlich ascites hepatoma represent mtDNA from hepatomas of two different batches of eight and 14 mice, respectively. The weight gain of the former batch of mice over the 20 day infection period was not determined. The weight gain of the latter batch over the same time period was approximately 31%. Entry 3 represents mtDNA from a group of 31 mice which underwent a 23% weight gain over an 11 day infection period. Entries a and b represent two different protein monolayer preparations at relative field densities of DNA molecules of approximately 1:8, respectively. Entry c represents the same preparation of DNA prepared for electron microscopy in the presence of formamide. Entries 1 and 2 for normal mouse liver represent mtDNA from the livers of two different batches of 15 adult mice. The entry for Ehrlich solid hepatomas represents mtDNA from hepatomas grown subcutaneously in the backs of 30 mice which underwent a weight gain of approximately 32% over a period of 13 days.

* 95% confidence limits calculated according to the procedure given in Clayton et al. (1968).

TABLE III
Frequencies of Different Forms of Circular DNA Molecules from Mitochondria of Two Rat Hepatomas and from Normal Rat Livers

| | Total circular molecules examined | Percentage of circular molecules as catenated | | | | Percentage weight of circular DNA as | | |
|------------------------------------|-----------------------------------|---|-----------|-----------|-----------|--------------------------------------|------------------|----------------------------|
| | | Dimers | Trimers | Tetramers | Pentamers | Supercoiled circles | Catenated dimers | Catenated higher oligomers |
| Chang rat hepatoma | | | | | | | | |
| Ascites, 7 day | 1114 | 4.6 ± 1.2* | 0.3 ± 0.3 | 0.1 ± 0.2 | 0 | 55.2 ± 2.9 | 8.7 | 1.1 |
| Solid | | | | | | | | |
| Intraperitoneal ^a | 1016 | 9.1 ± 1.8 | 1.1 ± 0.6 | 0.4 ± 0.4 | 0.1 ± 0.2 | 52.1 ± 2.9 | 16.2 | 4.7 |
| Subcutaneous ^b | 1026 | 6.0 ± 1.5 | 1.2 ± 0.7 | 0.1 ± 0.2 | 0.1 ± 0.2 | 81.8 ± 2.3 | 11.1 | 4.1 |
| Subcutaneous ^c | 1069 | 2.7 ± 1.0 | 1.0 ± 0.6 | 0 | 0 | 78.7 ± 2.4 | 5.2 | 2.7 |
| Subcutaneous ^d terminal | 1174 | 0.9 ± 0.5 | 0 | 0 | 0 | 71.0 ± 2.6 | 1.9 | 0 |
| Novikoff rat ascites hepatoma | | | | | | | | |
| 5 day | 1157 | 4.5 ± 1.2 | 0.5 ± 0.4 | 0.1 ± 0.2 | 0 | 80.5 ± 2.2 | 8.5 | 1.7 |
| 7 day | 1032 | 4.3 ± 1.2 | 0.1 ± 0.2 | 0.1 ± 0.2 | 0.1 ± 0.2 | 74.6 ± 2.6 | 8.1 | 1.2 |
| 8 day (a) | 1104 | 3.9 ± 1.1 | 0.1 ± 0.2 | 0 | 0 | 91.7 ± 1.6 | 7.8 | 0.3 |
| 8 day (b) | 1057 | 4.2 ± 1.2 | 0.1 ± 0.2 | 0.1 ± 0.2 | 0 | 90.5 ± 1.7 | 8.0 | 0.6 |
| Normal rat liver | | | | | | | | |
| (1) | 1039 | 4.0 ± 1.2 | 0.1 ± 0.2 | 0 | 0 | 51.3 ± 2.9 | 7.8 | 0.3 |
| (2) | 1292 | 1.1 ± 0.6 | 0 | 0 | 0 | 93.0 ± 1.4 | 2.0 | 0 |

The superscript letters associated with the solid Chang rat hepatoma entries indicate the following: a, an intraperitoneal tumor weighing 71 g; b, two subcutaneous back tumors averaging 35 g; c, a subcutaneous back tumor weighing 53 g; d, a subcutaneous back tumor weighing 200 g harvested 15 min post mortem. Entries a and b for the Novikoff ascites hepatoma taken 8 days after infection represent two different protein monolayer preparations at relative field densities of DNA molecules of approximately 1:6, respectively. Entries 1 and 2 for normal rat liver represent mtDNA from livers of two different batches of five rats * 95% confidence limits calculated according to the procedure given in Clayton et al. (1968).

mtDNAs prepared for electron microscopy in the presence of formamide).

The frequencies of the different oligomeric forms of mtDNA molecules from rat hepatoma cells and from normal rat liver cells are shown in Table III. MtDNA from both the Novikoff hepatomas and Chang hepatomas taken from living rats were distinguished from normal rat liver mtDNA by the presence of tetrameric and pentameric forms (Fig 5). Substantially more mtDNA from these Chang hepatomas was in the trimeric form than in either Novikoff hepatoma or normal rat liver mtDNAs. The frequencies of catenated dimers was about the same in Novikoff hepatoma mtDNAs (average 4.2%; range 3.9–4.5%) as in one of the

normal rat liver mtDNAs (4.0%). The frequencies of dimeric forms of Chang hepatoma mtDNA molecules from living animals varied from 2.7–9.1%.

In one Chang hepatoma removed 15 min post mortem, the frequency of catenated dimers was lower than that found in either of the normal rat liver mtDNAs examined, and no higher oligomeric forms were observed. The absence of oligomeric forms seems more likely to be related to the stage of tumor growth than to random hydrolysis by DNase since 71% of the circular DNA in this preparation appeared as supercoiled circles (Table III).

The frequencies of the different oligomeric forms

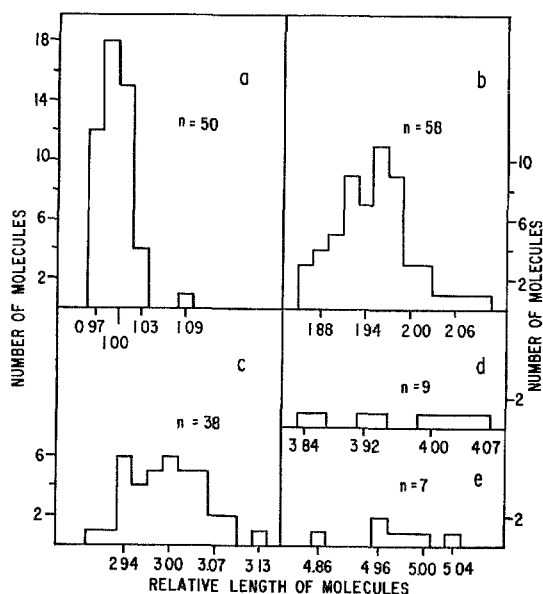


FIGURE 1 Frequency distributions of the lengths of circular molecules classified as monomers (a), dimers (b), trimers (c), tetramers (d), and pentamers (e), in mtDNA of an Ehrlich mouse ascites hepatoma harvested 20 days after infection. The lengths in (a) were normalized by the mean length of $4.86 \mu\text{m}$ ($\pm 0.02 \mu\text{m}$). The lengths in b-e were also normalized by the mean length of the monomers in (a).

in two electron microscope preparations of a single sample of Novikoff hepatoma mtDNA which had relative field densities of approximately 1:6 were again found to be similar (Table III) indicating that in this mtDNA also, the observed associations of molecules was not due to simple overlapping. In all of the mtDNAs examined, dimeric forms were observed comprising all three possible combi-

nations of supercoiled and open circular molecules (i.e., two supercoiled; one supercoiled, one open; two open). Similarly, all four possible combinations for trimers and all five possible combinations for tetramers of supercoiled and open circular molecules were observed. Also, various combinations of supercoiled and open circular molecules were found to make up the pentamers, hexamers, and septamers.

In some of each oligomeric form, the component circular molecules were arranged in a chain, where a single molecule was clearly never interlocked with more than two others (Fig. 5). In some cases, however, each of the component molecules could have been interlocked with all of the other molecules in the group (Fig. 4). Forms apparently intermediate to these two extreme cases were also observed; for example, a tetramer in which each of three circles could have been joined to each of the other two, but in which a fourth circle was only joined to one of the remaining three.

Not a single double-sized circular molecule (the circular dimers of Clayton and Vinograd, 1967) was observed in any of the mouse or rat mtDNAs examined in the present study.

DISCUSSION

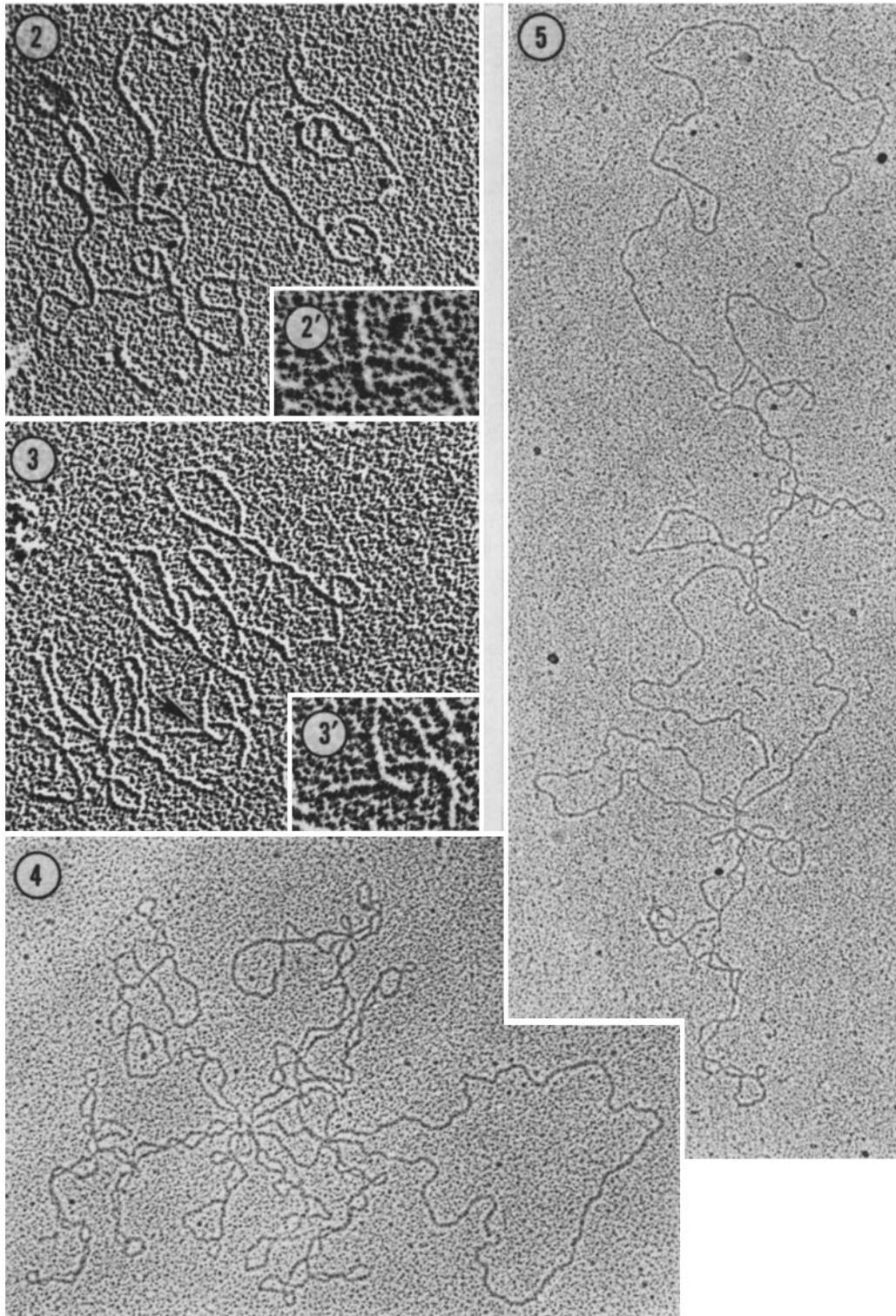
Each of the rodent tumor mtDNAs examined was distinguished from mtDNA of normal livers of the respective host animal by the presence of tetrameric and pentameric, and in the case of the Ehrlich hepatoma, higher oligomeric forms. It is clear from our data, however, that a high proportion of higher oligomeric forms is not a feature common to all tumors. Also, the frequency of these forms is not positively correlated with the speed of tumor growth. Novikoff ascites hepatoma, the mtDNA of which differs very little from normal rat

FIGURES 2 and 3 Electron micrographs of dimeric forms of circular DNA molecules from mitochondria of Ehrlich mouse ascites hepatoma cells, which have been first shadowed from one direction and then rotary shadowed. Each dimer comprises two circles, approximately $5 \mu\text{m}$ in contour length, which appear to be interlocked (arrows). The insets (2' and 3') show the regions of interlocking of the two dimers at higher magnifications. Fig. 2, $\times 45,000$; Fig. 3, $\times 49,000$; Insets, $\times 80,000$.

FIGURES 4 and 5 Electron micrographs of rotary shadowed mtDNA molecules.

FIGURE 4 A pentameric form apparently comprising four supercoiled and one open circular molecules. Each circular molecule could be attached to more than two others in the group. From a preparation of Ehrlich mouse ascites hepatoma mitochondria. $\times 44,500$.

FIGURE 5 A tetrameric form apparently comprising two supercoiled and two open circular molecules arranged in a chain. From a preparation of Novikoff ascites hepatoma mitochondria. $\times 43,500$.



liver mtDNA with respect to oligomeric forms, kills rats in 6–8 days, having increased in cell mass by at least 70 times. The highest frequency of oligomeric forms was found in mtDNA from mouse Ehrlich hepatoma which takes at least twice as long to kill its host, having increased proportionally in cell mass about the same as the Novikoff hepatoma.

It seems unlikely considering the low frequencies of catenated oligomers in Novikoff hepatoma mtDNAs, that these forms have a basic causal effect in the production of malignant cell growth in rodents. This is worth mentioning, because of the proposal of Clayton et al (1969) and Clayton and Vinograd (1969), that a significant relationship exists between the formation and presence of the circular dimer and neoplasia in man.

The present finding that up to 39% of Ehrlich hepatoma mtDNA was in the form of catenated dimers and higher oligomers is in marked contrast to the value of 9.4% found by Nass (1970) for Ehrlich mouse ascites mtDNA. The different values might result from use of different strains of either tumor cells or mice in the two experiments. In the present case, the amount of this mtDNA in the form of higher oligomers was between 88 and 192 times that found in normal mouse liver.

The fate of catenated dimeric and higher oligomeric forms of mtDNA is not known. The present observation that multiple forms were in low frequency in a terminal Chang hepatoma mtDNA in which replication may have ceased, but in which there had apparently not been extensive random hydrolysis by DNase (71% of the DNA was in the form of supercoiled circles) would be explained if catenated oligomers could separate into their component circular molecules.

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