CLOSED CIRCULAR DNA IN ANIMAL CELLS

I. COMPLEX MITOCHONDRIAL DNA IN NORMAL AND MALIGNANT TISSUE AND THE IN VIVO EFFECTS OF DRUGS ON THE SUPERHELIX DENSITY OF MITOCHONDRIAL DNA

II. SMALL POLYDISPERSE CIRCULAR DNA OF HELA CELLS

III. SEQUENCE HETEROGENEITY IN CLOSED SIMIAN VIRUS 40 DEOXYRIBONUCLEIC ACID

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The subbasement contains no windows to let in the sunshine; those who live there must generate their own. To those many people who have given of themselves to brighten my experience there, my sincere thanks.

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My gratitude for all the things my parents have done for me cannot be expressed here in words.

ABSTRACT

Part I of the thesis is concerned with mitochondrial DNA (mtDNA) of animal cells. Complex mtDNA includes catenated oligomers, two or more of the basic 5-μ circles linked as in a chain, and circular dimers, 10-μ circles composed of two monomer genomes in tandem. The mtDNA of human placenta and several organs of rabbits, guinea pigs, and mice was found to contain 6 to 9% catenated dimers and 0.5 to 2% higher oligomers. Circular dimers were absent or below 0.2%. In contrast, the mtDNA from 12 of 15 human tumors contained the circular dimer in frequencies from 0.2 to 9%, in addition to catenated molecules in the above frequency ranges. Two lines of mouse L cells were found to contain circular dimers in frequencies of 5 and almost 100%. These frequencies were not changed when cells were treated with cycloheximide or maintained at high cell density.

Ethidium bromide was shown to inhibit mtDNA synthesis in HeLa and SV3T3 cells, but not to affect base composition or complexity of the pre-existing mtDNA. With increasing treatment time or dosage, the pre-existing mtDNA undergoes a gradual change in the superhelix density from about -0.025 superhelical turns per ten base pairs to maxima of -0.088 in HeLa and -0.114 in SV3T3 cells. The nicking-closing cycle demonstrated by these results operates at least every 30 minutes and was shown not to be an artifact of mtDNA isolation. The change is reversible, and can be demonstrated with other known intercalators. Similar changes in superhelix density were found in the mtDNA of livers, spleens, and kidneys of mice treated with ethidium bromide. It is

postulated that the effect on superhelix density is the result of in vivo intercalation of the drugs into mtDNA.

Part II is a study of the small polydisperse closed circular DNA in HeLa cells. This DNA was found to range in size from 0.05 μ to greater than 2 μ, with an average length of 0.32 μ and a weight average molecular weight of 1.0×10^6. This latter value was corroborated in sedimentation studies in neutral and alkaline solvents. The buoyant density, 1.692 g/ml, indicates a G+C content of 38 mole %. The separated strands do not have detectably different buoyant densities at pH 12.5. The superhelix density of the DNA (-0.039 to -0.045) is significantly greater than that of HeLa mtDNA. Renaturation kinetics studies have shown that the circles are not composed of varying numbers of a sequence the size of the smallest molecules in the population. The DNA may be prepared from whole cell extracts or cytoplasmic fractions; it is not associated with purified mitochondria or nuclei. There are a minimum of 50 circles per growing cell; treatment with cycloheximide results in a 20- to 30-fold increase. Labeling experiments showed that the cycloheximide-induced small circles are not newly replicated, but are formed from pre-existing DNA.

Part III is a study of sequence heterogeneity in closed circular SV40 viral DNA. Denatured singly nicked DNA was reannealed and the heteroduplexes formed were examined for regions of nonhomology by formamide-protein film electron microscopy. Substituted and deleted sequences longer than about 50 nucleotides are detected by this method. DNA from viruses passaged twice at multiplicities of infection much

less than one p.f.u./cell contained 2% deletions and no detectable substitutions. In contrast, DNA from viruses grown by several passages of undiluted lysates or by infecting cells with stock virus at 5 p.f.u./cell contained 13 and 11% deleted molecules and 12 and 7% substituted molecules, respectively. The substitutions appear to have arisen as the result of integration of SV40 into chromosomal DNA, followed by excision of molecules containing stretches of chromosomal DNA. On the average, the substituted sequence is somewhat shorter than the native SV40 sequence it replaces.

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PART I

COMPLEX MITOCHONDRIAL DNA IN NORMAL AND MALIGNANT TISSUE AND THE IN VIVO EFFECTS OF DRUGS ON THE SUPERHELIX DENSITY OF MITOCHONDRIAL DNA

Chapter 1.

General Introduction

Early reports that mitochondria isolated by differential centrifugation contained nucleic acids as judged by colorimetric tests (1) were regarded as demonstrations of the contamination of such preparations by nuclear DNA. Although this interpretation was probably correct, several years later electron microscopy provided histochemical evidence for DNA in the mitochondria of a wide variety of plants, animals, and unicellular eukaryotes (2). That mitochondria might contain a genetic system was also suggested by respiratory-deficient mutants of yeast (3) and Neurospora (4) which followed the rules of cytoplasmic inheritance.

The first demonstration that the DNA extracted from isolated mitochondria was a unique species (5) took advantage of the fact that mitochondrial DNA (mtDNA) of Neurospora crassa has a buoyant density in neutral cesium chloride which is 11 mg/ml less than that of the nuclear DNA. It was also established that mtDNA was not degraded when the mitochondria were incubated with DNase. Within a few years the mtDNA from a large number of plants, animals, and unicellular eukaryotes was isolated and characterized. That all mitochondria contain DNA is now firmly established and this DNA has been the subject of several reviews (6-9).

Although the buoyant densities of the mtDNA from a considerable number of plants have been determined (they are generally 10 to 12 mg/ml denser than the corresponding nuclear DNA's), only a few studies of the physical size of these DNA's are available. Heterogeneously sized linear fragments have been seen which range up to 25 μ

for Neurospora crassa (10), to 35 μ for the voodoo lily (Sauromatum venosum) (8), and up to 60 μ for the red bean (Phaseolus vulgaris) (11). No evidence for circular DNA was seen in the mtDNA of the cellular slime mold Dictyostelium discoideum (12), and the mtDNA for the acellular slime mold Physarium polycephalum has been reported to be linear (13).

Because of the size heterogeneity of these linear molecules, the possibility remains that the genome is of constant size, possibly circular, and easily degraded. Indeed, a mutant of Neurospora which is lacking a cell wall has been shown to contain a closed circular species of about 20 μ, originating from a mitochondrial fraction (14). In yeast, the subject of many conflicting reports concerning the size of its mtDNA, 25-μ circles have now been seen in preparations of mitochondria lysed directly on electron microscope specimen grids (15). It is likely that the smaller circles seen previously in yeast mitochondrial preparations do not represent the native mtDNA genome (see Discussion, Part II, this thesis).

Heterogeneously sized linear molecules have been seen in several studies of the mitochondrial DNA of Euglena gracilis, the latest of which (16) also contains the report of 40-μ circular DNA in the chloroplasts.

The only report of homogeneously sized linear mtDNA is from a study of Tetrahymena (17), in which a population of linear molecules about 18 μ long was seen.

In striking contrast, the mitochondrial DNA from multicellular animals consists predominantly of closed circular molecules with a

contour length of about 5 μ, indicating a molecular weight of about 10^7. This observation has been made with insects, echinoderms, fish, amphibians, reptiles, birds, and several species of mammals. The actual contour lengths reported vary between 4.5 and 5.9 μ. Some of the length differences reported may be due to different conditions in different laboratories, but in two cases length differences have been confirmed by demonstrating a bimodal distribution of the measurements obtained with a mixture of mtDNA from two sources (8, 18). In addition to the 5-μ circles, two complex forms of mtDNA have been described: catenated forms--oligomers in which two or more 5-μ circles are topologically interlocked (19)--and circular oligomers in which the contour length of circles are multiples of the 5-μ unit (20). These complex forms will be discussed in detail in later chapters.

The closed circular nature and size constancy of animal mtDNA has allowed extensive physiochemical characterization. MtDNA displays the special properties of closed circular DNA. A high buoyant density in CsCl at pH 12.5 has not yet been reported. This is due to the sensitivity of this DNA to single-strand scissions at this pH, a phenomenon not yet understood. Under these conditions the complementary strands separate and two species with distinctly different buoyant densities appear. These density differences in mammalian mtDNA are great enough to allow complete separation and isolation of the complementary strands, a property which has allowed hybridization experiments with the individual strands. The density differences are interpreted to mean that one of the complementary strands (the heavier strand) has a higher

content of G+T than does the other (21). This bias has also been shown by direct determination of the base compositions of the separated strands of HeLa mtDNA (22).

The overall G + C content of mtDNA, as determined by neutral buoyant densities, varies from species to species and may be less than, equal to, or greater than that found for the corresponding nuclear DNA's. Base compositions of mtDNA of related organisms are generally similar.

The single sharp band found when mtDNA from a single species is centrifuged to equilibrium in neutral cesium chloride rules out gross base composition heterogeneity. Renaturation kinetics studies of chick liver (23) and guinea pig liver (24) mtDNA's have demonstrated that the base sequence length is equal to the physical length. Base sequence homogeneity of mtDNA from a single source is indicated by experiments in which renatured mtDNA is examined by electron microscopy. This technique allows the visualization of sequence heterogeneity in sequences longer than about 100 base pairs. No such heterogeneity has been seen in renatured human, mouse and toad mtDNA (25, 26). All these observations indicate that the total coding capacity of animal mitochondrial DNA is about 15,000 base pairs.

It has been suggested that the uniform small size of animal mtDNA represents the minimum size necessary for the fulfillment of certain functions which for some reason have not been taken over by nuclear genes and which have remained the same throughout almost all animal evolution (24, 27). The information coded for by mtDNA is just now becoming elucidated.

Experiments by Borst (28) suggested that the RNA transcribed in vivo in rat liver mtDNA is complementary only to the heavy strand. Investigations of HeLa cell mtRNA at first suggested that the heavy strand is almost completely transcribed, while only a small portion of the light strand is copied (29, 30). A later report (31) presented evidence that the transcription is to a large extent symmetrical, but the light strand transcript is rapidly degraded or removed from the mitochondria.

Some of the products of the transcription of mtDNA have recently been identified. Two high molecular weight discrete species have been identified in HeLa cells (32-35), cultured hamster cells (36) and Xenopus laevis oocytes (37). In HeLa cells, these two components have been shown to be transcripts of the heavy strand of mtDNA. Hybridization experiments and electron microscopy of RNA-DNA hybrids have demonstrated that the DNA codes for one copy of each, that the two genes are located adjacent or within 500 base pairs of each other, and that about 20% of the mtDNA is used to code for the two RNA's (35, 38, 39). These two species have also been shown to comprise the RNA of the 60 S ribosome-like particles found in the mitochondria in HeLa cells (40) and Xenopus ovaries (41).

Some of the transcription products of mtDNA are undoubtedly mitochondrial-specific transfer RNA's. In rat liver, five species of mitochondrial tRNA's have been identified and are presumably coded for by mtDNA (42, 43). One of these has been shown to hybridize to the mtDNA (44). N-Formylmethionyl tRNA has been found in the mitochondria

of rat liver (45) and HeLa cells (46). A discrete mtRNA species sedimenting at 4 S has been demonstrated in HeLa cells (30, 35). Hybridization experiments indicate that the heavy strand of mtDNA of HeLa cells codes for about eight genes of tRNA size. Electron microscopy indicates that they are probably not all in one cluster (39). It is possible that the light strand also codes for some tRNA-sized RNA.

These three discrete RNA species account for 25% of the coding capacity of the mtDNA. It seems unlikely that the remaining 75% of the RNA which is transcribed is not functional; otherwise one would suspect that the size of mtDNA would be much smaller. This RNA sediments heterogeneously in sucrose, and is probably some kind of messenger RNA. Presumably, some of the messages direct the synthesis of some vital mitochondrial proteins, using the synthesizing machinery in the mitochondria. Since the vast majority of mitochondrial proteins must be coded for in the nuclear DNA (mtDNA is much too small to code for very many proteins), it remains to be explained why some mitochondrial proteins would need to be coded for by mtDNA. It is not known whether any externally made messenger enters the mitochondrion for protein synthesis.

It has been suggested that some of the mitochondrial RNA might be exported to the cytoplasm for translation (27). Definitive proof of this is difficult due to the ever-present possibility that mtRNA found in the cytoplasm originates from mitochondria damaged during cell fractionation.

It is difficult to determine whether non-mitochondrial proteins are coded for by mtDNA, since animal cells cannot dispense with

mitochondrial function. In yeast cells, where this is possible, there is some evidence that functions not directly concerned with mitochondrial function may be coded for by the mitochondrial DNA. Some respiratory mutants perpetuate and transcribe a mitochondrial DNA which contains only enough G+C to code for a few polypeptide chains if all the G and C were in a small region. It is difficult to understand why the DNA would be perpetuated, unless this transcription product is vital to the cell. Unfortunately, since the mtDNA of yeast is five times that in animal cells, this function may have been assumed by animal cell nuclear DNA.

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Chapter 2.

Complex Mitochondrial DNA in Normal Tissue

INTRODUCTION

The interaction between the intercalating dye, ethidium bromide, and closed circular DNA, first used to study the number of superhelical turns in such DNA (1, 2), soon became a powerful tool for the preparative isolation of such DNA from solutions also containing nicked or linear DNA. The complex between the dye and closed circular DNA has a greater buoyant density than the complex with nicked or linear DNA and thus the two different forms of DNA can be easily separated in a cesium chloride density gradient which contains an appropriate concentration of the dye. When this preparative method was used to isolate the closed circular DNA from whole cell extracts of HeLa cells, two forms of closed circular DNA were seen which had not been previously reported in purified DNA samples (3). These were a species of small DNA, heterogeneous in size, which formed the basis for the studies in part II of this thesis, and molecules whose contour lengths were multiples of the 5 μm mitochondrial DNA (mt-DNA) molecules.

Nass (4) had previously reported molecules of about 10 μm in preparations of mitochondria which were osmotically shocked directly onto protein films and immediatly transferred to electron microscope specimen grids. She attributed these to superposition of molecules from the same mitochondrion. The multiple length molecules reported by Radloff et al. could always be seen as two or more tangent 5 μm circles. Although the appearance of these molecules even when the density of DNA on the grid was low seemed

to rule out overlapping monomers as an explanation, some kind of molecular aggregation prior to grid preparation could not be ruled out.

Before the nature of the oligomeric molecules in HeLa cells was understood, the existence of circular mitochondrial DNA with a contour length of 10 μm was discovered in the mt-DNA isolated from the leukocytes of a patient with chronic granulocytic leukemia. These circular dimers could be seen as circles free of any cross-over points (5).

A clue to the nature of the oligomers in HeLa cells came when several micrograms of HeLa mt-DNA were subjected to CsCl-ethidium bromide centrifugation and a band of DNA was observed midway between the closed and open DNA bands. This suggested that two independent circles were connected in such a way that one of them could be closed circular after the other had suffered a single strand scission. Electron microscopy revealed that this middle band was indeed enriched in the dimer molecules previously seen in HeLa cells. Electron microscopy also revealed that the nature of the connection was a topological bond; that is the circles appeared as the links of a chain. These molecules, which may be made up of several circles linked in this way, are designated catenated oligomers (6).

In subsequent investigations, the catenated oligomers were found to occur in both normal and leukemic human leukocytes (7), mouse myeloma cells (8), and rat ascites tumor cells (8).

The fact that circular dimer molecules were seen only in

the mt-DNA from leukemic cells while catenated molecules were found in several other cells, most of which were associated with malignancy in some way or another, prompted a study of the mt-DNA of several tissues of rabbits, mice, and guinea pigs as well as further study of the mt-DNA of normal human leukocytes. The results of this study, which required an investigation of the proper methods for accurately determining frequencies of complex mt-DNA by electron microscopy, are given in the following publication.

Occurrence of Complex Mitochondrial DNA in Normal Tissues

by

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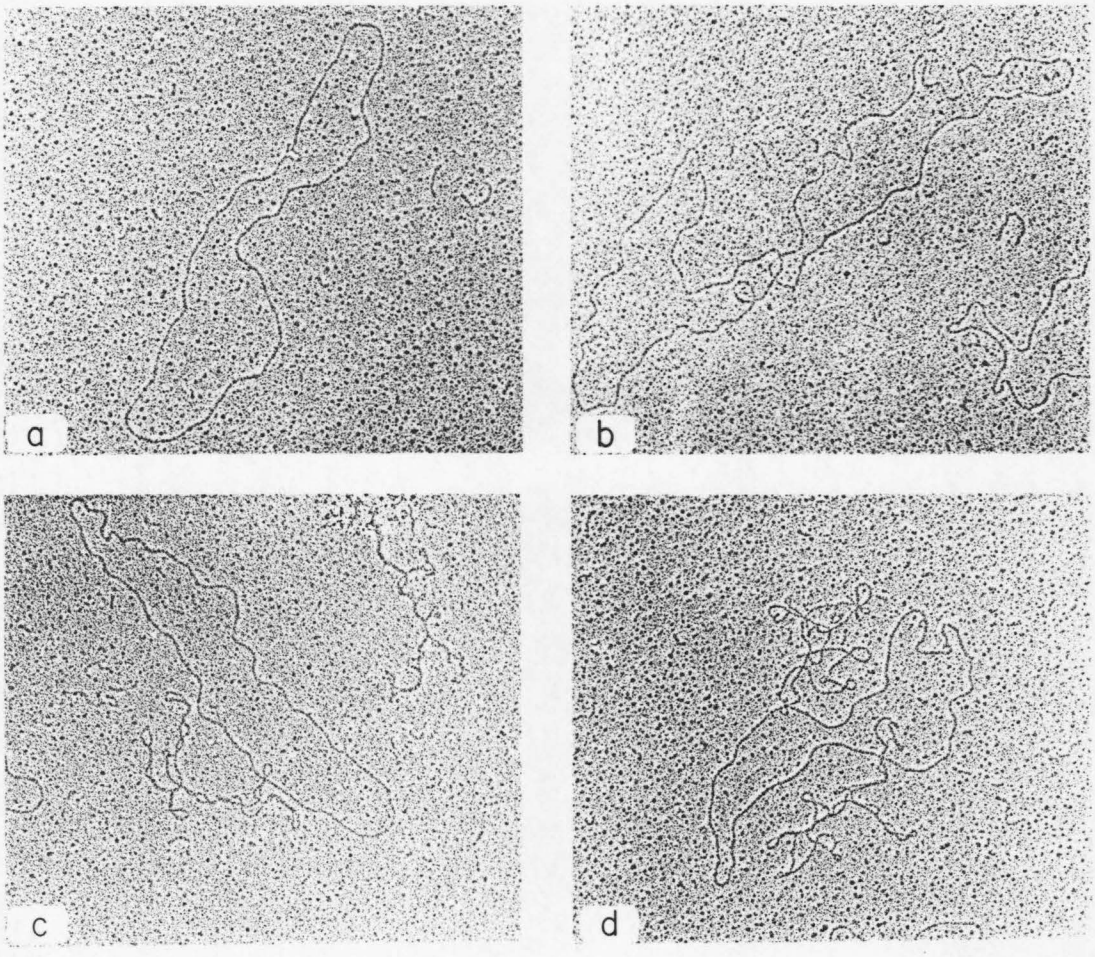
The following paper was published in Nature, 220 (5171), 976-979 (1968), and is included here with the permission of the publisher.

Publisher's Abstract

Mitochondrial DNA isolated from several mammalian organs contains from 6 to 9 per cent catenated dimers and 0.5 to 2 per cent catenated higher oligomers. Multiple length circular molecules, such as occur in leukaemic leucocytes, were not detected.

The DNA in the mitochondria of many higher organisms is now known to occur in the form of closed circular duplex molecules with contour lengths of approximately 5μ and a molecular weight of about 10 million daltons[1]. We have recently discovered that human mitochondrial DNA (M DNA) occurs in two larger circular forms which we refer to as complex forms. The catenated oligomers are molecules that consist of two or more interlocked circular duplexes connected to each other like links in a chain (Fig. 1). The circular oligomers are circular duplexes with a contour length that is a multiple of the monomer length. White blood cells from patients with chronic granulocytic leukaemia contained catenated oligomers and circular dimers[2]. HeLa cells, in contrast, contained catenanes but no circular dimers were seen[3]. This disparity among human cells prompted us to examine a number of normal

Fig. 1. Representative electron micrographs of mitochondrial DNA isolated from rabbit bone marrow. Grids were prepared as described earlier and examined in a Philips EM300 electron microscope[2]. a, Monomer length mitochondrial DNA; b, catenated dimer, fully relaxed; c, catenated dimer with one relaxed submolecule; d, catenated trimer with one relaxed submolecule. The contour length of the monomeric molecules and submolecules is approximately 5 μ.


a
b
c
d

tissues for the occurrence of the two types of complex mitochondrial DNA, and to develop procedures for reliably estimating the frequency of each type in an M DNA preparation.

We have found that the mitochondrial DNAs obtained from rabbit brain, kidney, liver and bone marrow, from guinea-pig brain and liver, from normal human leucocytes, and from 13 day old mouse embryos contain 2-9 per cent catenated dimers but do not contain detectable circular dimers. These results are similar to those obtained in previous studies of HeLa cell M DNA (ref. 3) and M DNA from the unfertilized sea urchin egg[4], but are in marked contrast to the previously reported observations of the presence of circular dimers at a frequency of 26 per cent in leucocytes of a patient with chronic granulocytic leukaemia[2]. All the M DNAs studied in this work contained higher catenated oligomers—trimers, tetramers and so on—at a frequency that varied between 0.5 and 1.8 per cent.

Scoring the Frequency of Complex Mitochondrial DNA

Mitochondrial DNA usually represents only a minor constituent of the total DNA in the cell. A very efficient separation process for the removal of the overwhelming amount of nuclear DNA is therefore necessary before analysis for the various complex forms can be undertaken. In our earlier work we removed most of the nuclear DNA with a cell fractionation procedure which yielded partially purified mitochondria. The DNA from such mitochondrial preparations was isolated and purified in a single step with the ethidium bromide-caesium chloride density gradient method[5]. The lower band, containing only closed circular

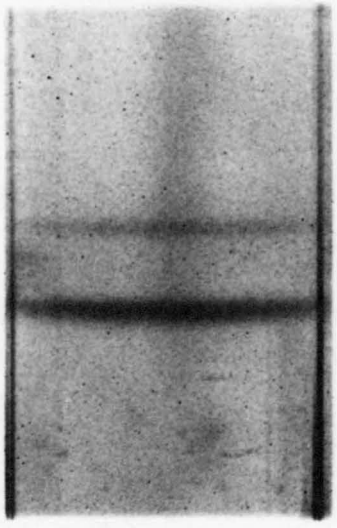
M DNA that was free of both nuclear DNA and nicked M DNA, was recovered for further study. The use of lower band DNA introduced an uncertainty in the result for the frequency of complex mitochondrial DNA molecules, because these latter molecules are larger targets than the monomers for any process which introduces single-strand scissions into DNA. We have now improved our procedure for the purification of mitochondria so that the ratio of the closed M DNA in the lower band to the nicked M DNA plus linear nuclear DNA in the upper band is reduced from approximately 10 to 0.2–1.0 as judged by a visual estimate of the fluorescence intensity of the bands (Fig. 2). Both bands and the intermediate caesium chloride solution between them were collected together to provide an unfractionated M DNA sample for electron microscope analysis. The remaining linear DNA does not interfere in the analysis. Occasional preparations in which the amount of DNA in the upper band exceeded the amount in the lower band were rejected.

To obtain quantitative estimates of the frequency of complex forms in a sample, we have examined a large population of molecules and distinguish the categories: monomer, circular dimer, catenated dimer and catenated higher oligomer. The required size of the sample was estimated with the relation[6]

$$\alpha = \pm 1.96\sqrt{(1 - f)/nf}$$

where f represents the mean obtained in a survey of n molecules and $f(1 \pm \alpha)$ represents the interval which contains the true mean at a level of confidence of 95 per cent. In the present work we have classified

Fig. 2. A caesium chloride-ethidium bromide density gradient showing two bands of mitochondrial DNA from rabbit brain. The centrifugation and photographic conditions were described previously[2, 3]. The contents of the middle one-third of the tube were collected, diluted to 5 ml., and concentrated into a pellet in an SW 50 rotor, 43,000 r.p.m., 10 h, 15°C. The total DNA from such preparations contained less than 5 per cent nuclear DNA as indicated by electron microscopy and analytical buoyant density centrifugation after removal of ethidium bromide.



about 2,000 molecules in each M DNA preparation so as to estimate the frequencies of complex forms which vary, for example, from 2 to 10 per cent. The corresponding ranges are ±0.64 and ±1.2 per cent if 2000 molecules are classified.

Photographic methods are too slow to be practical for classifying such large numbers of molecules. We have therefore classified the molecules as they appear imaged on the fluorescent screen in the electron microscope. A general requirement for the scoring procedure is that circular molecules be free of extensive twisting. Highly twisted catenated dimers cannot be distinguished from twisted circular dimers even though it is possible to distinguish twisted monomers from twisted dimers. Our previously described procedure[2, 3] for preparing specimen grids usually results in only a small fraction of highly twisted molecules. Specimen grids which contained more than 10 per cent of such molecules were rejected. The surface concentration of molecules must be low enough, less than about 400 per 300 mesh grid hole, for the eye readily to follow the contour of the molecule. The contrast must be adequate for the examination of the image on the fluorescent screen and "flower patterns" should be infrequent. The linear DNA mass concentration should not exceed 50 per cent of the total DNA.

The specimen grid was examined at a magnification of 5,400 beginning at a corner grid hole. The first grid hole was scanned completely in a systematic manner and was followed by similar scans of the laterally adjacent grid hole. Scanning was continued until an adequate number of molecules had been classified or until all holes on the grid had been scanned. The results were tabulated on an eight unit blood

cell counter as monomer, circular dimer, catenated dimer, catenated higher oligomer and as ambiguous molecules.

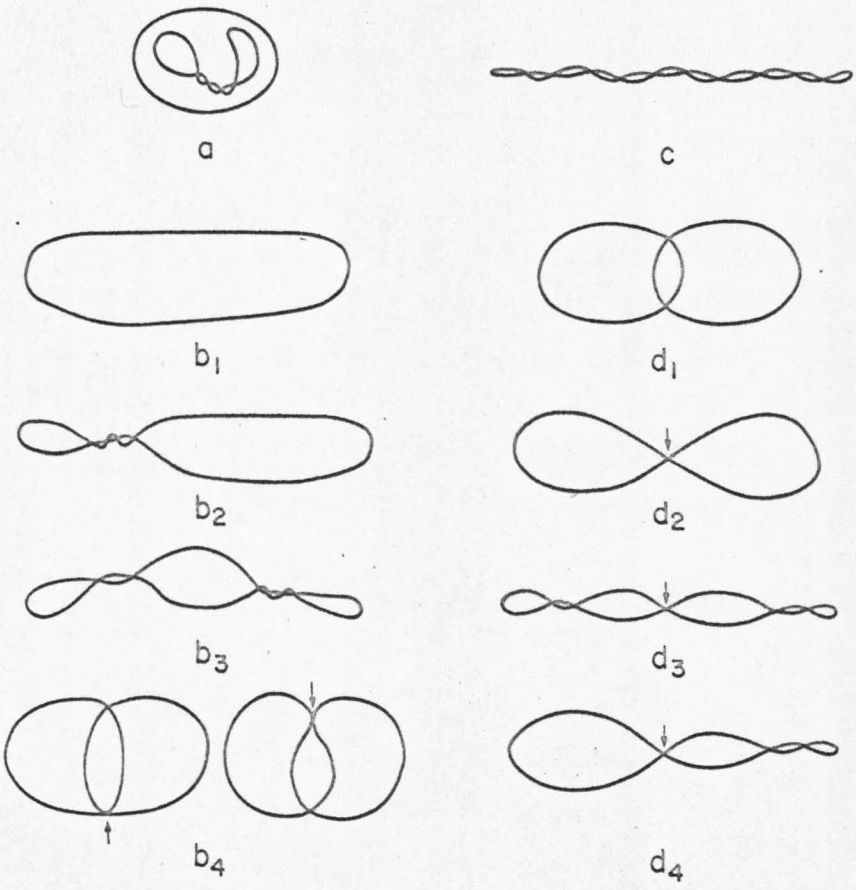
Decision making was in two stages. A decision was made between monomer, dimer and higher oligomer, and then between circular oligomer and catenated oligomer. The investigator readily learns, from the results of contour length measurements of photographed molecules with different shapes and differing extents of convolution, to distinguish monomers, dimers and higher forms. A basic problem is to ensure that overlapping monomers are not scored as dimers. We have reported earlier that molecules on a surface layer often appear to repel each other[3] and do not overlap on specimen grids unless very high DNA concentrations are used. Forms such as those illustrated in Fig. 3a, in which there is clearly no contact between two approximately concentric molecules, were not uncommon. If there was adventitious overlapping, the frequency of catenanes should be expected to be a steep function of the surface density of molecules. A preparation of rabbit kidney M DNA was examined on specimen grids with three different surface densities: approximately 500, 150 and 75 molecules per 300 mesh grid holes. The frequencies of total catenated oligomers in the three samples of approximately 1,000 molecules were 7.2 ± 1.6 per cent, 7.1 ± 1.5 per cent and 6.2 ± 2.0 per cent. The individual results do not differ significantly from the mean 6.8 ± 1.1 per cent, so we conclude that the extent of adventitious overlapping is small. Specimen grids with surface densities of more than 300 molecules per grid hole are difficult to score and were normally not used.

Fig. 3 (b, c and d) represents dimeric molecules and illustrates the problems encountered in distinguishing catenated dimers and circular dimers. Fig. 3 (b_1–b_3) represents unambiguous circular dimers because no crossover exists that could divide the dimer into two monomers. Dimers which contain a crossover (arrow) as in Fig. 3 (d_2–d_4) are regarded as catenanes and not as circular dimers, because we would have expected to have seen some unambiguous forms of circular dimers had they been present. These were not seen. Similarly, if circular dimers appeared in forms such as those illustrated in Fig. 3 (d_2–d_4) we would have expected to find examples of such kinds of molecules of monomer length. Because these are only infrequently seen, circular dimers of this form can represent only a small fraction of circular dimers present in the sample. Dimers with the pulled out form illustrated in Fig. 3 (d_2–d_4) were reported in preparations of HeLa M DNA (ref. 5) and were later shown to be catenated dimers[3]. An M DNA dimer of the type shown by Suyama and Miura[7] from monkey liver would be scored as a catenane in this laboratory.

Molecules such as in Fig. $3d_1$ are scored as catenated dimers even if the detailed nature of the overlapping is not discernible. In order for a circular dimer to appear in this conformation it would have to fold as in Fig. $3b_4$. We think this unlikely because tangential segments (arrow) are rarely seen in preparations known to contain circular dimers and circular monomers are never seen folded in such a manner as to appear to be a catenated dimer with a total contour length of 5μ.

Dimeric molecules in the form represented in Fig. 3c are classified as ambiguous because of extensive twisting. Such forms, if

Fig. 3. Representations of circular DNA conformations. a, Monomers; b, circular dimers; c, ambiguous dimer; d, catenated dimers.


a
c
b1
d1
b2
d2
b3
d3
b4
d4

they do not exceed 10 per cent, are apportioned according to the frequencies obtained with unambiguous dimers. No unambiguous circular dimers were seen in this study, so ambiguous dimers were scored as catenanes.

The variation of the results between four of the authors of this study was checked in a set of measurements with a given population of rabbit brain M DNA on one grid hole (Table 1). The results demonstrate that individual investigators classify the DNA molecules in essentially the same way.

The mitochondrial DNA prepared in this study was examined in buoyant CsCl in the analytical ultracentrifuge in order to verify the purity of the DNA and to determine the buoyant densities of these DNAs. The buoyant densities are presented in Table 2. The details of the experiments are given later in this report.

The results of our study of the frequency of complex M DNA in normal tissues are given in Table 3. The frequency of catenated dimers varies between 5.9 and 9.1 per cent among the various organs from rabbit and guinea-pig. The frequency of the higher catenanes varies from 0.6 to 1.8 per cent. These results may be restated as the percentage of the 5μ circles that are catenated to form dimers or higher oligomers. In these units the catenated dimers vary from 10.9 per cent for rabbit kidney to 16.6 per cent for rabbit brain M DNA. Higher catenanes were assumed to be trimers in the latter calculations. We conclude from the quantitative analyses of the M DNA prepared from the normal tissues listed in Table 3 that catenated dimers and catenated higher oligomers are normal constituents of M DNA. Data obtained with rabbit

Table 1. CLASSIFICATION OF COMPLEX M DNA FORMS IN THE SAME POPULATION OF RABBIT BONE MARROW M DNA BY FOUR INVESTIGATORS

Investigator	Monomers	Circular dimers	Catenated dimers	Catenated trimers
D. A. C.	175	0	19	6
J. M. J.	175	0	18	7
C. A. S.	175	0	19	6
M. R. T.	175	0	19	6

Table 2. BUOYANT DENSITIES OF MITOCHONDRIAL AND NUCLEAR DNAs ISOLATED IN THIS INVESTIGATION

Source	Buoyant density*, θ (g/ml.) Mitochondrial	Nuclear
Rabbit: brain, kidney, liver, bone marrow	1.696	1.692
Guinea-pig: liver	1.695	1.694
Human leucocyte: normal and leukaemic[2]	1.700	1.690

* Referred to E. coli DNA, 1.704 g/ml. and calculated with the buoyant density gradient[2] in experiments with crab dAT DNA, $\theta = 1.670$, or M. lysodeikticus DNA, $\theta = 1.725$ g/ml.

Table 3. FREQUENCY OF COMPLEX MITOCHONDRIAL DNA FORMS IN VARIOUS TISSUES

Tissue	Catenated dimers %	Catenated higher oligomers %	Circular dimers %	Molecules scored
Rabbit				
Brain*	9.1 ± 1.3‡	1.2 ± 0.5	0.0	1,762
Marrow*	8.1 ± 1.2	1.5 ± 0.6	0.0	1,845
Kidney*	5.9 ± 0.9	0.6 ± 0.3	0.0	2,817
Liver†	(4.8 ± 0.8)	(0.4 ± 0.2)	0.0	2,855
Guinea-pig				
Brain*	7.6 ± 0.9	0.8 ± 0.3	0.0	3,287
Liver*	7.7 ± 1.1	1.2 ± 0.5	0.0	2,210
Human				
Leucocytes†	(1.7 ± 0.5)	(0.0)	0.0	2,707
Mouse				
Embryo*	5.6 ± 0.9	1.8 ± 0.5	0.0	2,300

* Total DNA from purified mitochondria.

† Closed DNA from purified mitochondria. These results in parentheses are regarded as minimum values for reasons explained in the text.

‡ Range of 95 per cent confidence interval calculated as described in text.

liver and normal human leucocyte M DNA isolated from lower bands in the EB-CsCl gradients are also included in Table 3. These results, which must be regarded as minimum values because some selection against higher molecular weight forms may have occurred, also show that catenanes are normal constituents of M DNA.

The circular dimer form was not detected in the entire course of this survey of the complexity of M DNA from normal tissue (Table 3). We estimate that our limit of detection is of the order of one to two molecules per 1,000 molecules classified. This result is in marked contrast with our previous observations[2] that the circular dimer form occurs at a high frequency in M DNA from leukaemic leucocytes. It should be noted that the circular dimers were absent from the M DNA from the rapidly growing tissues—rabbit bone marrow and mouse embryo (Table 2). An examination of 400 molecules of M DNA from sea urchin gastrulae (S. purpuratus) also failed to reveal the circular dimer form.

The frequency of catenanes does not seem to be especially enhanced in rapidly dividing tissue. The frequencies in rabbit bone marrow, 8.1 per cent, and 13 day old mouse embryos, 5.6 per cent, are in the same range as in rabbit brain and kidney and guinea-pig brain and liver. These comparisons must be regarded as tentative, because we have not as yet investigated the variability of the frequency of catenanes among individual animals.

Preparation of Mitochondria and Mitochondrial DNA

Brains from four rabbits were minced in cold 0.25 M sucrose, 0.01 M tris, pH 7.5, 0.01 M KCl, 0.005 M EDTA and washed twice. The tissue was suspended in 0.21 M mannitol, 0.07 M sucrose, 0.001 M tris, pH 7.5, 0.0001 M EDTA and homogenized with a loose fitting "Teflon" homogenizer at 4° C until the homogenate appeared to be free of particulate material. The homogenate, filtered twice through cheese cloth, was sedimented at 1,000g for 10 min. The supernatant was sedimented a second time to remove remaining nuclei and cell debris. A pellet containing mitochondria was obtained in a Sorvall SS 34 rotor, 10,000 r.p.m., 10 min. The pellet was resuspended in 5 ml. of mannitol-sucrose medium and layered on to a step gradient of equal volumes of 0.75 M, 1.0 M, 1.30 M, and 1.75 M sucrose, each containing 0.1 M tris, pH 7.5, 0.01 M KCl, 0.005 M EDTA, and centrifuged for 1 h at 50,000g, 4° C, in a swinging bucket or a fixed angle rotor. The material at middle interface was collected with a pipette, diluted with mannitol-sucrose media and resedimented at 10,000 r.p.m. as described. The pellet was washed once and resuspended in 10 ml. 0.25 M sucrose, 0.01 tris, pH 6.7, and 0.005 M $MgCl_2$. DNase I, 100 μg, Sigma Chemical Company, was added and the mixture incubated at 25° C for 30 min. The digestion was stopped by addition of 0.2 ml. 0.5 M EDTA and chilling to 0° C. The mitochondria were sedimented to form a pellet which was washed once and the DNA extracted with SDS and banded in CsCl-ethidium bromide gradients as described by Radloff et al.[5].

These procedures were also used for rabbit kidney and bone

marrow, guinea-pig brain and liver, and the 10-12 day mouse embryos except as noted below. The mouse embryos were freed of the embryonic sacs and heads and limbs discarded before mincing. The homogenization and wash solution was 0.25 M sucrose, 0.01 M tris, 0.001 M EDTA, pH 7.2. This solution was also used in the preparation of total mitochondrial DNA from guinea-pig liver and brain.

In the preparation of the rabbit liver mitochondrial DNA, the first mitochondrial pellet was subjected to nine cycles of suspension in MS buffer with a Thomas loose fitting homogenizer, followed by a 1,000g spin (pellet discarded) and another 10,000g spin. No sucrose gradients or DNase treatments were used. The closed band in an ethidium bromide-CsCl gradient was used for scoring. The upper band was examined and shown to contain less than 10 per cent circular material. Mitochondrial DNA from normal human leucocytes was isolated as described previously[2].

Examination of Preparations in the Analytical Ultracentrifuge

The rabbit liver M DNA was isolated from a lower band in an ethidium-CsCl gradient, freed of dye by chromatography through a small Dowex 50 column and centrifuged as previously described[2]. A single symmetrical band was obtained. A comparable experiment with rabbit liver nuclear DNA formed a band skewed toward the dense side with a maximum 4 mg/ml. less dense than M DNA. A synthetic mixture of equal parts of nuclear and M DNA gave a single broad band. The combined upper and lower bands from purified mitochondria from rabbit brain, kidney, and bone marrow formed single bands in dye-free

CsCl that were skewed to the light side in rough agreement with the known 10-20 per cent contamination by nuclear DNA seen in the electron microscope. The buoyant densities corresponding to the band maxima were the same as for rabbit liver M DNA. Comparable results were obtained for the nuclear DNAs from all rabbit organs. The guinea-pig liver M DNA isolated from the bottom band in EB-CsCl gradients formed a symmetrical band 1 mg/ml. denser than the nuclear DNA, with a buoyant density in agreement with those previously reported for the corresponding DNAs from leukaemic leucocytes.

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ADDITIONAL RESULTS

(a) The problem of overlapping monomers

According to the scoring rules outlined in the preceeding publication, any figure which appears as two monomer circles tangent at a point is scored as a catenated dimer. It was argued that such figures are not overlapping monomers because at the concentrations of DNA used molecules seem to repel each other, and because the catenated dimer frequency remained constant as the density of the DNA upon the grid was varied. To check further the extent of monomer overlap, specimen grids were prepared from PM-2 viral DNA. This DNA has a molecular weight of 6×10^6, closer to that of mt-DNA than any other readily available closed circular DNA, and the general appearance of PM-2 molecules on the specimen grids is very similar to that of mt-DNA. Closed circular DNA from purified virions was enzymatically nicked prior to grid preparation. Because of the viral origin, it is assumed that the sample contained no catenated dimers.

Grids prepared from 0.2 μg DNA had approximately the same DNA density as those used for the determination of the frequency of complex mt-DNA in the preceding publication. One thousand molecules were scored on each of two grids. The frequency of apparant catenated dimers was 0.8%; that of apparant catenated trimers was 0.03%. Only one third of the molecules scored as catenated dimers appeared as two tangent or overlapping monomers (fig. d_1 and d_2 of fig. 3 in the preceeding publication). The other molecules scored as catenated were tangled and would

have been scored as ambiguous molecules had they been in the mitochondrial DNA samples.

To ensure that the apparant dimers were not real dimers, 3 μg of the sample was used in an analytical band velocity experiment. If dimers had been present, they would have made up about 1.5% of the mass, or 0.05 μg in the velocity experiment, an amount which would have been detectable as a fast moving band at the sensitivity routinely used with the photoelectric scanner on our analytical ultracentrifuge. No such band was observed.

It is concluded that the contribution of accidental overlap of monomers to the frequency of carenated oligomers is very small when compared to the frequencies of such molecules usually detected in mitochondrial DNA. A slightly larger, but still small contribution to the error is made by tangled molecules which are scored as ambiguous catenated molecules.

(b) Molecular weight discrimination

A problem not considered in the preceeding publication is the possibility that molecules of different molecular weight might be transferred from the spreading sample to the specimen grid at different efficiencies. The difficulty of obtaining purified samples of monomers and dimers precludes a direct examination of this possibility with mitochondrial DNA. However, circular molecules of different size can be prepared from two different sources and mixtures of these can be examined by electron microscopy. This was done using PM-2 and SV40 viral DNA, whose molecular weights are

in the ratio of two to one. The DNA used was originally closed circular, purified by cesium chloride-ethidium bromide density gradient centrifugation of viral extracts.

Samples of the DNA's were prepared at the same concentration, as judged spectrophotometrically. The PM-2 DNA was closed circular; the SV40 had been nicked to about one nick per molecule. Specimen grids were prepared such that almost all the molecules were relaxed. Various mixtures of the two DNA samples were prepared and all the spreading samples were prepared using the same volumes of each of the various components. In the mixtures with small frequencies of PM-2 DNA, a diluted sample was used to prepare the mixtures. This dilution was done with volumes which were large compared to the volumes used to make the mixtures.

The results of the scoring of grids prepared from six such mixtures (Table 1) demonstrate that over a wide range of frequencies, the frequency of each component in the sample is accurately determined by electron microscopy. It is not known whether this is true for molecules with molecular weights greatly different from the ones used in this experiment.

Table 1

Scoring of the PM-2 - SV40 Mixtures

PM-2 Expected Mass %	PM-2 Observed Mass %	Molecules Scored
2	2 ± 0.7	3000
18	19 ± 2	2000
33.3	34 ± 2	2000
50	51 ± 2	2500
66.7	66 ± 1	2500
80	80 ± 1.5	2000

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Chapter 3.

Complex Mitochondrial DNA in Human Tumors

by

Charles Allen Smith and Jerome Vinograd

The following paper has been prepared for submission to Cancer Research.

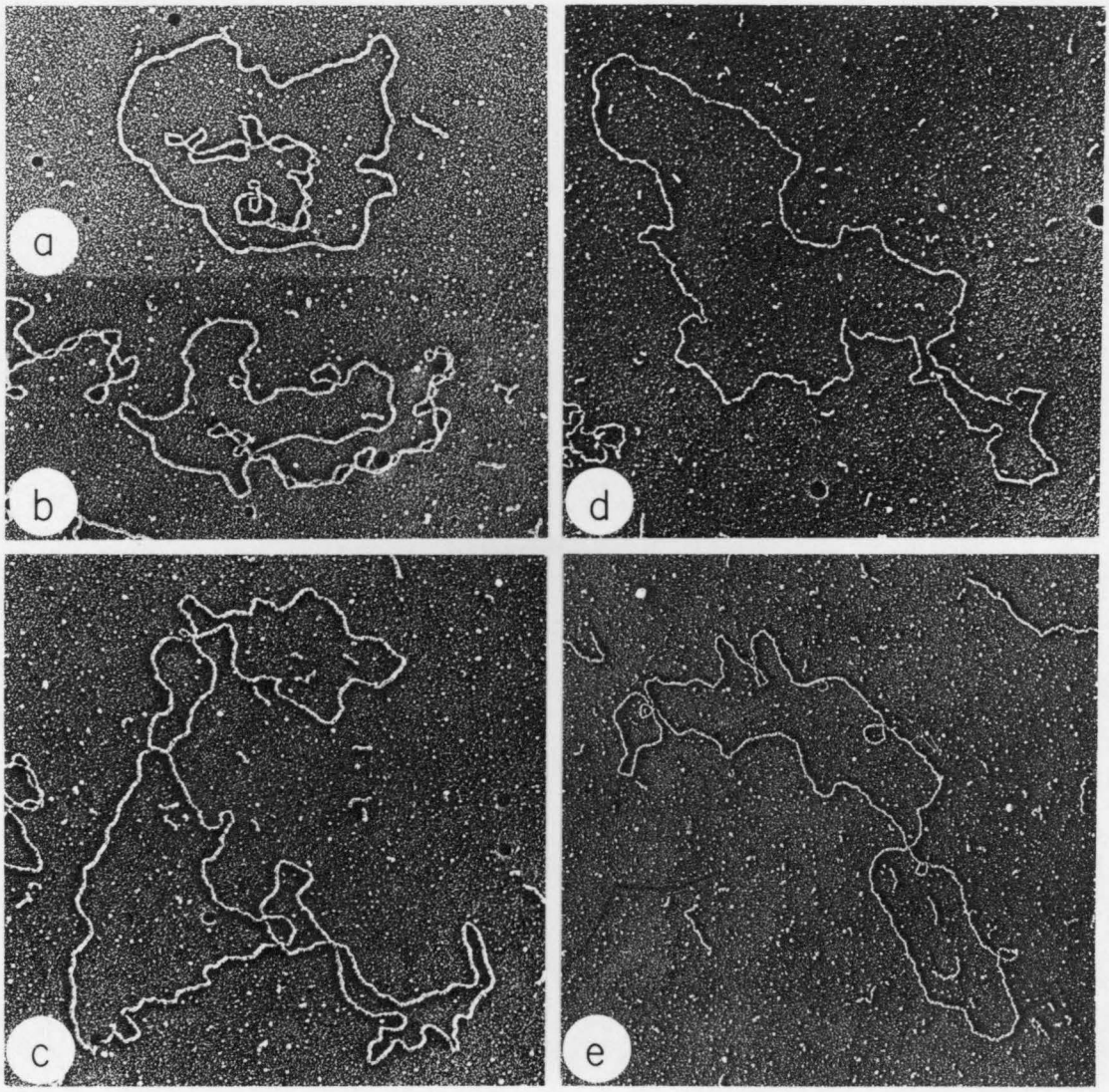
SUMMARY

The frequencies of complex mitochondrial DNA forms have been determined in the DNA extracted from a number of human solid tumors. The catenated forms in all but one sample were within the ranges of frequency reported previously in animal tissue. One sample contained 14% catenated dimers and 10% catenated higher oligomers. Circular dimers were present in 12 of 15 samples, ranging in frequency from 0.2 to 9%. No circular dimers were seen in a sample of human placenta. The results extend previous studies in which circular dimers were found to be present in leukocytes of patients with granulocytic leukemia, but not in leukocytes from non-malignant proliferations.

INTRODUCTION

It is now well established that the mitochondria in eukaryotic cells contain DNA. The mtDNA[2] in several plants has been reported to be linear and variable in size from organism to organism, although these may be degradation products of large circular molecules. On the other hand, the bulk of the DNA in mitochondria of animal cells from sea urchins to humans is in the form of a closed circular duplex (7) with a molecular weight of approximately 10^7 daltons (1, 2, 12, 16). A variable proportion of mtDNA in animal cells is in the form of complex mtDNA. Such complex mtDNA occurs in two forms: catenanes, in which two or more monomer-length circles are topologically bonded as the links in a chain (8), and circular dimers, circular molecules of twice the monomer size and consisting of two monomer genomes in a tandem arrangement (5) (Fig. 1). Catenated molecules, first seen in HeLa cells (8), have been demonstrated in the mtDNA of many animal cells studied (3, 6, 14), including several tissue culture lines (10, 13, 17). In tissues from laboratory animals, catenated dimers, containing two monomer circles, occur in frequencies of 5—9% of the mtDNA molecules; catenated higher oligomers, containing 3 or more monomer circles, make up from 0.5—2% of the mtDNA molecules. The mtDNA of cells in tissue culture may contain somewhat higher frequencies of catenated molecules. In contrast, circular dimers, which comprise 12—50% of the mtDNA in leukocytes of patients with granulocytic leukemia (3), were not detected in normal human leukocytes, leukocytes from patients with non-malignant proliferations of granulocytes (4), or

Fig. 1. Electron micrographs of mitochondrial DNA molecules from human tumors. a, A supertwisted monomer lying entirely within a relaxed monomer. b, Catenated dimer containing one relaxed and one supertwisted submolecule. c, Catenated trimer. d, Circular dimer with no crossover points. e, Circular dimer with two crossover points, neither of which divides the molecule into two equal parts.


a
b
d
c
e

in a variety of tissues from healthy laboratory animals (6). They have been seen in only a few tissue culture cells (9, 10). Recently, circular dimers have been reported in some viral-transformed cell lines and in human thyroid tissue, excised for various medical reasons (13).

It was shown that the frequencies of the circular dimer in the leukocytes of patients with granulocytic leukemia were reduced when patients were treated with antileukemic drugs (4). This and the fact that circular dimers were absent in all other tissues studied at the time this investigation was begun suggested a correlation between this mtDNA form and human neoplasia. In order to further study this correlation, the mtDNA of human solid tumors was investigated.

Leukemia offered several advantages for the study of mtDNA. Malignant leukocytes could be obtained without surgery, easily separated from other cell types, typed directly for degree of malignancy, and used for mtDNA preparation. In studying solid tumors, we relied upon the cooperating surgical pathologists to provide us with malignant tissue. Our criteria for the use of the freshly excised tumor samples were that preliminary observations indicate a high probability of malignancy and that the sample be resonably free from non-malignant tissue and grossly similar to the sample taken for pathological examination. It was also necessary that the sample have a soft consistency. A copy of the final pathology report was made available for tumor samples tested. To provide a control, mtDNA was prepared from a human term placenta.

The results of this preliminary survey extend the observation of the circular dimer form in human neoplastic cells. The presence of

the dimer was unambiguous in 12 of 15 cases in which analysis was possible. However, the frequencies found were all well below those seen in granulocytic leukemia, several being at our level of detection. It is not known whether these low frequencies are low due to tumor type or physiology, result from selective loss of malignant cells or their mitochondria, or are the result of dilution by mtDNA of contaminating non-malignant tissue.

MATERIALS AND METHODS

Human Tissue. Samples of tumor tissue from surgical excisions were provided by several pathologists in the area (see Acknowledgments), usually within 4 hr after excision. The samples were stored in either MS buffer (see below) or standard physiological saline, at 4°. A human term placenta was obtained 1 hr after removal by caesarian section.

Preparation of Mitochondria. Tumor tissue was minced into pieces approximately 3–5 mm^3 and washed extensively in MS buffer (0.21 M Mannitol, 0.04 M sucrose, 0.001 M EDTA, 0.01 M Tris, pH 7.5) or TD buffer (0.14 M NaCl, 0.005 M KCl, 0.007 M Na_2HPO_4, 0.025 M Tris, pH 7.5) to remove blood and fatty tissue. The tissue was then homogenized by a method which varied with the softness of the material. For tissue with some fibrous content, a large Dounce glass homogenizer fitted with a motor-driven nylon pestle was used to make a homogeneous suspension. If necessary, this suspension was filtered through cheesecloth to remove fatty tissue which does not homogenize. Usually some of this material collected on top of the pestle and was first washed back down into the suspension and later removed from the top, where it had collected again. Small samples were first homogenized in a small motor-driven Thomas homogenizer. Samples were then extensively homogenized in either the Dounce or a homogenizer of the Potter type fitted with tight-fitting teflon pestles. Homogenizations were usually done in MS; in some cases they were done in 0.01 M Tris (pH 7.5), 0.01 M NaCl, 0.001 M EDTA. In these cases sucrose was added to 0.25 M immediately following homogenization. Light microscopy at 400X using phase optics

was employed to monitor the homogenization. Exact determination of the extent of homogenization could not be done due to the wide variety of cell types in such masses. Re-homogenization was sometimes carried out if the amount of crude mitochondria obtained was much less than expected for the size of the starting sample. Homogenates were centrifuged at 2500 rpm for 5 min at 4° in an International centrifuge to remove cells, nuclei and debris. After another similar spin, the supernatant was centrifuged at 12,000 rpm for 20 min in a Sorvall SS34 rotor to pellet mitochondria, which were then washed once with MS buffer.

The placenta was extensively washed with TD and large pieces of tissue were cut away from the membrane. These pieces were minced and washed extensively, then homogenized in the Dounce glass homogenizer. All further operations were as described for the tumor samples.

Purification of Mitochondria and Preparation of mtDNA. In all cases, sucrose gradients were used to purify mitochondria. Mitochondrial pellets were resuspended in MS buffer (5 ml per sucrose gradient) and layered atop a step gradient consisting of 1.75, 1.5, and 1.0 M sucrose layers, each containing 0.001 M EDTA, 0.01 M Tris (pH 7.5). Centrifugation was in a SW 25.1 or SW 27 rotor at 22,000—25,000 rpm for 45—60 min at 4°. The material at the lower interface was collected and lysed after pelleting. This is a minor fraction of the mitochondria and is used only for preparation of closed DNA. The major portion of mitochondria, appearing at the center interface, was collected and pelleted. In most cases this fraction was then treated with DNase I as previously described (6). This step was omitted in cases in which only a small mitochondrial pellet was recovered. Lysis

of the mitochondria and preparation of the DNA with CsCl-EthBr[2] density gradient centrifugation have been described (15, 17). In cases in which DNase treatment had reduced the form II DNA to less than 50%, both fluorescent bands were collected. In other cases only the closed DNA was taken for analysis.

Electron Microscopy. DNA from the CsCl-EthBr gradients was used directly for electron microscopy when concentrations warranted. If little or no material was seen, the region in which form I DNA would band was collected and the DNA concentrated by pelleting overnight at 35,000 rpm, 20°, in polyallomer tubes. All but 0.2 ml was drawn off and the tubes were agitated on a Vortex mixer and allowed to stand for several hours to resuspend the DNA. Preparation of electron microscope specimen grids and the scoring rules for classifying molecules have been previously described (8). In cases in which the frequency of the circular dimer was very low, all examples of the form seen were photographed and measured.

RESULTS

Frequencies of Complex DNA. Mitochondrial DNA was prepared from a total of 21 human tumor samples, and from one human placenta. The results from 6 of the samples are not presented. All 6 samples were small and little mtDNA was recovered. In one case the DNA was exhausted before electron microscope grids suitable for scoring were obtained. In 3 cases a limited number of molecules were scored on grids which were suitable, and the frequency of catenated dimers was less than 2%, as compared with the usual frequency of 5—9%. In these cases only the closed mtDNA from the lower bands in EthBr-CsCl gradients was used for analysis. A process which nicks the closed mtDNA before isolation of closed DNA has the effect of removing dimer molecules (both catenated and circular) from the closed DNA twice as fast as monomer molecules, due to the increased target size for nicking in the dimers. The low frequency of catenated dimers is taken to be indicative of extensive nicking, which would have also lowered the frequency of circular dimers by a factor of 3 to 5, so that dimers in the 0.2—0.4% frequency range would have become undetectable. Two samples did not afford enough molecules for adequate estimation of frequencies in the 0—0.5% range.

The scoring results for the remaining 15 samples and the placenta are given in Table 1. The samples are grouped as tumors of the germ line, carcinomas, sarcomas, and tumors which do not easily fall into these categories. They are listed in order of decreasing frequency of the circular dimer.

Table 1

Complex mitochondrial DNA in human tumors

Sample‡	Frequency of forms (%)†				
	Monomers	Circular dimers	Catenated dimers	Catenated oligomers	Molecules classified
Malignant tissue					
Teratocarcinoma, ovary (LB)[b]	83.0 ± 2.5	8.9 ± 1.8	6.2 ± 1.6	1.9 ± 0.9	884
Seminoma, testis (LB)[a]	86.4 ± 1.8	0.3 ± 0.2	9.6 ± 1.5	3.7 ± 1.0	1445
Granulosa cell carcinoma, ovary[a]	70.5 ± 2.6	5.1 ± 1.2	14.2 ± 1.9	10.2 ± 1.7	1164
Squamous cell carcinoma, metastatic, groin[b]	90.7 ± 1.5	2.6 ± 0.8	5.9 ± 1.2	0.8 ± 0.5	1400
Squamous cell carcinoma, foot (LB)[c]	91.4 ± 1.5	1.1 ± 0.6	6.1 ± 1.3	1.4 ± 0.6	1283

. . . Table 1, continued.

Adenocarcinoma, salivary gland[d]	93.0 ± 1.1	0.8 ± 0.4	5.4 ± 1.0	0.8 ± 0.4	1953
Carcinoma, lung[a]	88.9 ± 1.3	0.2 ± 0.2	8.5 ± 1.2	2.4 ± 0.6	2040
Carcinoma, breast[c]	87.8 ± 1.1	0	9.2 ± 1.0	3.0 ± 0.6	3175
Neurofibroma or fibrosarcoma (LB)[c]	90.6 ± 1.8	1.8 ± 0.8	6.3 ± 1.5	1.3 ± 0.7	1011
Rhabdomyosarcoma, alveolar[b]	88.6 ± 1.2	0.4 ± 0.2	8.6 ± 1.0	2.4 ± 0.3	2526
Liposarcoma, breast (LB)[b]	96.8 ± 0.6	0.2 ± 0.2	2.8 ± 0.6	0.2 ± 0.2	2700
Glioblastoma, brain[e]	88.9 ± 1.4	0.4 ± 0.4	8.5 ± 1.7	2.2 ± 0.9	1000
Osteogenic sarcoma, tibia[b]	93.9 ± 1.2	0	4.9 ± 1.0	1.2 ± 0.5	1500
Mesothelioma, omentum (LB)[b]	96.5 ± 1.0	0.4 ± 0.3	3.0 ± 0.8	0.1 ± 0.1	1500

. . . Table 1, continued.

Myeloma, metastatic, ileum (LB)[d]	94.5 ± 0.8	0	4.9 ± 0.8	0.6 ± 0.3	2500
		Normal tissue			
Placenta[a]	94.7 ± 0.8	0 (< 0.1)	4.7 ± 0.8	0.6 ± 0.3	3000

† The errors given are the statistical sampling errors at the 95% confidence limit.

‡ (LB) designates those scorings done on closed circular DNA only.

Diagnoses were provided by the surgical pathology department of the hospitals indicated: [a] Huntington Memorial Hospital, Pasadena, Calif.; [b] University of Southern California-County Medical Center, Los Angeles, Calif.; [c] City of Hope Medical Center, Duarte, Calif.; [d] White Memorial Hospital, Los Angeles, Calif.; [e] St. Luke Hospital, Pasadena, Calif.

The tumors present a wide range of values for all types of complex mtDNA. The circular dimer is unambiguously presnt in 12 of the 15 cases. In 6 of these, however, the frequency is very close to our level of detection. In only two cases was the level above 5%, and in no case was a frequency seen which approaches frequencies in cases of granulocytic leukemia. In 3 samples circular dimers were not detected. The placental mtDNA confirms our earlier conclusions that the circular dimer is absent from normal tissue. One circular dimer was seen in scoring 3000 molecules, which indicates that whatever process results in dimer formation it is under tight control in non-malignant tissue. Finding one molecule also gives some assurance that the investigator scoring does see the circular dimer even if it is present at very low frequencies. The catenated dimer and oligomer frequencies seen in the placental sample are in accordance with frequencies detected in other animal tissue.

The frequencies of catenated dimers and higher oligomers in 12 of the tumor samples fall within the ranges seen in tissues from laboratory animals. Two samples had substantially lower frequencies of catenated dimers and very low frequencies of catenated higher oligomers. In both of these cases, only closed circular DNA was analyzed. Substantial nicking is a probable explanation for the low values. One sample, the granulosa cell carcinoma, contained very high frequencies of complex DNA. In addition to circular dimers, this sample contained the highest level of catenated dimers seen in tissue <u>in situ</u> and the highest level of higher oligomers ever seen, roughly 10% by number. A

more detailed analysis gives 5.2% trimers, 2.3% tetramers, 1% pentamers, and 1% oligomers containing more than 5 submolecules. It was shown that these were not electron microscope artifacts by roughly fractionating the sample with a preparative CsCl velocity experiment. These large forms were found near the bottom of the the gradient and the material near the top contained very few such molecules. The molecules do not give the appearance of aggregation at a single point when seen in the electron microscope. In addition, the sample contained about 0.5% molecules which appear to be circular dimers catenated to monomers. Because of the possibility of occasional overlap, the true nature of these molecules remains unclear. They were not seen in any quantity, however, in samples from leukemic leukocytes containing 12—50% circular dimers (4).

Buoyant Properties of the mtDNA. Prior to this work, values obtained in this laboratory for buoyant densities of human mtDNA were from leukemic cells (5) or from HeLa cells (17), which have been growing in culture for many years. The neutral buoyant densities for one tumor sample and the placental DNA were found to be in agreement with previously obtained values. The buoyant separations in alkaline CsCl of the separate single strands for one tumor sample and for the placental DNA were also determined and compared in the same manner. The absolute values obtained for the separated stands in alkali for the placental sample were each 0.001 g/cc lighter than the values obtained previously. This is considered within experimental variation when a single determination is made.

DISCUSSION

The results of this study extend the correlation between human neoplasia and the presence of the circular dimer form of mitochondrial DNA in the neoplastic cells. Detailed interpretation of the variation of frequencies of the form, as well as the meaning of the fact that three samples were found to be free of circular dimers, is made difficult by several factors.

1. Normal tissue. The frequencies found are very sensitive to the presence of normal tissue in the sample. All tumors contain some normal vascular and connective tissue, but this amount is variable. It is difficult to assess this contribution and, indeed, no direct attempt was made to do so. Detailed microscopic examination was of necessity done on other portions of the tumor, and we rely on the pathologist's gross examination for the assurance that our sample was not heavily contaminated with non-tumor cells. Some selection may have taken place in the homogenization of the sample. It was presumed that the softer tumor tissue would be more easily homogenized than the connective or vascular tissue, but in certain cases this may not have been valid. The sample from osteogenic sarcoma, for example, was very difficult to homogenize due to small particles of bone present in the tumor and to the bone which was removed with the tumor. It is possible that mitochondria from tumor tissue are more sensitive to the fractionation procedures and DNase treatments used, although we do not regard this as a strong possibility.

2. Tumor physiology. Tumors vary in size, growth rate, oxygen

supply, and age. Results by Nass (11) indicate that at least in one line of cultured cells the level of circular dimer may be influenced by the growth conditions of the cells. If this were to be the case generally, the fluctuation in dimer levels seen here could be strongly affected by such factors.

3. Degree of differentiation or malignancy. Pathologists often classify tumors on the basis of the differentiation of the cells in the tumor and generally accord less differentiated cells more "malignancy." No obvious correlation can be made in the cases surveyed here.

4. Type of neoplasia. The correlation between neoplasia and the circular dimer may be determined by the cause of the cancer itself, or may be in part determined by the type of cell which gives rise to the malignancy. In the absence of a general model for all the different types of cancers, and without several samples for each kind of malignancy, we cannot begin to analyze the variations seen here.

The correlation between human neoplasia and the circular dimer form has recently been called into question by results of Paoletti and Riou (13). They found the circular dimer in seven human thyroids excised for a variety of medical reasons. The dimer frequency ranged from 11—48% by mass. In three cases adenomas were present, one case involved an epitheloma, one patient was suffering from acute lymphoblastic leukemia, and in two cases the thyroid was removed due to thyroid condition. Although malignancy is not involved in most of these cases, none of them can be thought of as normal tissue. It is possible that the circular dimer correlation reflects a metabolic state

which is also present in these thyroid conditions. Until the mtDNA of human tissue not involved with a pathological condition is examined, the question of whether circular dimers are present in normal tissue remains undecided. Circular dimers were absent from normal human leukocytes and placental tissue.

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Footnotes

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[2] The abbreviations used are: mtDNA, mitochondrial DNA; EthBr, ethidium bromide.

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Chapter 4.

Complex Mitochondrial DNA of Mouse L Cells

INTRODUCTION

Catenated mitochondrial DNA has been shown to occur in several tissues from various laboratory animals (1), sea urchin eggs (2), normal and neoplastic human tissue (3, 4, 5), and several cell lines (6, 7, 8). The circular dimer form of mtDNA was seen only in human neoplasms, which suggested a correlation between the circular dimer and some aspect of the neoplastic cell. Study of the relationships between the circular dimer and other forms of mtDNA and cellular functions was not possible because of this rather restricted location. Cell lines were examined for the presence of this form in the hopes that the manipulations possible with cultured cells could be used to advantage in studying the origin and cellular role of the circular dimer.

Two lines of mouse L cells were investigated. These cells are among the oldest culture lines, originally derived from subcutaneous tissue of a mouse which had been treated with methylcholanthrene (9). The cells were originally highly malignant, and with time their tumor producing capacity in adult mice has declined, but tumorigenicity in newborn mice remains high (10). One of the L cell lines was found to contain a low frequency of circular dimers; another line was found to contain this form almost exclusively.

While this investigation was in progress, Nass (11) reported that the frequency of circular dimers in a line of L cells could be increased from about 8% to over 60% by manipulating culture conditions or by treating cells with cycloheximide. Previous

investigations in this laboratory (11) had shown that cycloheximide had no effect on the complexity of HeLa cell mtDNA. However, because HeLa cells contain no circular dimers, it was thought possible that only cells initially containing this form could be induced to produce them in high frequency. Some of the experiments reported by Nass were repeated with the L cells in this laboratory. All results were negative. The cells containing almost 100% circular dimers were also treated with a variety of cytotoxic drugs used in the treatment of cancer. None affected the frequency of circular dimers. The characterization of the L cell mtDNA by electron microscopy, and the attempts to manipulate circular dimer frequencies were done in collaboration with M. Teplitz and D. Clayton.

MATERIALS AND METHODS

Cells and growth conditions

Clone 929 of mouse L cells was obtained from the American Type Tissue Culture Collection, Rockville, Md. The cells were first grown on plates in Eagle's medium supplimented with 10% calf serum, and later adapted to growth in suspension in Eagle's phosphate medium, also supplemented with 10% calf serum.

Another line of L cells, now called L_D cells (13) was obtained from Dr. Carl Schildkraut, Albert Einstein College of Medicine, New York. These cells were grown in the same media as the 929 cells.

Methods

The methcds for isolation and purification of DNA (5), electron microscopy of the mtDNA for the determination of the frequencies of the various forms (1), and the use of the analytical ultracentrifuge for the determination of buoyant densities and sedimentation coefficients (5) have been described.

RESULTS

(a) Electron microscopy of the mtDNA

Total mtDNA from clone 929 cells contained 87% monomers, 7% catenated dimers, 1% catenated higher oligomers and 4% circular dimers, as determined by electron microscopy. In contrast, the L_D cells contained no detectable monomers. The majority of the molecules seen were unambiguous circular dimers. Approximately 20% of the molecules seen were ambiguous and could have been catenated dimers. About 10% of the molecules appeared to be dimers catenated from 10μ-circles.

(b) Sedimentation properties

The DNA from clone 929 mitochondria which had been incubated with DNase to digest nuclear DNA was fractionated into closed and nicked DNA with a CsCl-ethidium bromide density gradient. When the nicked DNA was subjected to analytical band sedimentation, a major band was seen sedimenting at 26S, the value reported for nicked monomers (14). A small band with a greater sedimentation rate was also observed, and is presumed to be nicked dimers. Two species with sedimentation coefficients of 35 and 48 S were observed when the closed DNA was sedimented. The 35 S species comprised about 80% of the DNA. These sedimentation coefficients are slightly lower than the reported values for monomers (37 S) and dimers (51 S), possibly due to concentration dependent sedimentation, or a slight mis-estimation of the density of the

sedimentation solvent. When the lower band DNA was sedimented through CsCl at pH 12.5, two fast-moving bands were observed which had uncorrected sedimentation coefficients of 75 and 105 S. These are the values of closed monomers and dimers; the mass ratio was similar to that observed in the sedimentation at neutral pH. A third band with an approximate sedimentation coefficient of 21 S was also observed. This represents the separated strands of DNA nicked before the run or during the initial exposure to alkali. The sedimentation coefficient corresponds to a single strand molecular weight of about 6×10^6. The calculation was made by converting the observed coefficient to a standard coefficient using the ratio of these two coefficients known for polyoma DNA, and calculating the molecular weight from the relation obtained by Studier (15). The sedimentation results confirm the determination made by electron microscopy that the bulk of the mtDNA of clone 929 cells is in the form of the monomer.

When the closed mtDNA from L_D cells was sedimented at neutral pH, the major component had a sedimentation coefficient of 51 S. A minor component sedimented at 34 S, as did all of a nicked sample of this DNA. When this closed mtDNA was sedimented in alkaline CsCl, a single fast-moving band was observed, which had an uncorrected sedimentation coefficient of 103 S. No band with the sedimentation coefficient of closed monomers was observed. The band of single strand DNA had a sedimentation coefficient of 25 S. This corresponds to a molecular weight of 9.3×10^6. These results

confirm the observation made by electron microscopy that almost all the mtDNA of the L_D cells is in the form of dimers. The single strand molecular weight suggests that the majority of the dimers are of the circular type.

(c) Buoyant properties

The buoyant densities of both nuclear and mitochondrial DNA of both lines were determined in neutral CsCl. The mtDNA of each line had a buoyant density of 1.692 g/ml. An approximate ratio of molecular weights was made by comparing the bandwidths of the bands of mtDNA from the two cell lines. The ratio obtained was 1.8. Nuclear DNA from both lines contained a major species with a density of 1.692 g/ml, and a minor species at 1.682 g/ml. The light satellite is characteristic of mouse DNA. All values were determined using a Micrococcus marker assumed to have a density of 1.725 g/ml, and the buoyant gradient of Vinograd and Hearst (16).

(d) Attempts to influence the frequency of circular dimers

The clone 929 cells had a frequency of circular dimers similar to that of the cells studied by Nass (11). Some of the experiments she reported were repeated with these L cells. As it was known that the circular dimer frequency in circulating leukocytes of leukemic patients was reduced after chemotherapy, the L_D cells were also treated with some chemotheraputic agents to see if any specific effect upon circular dimer frequency could be found.

i) Cell crowding. Nass harvested cells and resuspended

them in fresh medium at concentrations similar to concentrations of cells in stationary phase. She reported that after two days of such treatment, the closed mtDNA contained over 60% circular dimers, whereas growing cells contained 8%.

Clone 929 cells were treated in a similar way, and the closed mtDNA extracted after two days of treatment and analyzed by analytical band sedimentation. The percent of DNA sedimenting at 51 S was no greater than that found previously in mtDNA of exponentially growing cells.

ii) Cycloheximide treatment. Nass also reported that a 24 hour treatment with cycloheximide at a concentration of 25 μg/ml resulted in a total dimer frequency of 63%. Both clone 929 and L_D cells were treated in this manner. Again, sedimentation velocity failed to demonstrate any change in the frequency of dimers in either cell line.

iii) Non-essential amino acids. Nass had reported that the circular dimer frequency increased when the cells were deprived of methionine or phenylalanine, and that the frequency was rapidly restored to the low value after the amino acids were restored. It was thought that the high frequency of circular dimers in the L_D cells might be due to a mutation which resulted in a requirement for an amino acid not normally added to cell growth medium. L_D cells were grown in the presence of all the normally non-essential amino acids, and the mtDNA examined by electron microscopy and sedimentation velocity. The frequencies of complex forms were

identical to those in mtDNA of cells grown simultaneously in medium without the amino acids. The sedimentation analyses of both samples also showed a minor component with a sedimentation coefficient of 70 S. This corresponds to a closed DNA with a molecular weight of 40×10^6, and probably represents molecules catenated from two circular dimers, as these have been seen in the electron microscope, whereas no circular tetramers have been seen.

iv) Treatment with cytotoxic drugs. Treatment of patients with granulocytic leukemia with the alkylating agent Myeleran, (1,4 bis(methane sulfonoxyl)butane), or the base analogue 5-mercaptopurine had been shown to reduce the frequency of circular dimers in the mtDNA of circulating leukocytes. L_D cells were treated with these two agents and also with the metabolic inhibitor of nucleic acid synthesis, fluorodeoxyuridine. No monomers were generated as judged by electron microscopy or analytical band sedimentation. The same result was obtained when the cells were treated with allopurinol, a drug which is sometimes used to remove uric acid from leukemics before chemotherapy.

DISCUSSION

Two different lines of mouse L cells were found to contain the circular dimer form of mitochondrial DNA. Clone 929 cells contain circular dimers in low frequency; similar results were reported for a line of mouse L cells by Nass. Another L cell contains almost 100% circular dimers . This cell was accordingly designated L_D. Early electron microscopic analysis of the mtDNA from these cells indicated that catenanes composed of two circular dimers might be present in these cells in frequencies similar to the frequencies of catenated dimers in cells whose major major mtDNA constituent is monomers. However, the first sedimentation analyses of the L_D mtDNA failed to show any evidence of such a species. These large molecules may have been lost from the samples before sedimentation, or in these molecular weight ranges the usual observation that molecules tend not to overlap on electron microscope grids may not hold. Later sedimentation analyses did reveal a component with the sedimentation coefficient expected for this species. It is possible that these catenated forms were increasing in frequency during the time of these investigations. It is difficult to determine accurately the frequency of catenated dimers of the more usual variety in these cells. When dimers are the major species in the population, the frequency of ambiguous molecules with dimer mass becomes much more significant than in the case in which the total dimer frequency is rather small. One attempt was made to purify catenated dimers by lightly nicking a

sample of L_D mtDNA and isolating the middle-band region of a CsCl-Ethidium bromide gradient containing this DNA. No catenated dimers were found by electron microscopy in this region. However the amount of DNA available was small and the experiment would have to be repeated before definite conclusions could be drawn.

It appears that at some point, the circular dimer replaced the monomer as the "simple" species of mtDNA in the L_D cells. We were unable to repeat this change with clone 929 cells under controlled conditions in the laboratory, using techniques which had been reported to vastly alter the circular dimer frequency in a line of similar cells. It should be noted that the conditions used by Nass to increase dimer frequency all inhibit the continued growth of the cells, whereas L_D cells containing all dimers or increased frequencies of dimers must have been able to grow at least as well as the original cells from which they came. Because there is a minimum cell concentration below which animal cells do not grow in culture, it is unlikely that a single cell with a high dimer frequency was selected at some point. More plausable possibilities are that some event altered the frequency of circular dimers in a group of cells which then either outgrew the other cells or were selected in some other way, or that some gradual alteration occurred over a period of time in all the cells in a culture, and that once the monomers were lost there was no pathway for their regeneration. These dimers in L_D cells apperantly replicate as dimers. No monomers have been seen, and two D-loops, thought to be involved

in initiation of DNA replication, have been seen diametrically opposed on dimer molecules from these cells (13). The details of how the mitochondrial DNA replication system accommodates molecules with two initiation sites may add much to our understanding of mtDNA replication.

Subsequent to these investigations, the mtDNA of the clone 929 cells growing on plates was found to have been converted almost exclusively to circular dimers. Attempts to repeat this conversion under controlled conditions by D. Clayton and L. Grossman have not succeeded. Circular dimers have also been observed in high frequencies in a line of SV40 transformed mouse 3T3 cells by J. Jordan. A gradual increase in frequency of the circular dimers was observed over several cell generations (17). The dimer frequency in these cells is also unaffected by maintenance at high cell density.

It is now established that mouse cells in culture can and do produce circular dimers. Further study and perhaps not a small amount of luck will be needed to realize the hopes for controlled manipulation of dimer frequency which prompted this investigation.

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Chapter 5.

Sedimentation Velocity of Doubly Nicked Catenated Dimers

INTRODUCTION

Hudson and Vinograd studied the sedimentation properties of the various forms of DNA which comprize the mitochondrial DNA (mtDNA) of HeLa cells. The closed circular 5-μ monomer circle had a standard sedimentation coefficient of 37.1 ± 0.3 S. The sedimentation coefficient of the nicked monomer was 26.1 ± 0.1 S. These values were in agreement with the expected values, calculated from the molecular weight of this form determined by electron microscopy and relations between sedimentation coefficient and molecular weight derived from the known values for other circular molecules. The covalently closed catenated dimer had a sedimentation coefficient of 51.3 ± 0.5 S, which is the expected value for a circle of twice the molecular weight of the monomer. This form occurs in the mtDNA of leukemic leukocytes; its sedimentation coefficient had been reported to be 51 ± 1.4 S (2). When partially nicked HeLa mtDNA was sedimented, species with standard sedimentation coefficients of 39.7 ± 1.3 S and 23.0 ± 0.4 S were observed. These were assigned to the catenated dimer with one and two nicked sub-molecules, respectively. The value for the doubly-nicked catenated dimer contrasted sharply with the reported, and expected, value of 33.4 ± 1 S for the nicked circular dimer.

This apparant difference in sedimentation coefficients between the two forms of dimeric mtDNA, when nicked, offered a physical method for the estimation of the frequencies of these forms in a sample of mtDNA. Previously, only electron microscopy had been used for this purpose, and not all molecules seen in the electron microscope may

be identified with confidence. In a study of the complex mtDNA of human tumors, several attempts were made to use sedimentation to demonstrate the presence of circular dimers in samples containing about 20% circular dimers, as determined by electron microscopy. In the sedimentation analyses of nicked DNA of these samples, no 23 S species were observed, even though the presence of catenated dimer was well established. This failure to detect the expected 23 S species led to a reinvestigation of the sedimentation coefficient of the doubly-nicked catenated dimer.

MATERIALS AND METHODS

Mitochondrial DNA

Two sources of mtDNA from HeLa cells were used. DNA was obtained from partially purified mitochondria obtained in the preparation of cell cytoplasm used in the study of the small polydisperse circular DNA of HeLa cells. The preparation of mtDNA from mitochondria has been described (3). Closed circular mtDNA was also a product of several preparations of total closed circular DNA from HeLa cells which had been treated with cycloheximide. The methods used to prepare the closed circular DNA and separate mtDNA from small circular DNA are described in Part II of this thesis. All DNA was originally isolated as closed circular with CsCl-ethidium bromide, or CsCl-propidium di-iodide density gradients.

Preparation of singly nicked catenated dimers

The mtDNA from the first source named above was made to contain 100 μg ethidium bromide/ml and irradiated with fluorescent room light for 12 hours. The dye was removed with a small column of Dowex-50 resin, and the DNA was rebanded in a CsCl-propidium di-iodide gradient. The tube was fractionated by drop collection and the region containing the band between the bands of closed and nicked DNA was taken. This should contain catenated dimers in which only one of the sub-molecules has been nicked. The closed circular DNA was re-illuminated, rebanded, and a second middle band collected. The DNA from the other source noted above was banded; it had been handled considerably

and a substantial middle band was present, which was isolated.

Preparation of doubly nicked catenated dimers

The pooled DNA from the middle bands was made to contain 100 μg propidium di-iodide/ml and illuminated for 24 hours eight inches from a 60 watt incandescent bulb. The DNA was banded in a CsCl-propidium di-iodide gradient. The upper band contained about 70% of the DNA, and was isolated. The dye was removed as above and the DNA was concentrated by centrifuging overnight in an SW50 rotor, 36000 rev/min, 20° C. All but the lower 0.1 ml of the solution was drawn off, and the pellet of DNA was resuspended in this volume.

Electron microscopy

The techniques for examining the DNA in the electron microscope, and the rules for classifying the molecules seen have been previously described (3).

Analytical band sedimentation velocity

The methods used were the same as those described by Hudson and Vinograd (1).

Estimation of the mass ratios in the band velocity experiments

Photoelectric scanner traces showing the band of interest at the center of the cell were magnified and displayed on a Nikon 6F projection microcomparitor. The magnified images were traced onto graph paper and the band areas calculated by counting the graph squares contained within them.

RESULTS

(a) Characterization of the nicked mtDNA by electron microscopy

97% of the DNA was circular. Catenanes containing more than two sub-molecules were estimated to comprize 5 to 10% of the mass. No circular dimers were seen. The ratio of mass of monomers to catenated dimers was determined by classifying 500 molecules, disregarding other forms. Of the two types, catenated dimers comprized 42 % of the mass.

(b) Sedimentation velocity experiments

Two analytical band velocity experiments were performed with the sample. In both cases, only two bands were seen. In one experiment, the amount of DNA utilized led to considerable skewing of the bands in a manner indicative of concentration-dependent sedimentation. The approximate sedimentation coefficients of the two species were 25 S and 36 S. A second experiment with a smaller amount of the sample was performed. The skewing was absent, and the two components observed had standard sedimentation coefficients of 25.5 ± 0.2 S and 35.4 ± 0.3 S. Of the two bands, the 35 S component comprized 40% of the mass. It is concluded that this component is the nicked catenated dimers.

DISCUSSION

The standard sedimentation coefficient of the doubly-nicked catenated dimer was found to be slightly greater than than that reported for the nicked circular dimer. This is in accord with the qualitative notion that the topological interlocking of the two circles results in a radius of gyration slightly smaller than that of the single circle of equal molecular weight. It is also close to the value predicted by an equasion recently presented by Wang (4), which allows the estimation of the sedimentation coefficient of a catenated molecule from the known sedimentation coefficients of its sub-molecules. The equation has been shown to give reasonable estimates of the sedimentation coefficients of catenanes artificially constructed from lambda DNA and some smaller circular DNA's.

After this study was completed, Brown and Vinograd prepared several species of complex HeLa mtDNA in substantial purity and examined their sedimentation properties. They report (5) a value of 36.5 ± 0.6 S for the doubly nicked catenated dimer. This value, obtained from several experiments, is slightly higher than the value reported here. Their value for the nicked monomer is also slightly higher than that reported here. If the value given here for the sedimentation coefficient of the doubly nicked dimer is corrected by the ratio of the two monomer values, it becomes 36.4 S. Brown and Vinograd also discuss the probable reasons for the earlier misestimation of the sedimentation coefficient of the doubly nicked catenated dimer.

Electron microscopy remains the only method for estimating the frequencies of both catenated and circular dimers in a sample which also contains monomers. In theory, a sample containing only catenated and corcular dimers could be analyzed by sedimentation of the nicked DNA in a denaturing solvent, where the frequency of dimer-length single strands might be determined. In the case of mitochondrial DNA, the alkaline lability rules out high pH as the denaturing solvent. At present there are no other convenient denaturing solvent systems.

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Chapter 6.

The Effects of Drugs on the Superhelix Density of Animal Mitochondrial DNA

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In vivo Effects of Intercalating Drugs on the Superhelix Density of Mitochondrial DNA Isolated from Human and Mouse Cells in Culture

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The degree of supercoiling (superhelix density) of closed circular mitochondrial DNA was found to increase three- to fivefold when the intercalating drug ethidium bromide was added to exponentially growing cultures of HeLa cells and to an SV40 virus-transformed line of mouse cells (SV3T3). This structural change was complete within ten hours in cells growing at a normal rate in the presence of 0·1 μg of the drug/ml., and within a shorter time at 1 μg/ml. Mitochondrial DNA synthesis, as indicated by incorporation of [^{32}P]orthophosphate and [^{3}H]thymidine, is essentially completely inhibited at the higher drug level and is greatly reduced at the lower drug level. The above results demonstrate that mitochondrial DNA is subject to nicking–closing cycles in the presence of the drug.

Four acridine derivatives, known to unwind DNA upon binding, have similar *in vivo* effects on the superhelix density of mitochondrial DNA. The ethidium analogue, propidium di-iodide, has no effect, but appears on the basis of fluorescence microscopy to be excluded from the cells. Actinomycin D also has no effect.

Ethidium bromide had no detectable effect on the base composition of mitochondrial DNA as indicated by buoyant density studies in neutral and alkaline cesium chloride. The drug had no effect on the distribution of complex mitochondrial DNA (circular dimers and catenated oligomers) measured with the electron microscope.

1. Introduction

Closed circular DNA's, when isolated from viruses, tissues, or cells in culture, all contain tertiary turns and are, therefore, described as *superhelical*, *supercoiled*, or *twisted* molecules (Vinograd, Lebowitz, Radloff, Watson & Laipis, 1965; Bauer & Vinograd, 1971).While the biological mechanisms responsible for the supercoiling of the isolated DNA's are still unknown, it is possible to group the conceivable mechanisms into two general and non-exclusive categories. (1) The DNA is supercoiled when the last phosphodiester bond is made. Such a mechanism requires a chemical interaction between the DNA and organizing substituents in the cell. (2) The DNA molecule is free of supercoils when closure occurs, but the duplex is in a partially unwound

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state with a reduced average angle of rotation of the bases along the duplex axis. Upon isolation of the DNA the average rotation angle increases to that of the *B* form of the duplex, and the supercoiling occurs to satisfy the requirements of the topological invariance in closed duplex DNA. The extent of supercoiling is an index of the winding number of the DNA when the axis is constrained to lie in a plane. This topological winding number, α, is an invariant which cannot be changed without nicking and reclosing one of the single strands in the circular duplex.

It has recently been shown that the superhelix density, σ_0, the number of tertiary turns per ten base pairs, may be arbitrarily regulated *in vitro* by closing nicked circular DNA with polynucleotide ligase in the presence of controlled amounts of the intercalating drug ethidium bromide (Hudson, Upholt, Devinny & Vinograd, 1969; Wang, 1969). This drug reduces the rotation angle in DNA and unwinds the duplex. Upon removal of the drug after closure, the rotation angle increases and a regulated amount of supercoiling occurs. The same drug has been found selectively to inhibit mitochondrial RNA synthesis in HeLa cells (Zylber, Vesco & Penman, 1969) and to act as a powerful inducer of petite mutants in yeast (Slonimski, Perrodin & Croft, 1968). It has been reported to modify the circular structure of kinetoplast DNA in trypanosomes (Riou & Delain, 1969). The foregoing results suggest that ethidium bromide is taken up by mitochondria, and have led us to ask whether the drug has any observable effect on the structure and base composition of animal cell mitochondrial DNA *in vivo*. We have found that, indeed, the drug induces large changes in the superhelix density of mitochondrial DNA. Since we have also found that new mitochondrial DNA synthesis is effectively inhibited by the drug, we conclude that pre-existing mitochondrial DNA is nicked and reclosed during the treatment with the drug. The buoyant density of the DNA in CsCl is unaffected, a result that indicates that significant base compositional changes do not occur. The frequency of complex forms, catenanes and circular dimers, in the mitochondrial DNA (Clayton, Smith, Jordan, Teplitz & Vinograd, 1968; Clayton & Vinograd, 1969) is also unaffected by the drug.

2. Materials and Methods

(a) *Isolation and purification of mitochondrial DNA*

(i) *Closed mitochondrial DNA from HeLa cells*

Suspension cultures in Eagle's phosphate medium (Grand Island Biological Co., Berkeley, Calif.) containing 5% calf serum were centrifuged at 1500 *g* and thereafter maintained at 0 to 4°C. Cultures containing ethidium bromide were grown in the dark. Cell pellets were diluted with a 7- to 10-fold vol. of buffer I (0·01 M-NaCl, 0·0015 M-$MgCl_2$, 0·01 M-Tris, pH 8·0), allowed to swell for 10 min, and disrupted with 2 to 5 strokes in a Dounce homogenizer. Sucrose was added to a concentration of 0·25 M, and crude mitochondria were isolated by differential centrifugation. The mitochondria were purified by sedimentation through 1·0 M-sucrose to an interface with 1·5 M-sucrose. They were then washed once with buffer II (MS of Clayton *et al.*, 1968); the mitochondria were resuspended and incubated at 20°C for 15 min in 1% sodium dodecyl sulfate. The mixture was then made 1·0 M in CsCl and chilled for 15 min. The density of the supernatant remaining after centrifugation for 15 min at 13,000 rev./min was adjusted to 1·57 g/ml. with solid CsCl; propidium di-iodide was added to approximately 400 μg/ml., and 3-ml. samples centrifuged for 24 hr at 38,000 rev./min at 20°C, in an SW50.1 rotor. The tubes were fractionated and the lower band of DNA collected. Propidium di-iodide was removed with a Dowex-50 resin column. Previously prepared nicked HeLa mitochondrial DNA was added for superhelix density determinations.

(ii) *Total mitochondrial DNA from HeLa cells*

The mitochondrial pellets from the sucrose gradients (see above) were taken up in buffer III (0·21 M-mannitol, 0·07 M-sucrose, 0·01 M-$MgCl_2$, and 0·01 M-Tris, pH 7·4) at 37°C. The mixture was incubated at 37°C for 30 min after the addition of 40 μg/ml. each of DNase I and RNase A. The reaction was terminated by adding EDTA to a concentration of 0·05 M and by chilling. Purification was then continued as in (i), except that both upper and lower bands were collected.

(iii) *Purification of HeLa mitochondrial DNA by velocity sedimentation*

This procedure was used to remove any residual isotope and small DNA fragments formed on DNase treatment. The DNA samples, dialyzed into buffer IV (0·1 M-NaCl, 0·01 M-Tris, 0·001 M-EDTA, pH 8·0), were layered onto 3 ml. of 1·3 g CsCl/ml. on a bottom layer of 1 ml. of 1·7 g CsCl/ml. in an SW50.1 cellulose nitrate tube. The 1·3 g/ml. solution contained propidium di-iodide or EthBr† at 200 μg/ml. or 100 μg/ml., respectively. The tubes were centrifuged at 38,000 rev./min for 3·5 hr, 20°C. The DNA in the lower part of the tube was isolated by drop-collection in ultraviolet light.

(iv) *Total mitochondrial DNA from SV3T3 cells*

Method (i) was not used with the SV3T3 DNA because the buoyant separations were often so small that isolation of the lower band was difficult. SV3T3 cells were grown, treated with EthBr, and harvested as described in method (i). They were resuspended in 0·002 M-Tris, 0·001 M-EDTA, 0·001 M-KCl, pH 7·4, for swelling and disrupted in a homogenizer with a motor-driven Teflon pestle. After addition of sucrose to 0·25 M, the crude mitochondria were isolated by differential centrifugation. The mitochondria were partially purified by sedimentation through 1·25 M-sucrose to an interface with 1·5 M-sucrose in a Sorvall swinging-bucket rotor. The mitochondria were concentrated by centrifugation and taken up in 0·25 M-sucrose, 0·01 M-$MgCl_2$, 0·005 M-NaCl, 0·005 M-Tris, pH 7·2, containing 30 μg DNase I/ml. and 100 μg RNase A/ml., and incubated for 30 min at 37°C. The reaction was stopped as in method (ii), and the mitochondria were washed 4 times with buffer II. The mitochondrial pellet was digested in 50 μg self-digested pronase/ml. for 30 min at 22°C. The digest was incubated for 15 min at 37°C in the presence of 1% sodium dodecyl sulfate and CsCl added to 1·0 M. The mixture was chilled for 30 min and centrifuged for 15 min at 14,000 rev./min. The M-DNA in the supernatant was concentrated by centrifugation for 10 to 12 hr at 38,000 rev./min. The pellet was digested with pronase for 15 min at 23°C. This digest was layered onto 4 ml. of 1·4 g CsCl/ml. on a bottom layer of 1·70 g CsCl/ml., both containing 100 μg EthBr/ml. The tube was centrifuged for 5 hr at 37,000 rev./min at 20°C. The DNA in the lower portion of the tubes was isolated by drop-collection, and rebanded as described in the legend to Fig. 1.

(v) *Closed mitochondrial DNA isolated by a modified Hirt procedure*

Pelleted cells were suspended in 3 vol. of buffer IV at 20°C. Samples of 1·5 ml. were spread onto plastic Petri plates (Hirt, 1966; Radloff, Bauer & Vinograd, 1967). Sodium dodecyl sulfate (0·8 ml. of a 2% solution) was added to each plate, which was mixed by gently "rocking" and then incubated at 20°C for 20 min. The viscous layers were scraped gently into a 50-ml. centrifuge tube, 7 M-CsCl added to a final concentration of 1·0 M, and mixed by pipetting with a wide-bore pipette. This solution was chilled to 0°C for 1·5 hr and centrifuged in a type 50.1 rotor at 30,000 rev./min for 30 min at 4°C. CsCl and propidium di-iodide were added to the supernatants and the tubes centrifuged as in method (i). Fractions of 1·0 ml. were collected from just below the main band in each tube after removal of the viscous upper band with a Pasteur pipette. The fractions were pooled and recentrifuged in a second CsCl–propidium di-iodide density gradient. Lower bands were isolated by drop-collection.

† Abbreviations used: EthBr, ethidium bromide; M-DNA, mitochondrial DNA.

(b) *Isolation and purification of nuclear DNA*

(i) *HeLa cells*

The pellet of undisrupted cells and nuclei obtained by low-speed centrifugation in method (i) of section (a), above, was taken up in buffer I and centrifuged for 2 min at 1000 *g*. The supernatant was centrifuged at 2000 rev./min for 5 min. The pellet was resuspended in 10 ml. of buffer I, mixed well, and sodium dodecyl sulfate added to 1%; the solution was mixed with vigorous pipetting and the mixture incubated for 30 min at 20°C. Sodium dodecyl sulfate was removed as in method (i), section (a), and one-third of the supernatant was centrifuged in a CsCl–propidium di-iodide density gradient. A small part of the band was removed with a pipette and purified as described in method (iii), section (a). The DNA was rebanded in CsCl and the fractions assayed for radioactivity as described in the legend to Fig. 4. No ^{32}P counts were observed outside the band defined by the [^{3}H]thymidine label.

(ii) *SV3T3 cells*

The pellets obtained by low-speed centrifugation of homogenates were further homogenized to disrupt the remaining cells. The nuclei in this homogenate were centrifuged through 1·5 M-sucrose to an interface with 2·2 M-sucrose. The interfacial material was resuspended and resedimented as above. The nuclei were digested with 100 μg heat-treated RNase A/ml., for 1 hr, and then with 50 μg pronase/ml. at 30°C for 6 hr. The lysate was incubated at 23°C for 12 to 16 hr after addition of sodium dodecyl sulfate to 2%. Sodium dodecyl sulfate was removed as in method (i), section (a), and the DNA further purified by CsCl–EthBr density gradient centrifugation. After removal of EthBr, the isolated DNA was dialyzed against 2 × SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate, pH 7·0).

(c) *Determination of superhelix density with fluorescence photography*

The superhelix density was determined by the buoyant separation method in the preparative ultracentrifuge as described by Gray, Upholt & Vinograd (manuscript in preparation) and Bauer & Vinograd (1970). The DNA sample was freed of EthBr or propidium di-iodide by passing it through a small Dowex-50 resin column or by dialysis against 1 M-NaCl, 0·01 M-Tris, 0·001 M-EDTA, containing 2% (v/v) Dowex 50. Dialysis was continued against 0·01 M-Tris, 0·001 M-EDTA. The DNA was diluted with the dialysis buffer to 0·6 ml. EthBr (0·1 ml. of a 10 mg/ml. solution in water), previously filtered through a 0·45 μ Millipore filter, was added and the solution density adjusted to 1·57 g/ml. with 7·0 M-CsCl. The solution volume was increased to 3·0 ml. with 1·57 g CsCl/ml. The CsCl solutions, also filtered through Millipore filters, contained 0·01 M-Tris, pH 8·0. The foregoing procedure is used to prevent precipitation of EthBr during the preparation of solutions and during subsequent centrifugation (Gray, Upholt & Vinograd, manuscript in preparation). The 3·0-ml. samples were centrifuged for 48 hr at 40,000 rev./min at 20°C, in an SW50.1 rotor. A reference tube containing a mixture of closed and nicked SV40 viral DNA was included in each experiment.

The tubes were illuminated with light at 365 nm and photographed with a camera system described by Watson, Bauer & Vinograd (1971). The distances between bands were carefully measured with a Nikon 6F projection microcomparator. The band positions were also measured with the bottom of the tube as a reference. The separations were measured with a pair of dividers when the bands were too faint to be seen on the screen. The distances between the divider points were measured on the microcomparator.

(d) *Analytical centrifugation, fluorimetry, and electron microscopy*

The analytical centrifugation procedures were the same as those described by Clayton, Davis & Vinograd (1970). Purified, nicked M-DNA in 2 × SSC was assayed fluorimetrically (LePecq & Paoletti, 1966) in a Hitachi Perkin-Elmer model MPF-2A fluorescence spectrophotometer. Nicked SV40 DNA was used as a standard. The procedures used in the electron microscope analyses were those described by Clayton *et al.* (1968).

(e) *Cell lines*

The HeLa cells, S3, have been maintained in this laboratory in suspension culture for several years. The SV40-transformed Balb/C 3T3 cells (SV3T3) were initially obtained from Dr G. Todaro, and were adapted for growth in suspension culture in this laboratory. Both cell lines were checked for the absence of mycoplasma at monthly intervals.

(f) *Enzymes and reagents*

DNase I and RNase A were obtained from the Sigma Chemical Company, St. Louis, Mo. Pronase was obtained from Calbiochem, Los Angeles, Calif. The optical grade cesium chloride was obtained from the Harshaw Chemical Company, Cleveland, Ohio. The ethidium bromide was a gift from Boots Pure Drug Co. Ltd., Nottingham, England. The propidium di-iodide was a gift from Calbiochem. All other chemicals were reagent grade.

3. Results

Figure 1 presents experiments which demonstrate the *in vivo* effect of ethidium bromide on the superhelix density of mitochondrial DNA in HeLa cells and in an SV40-transformed line of 3T3 Balb/C mouse cells. These cell lines were chosen because they contain different distributions of complex M-DNA. The separations between the bands of closed and nicked DNA in a buoyant cesium chloride density gradient containing EthBr are inversely related to the absolute value of the superhelix density, $|\sigma_0|$ (Hudson *et al.*, 1969). The experiments were performed in a standard way as described in Materials and Methods. The distances between the bands could, therefore, be used to compute the superhelix density (Bauer & Vinograd, 1970; Gray, Upholt & Vinograd, manuscript in preparation) with the relationship

$$\sigma_0 = \sigma_0^* + 0{\cdot}12\left(\frac{1}{f}\frac{r\Delta r}{r^*\Delta r^*} - 1\right) \qquad (1)$$

where r and Δr are the mean distances from the center of rotation and the separations between the bands, respectively. The quantity f is a factor (Bauer & Vinograd, 1970) very close to unity in these experiments. The asterisk refers to a reference DNA, in our case, viral SV40 DNA in another tube (Fig. 1(a)) or in the same tube (Fig. 1(b)). Addition of EthBr to the culture decreases the separation between the fluorescent open and closed bands (Fig. 1(a)) of HeLa M-DNA from 3·3 mm to 1·85 mm, and of mouse M-DNA from 3·6 mm to 1·2 mm. These shifts represent changes in the absolute value of the superhelix density, $|\sigma_0|$, according to equation (1), of 0·055 and 0·087 for HeLa and SV3T3 cells, respectively. A similar result, 0·085, was obtained with the above SV3T3 DNA, which was labeled with [^{3}H]thymidine, in an experiment with added ^{32}P-labeled SV40 DNA (Fig. 1(b)). It may be seen in this Figure, as well as in Figure 1(a), that a small fraction of the material did not change in superhelix density.

The absolute values of the superhelix densities induced by EthBr *in vivo* are considerably larger than any previously encountered in closed DNA obtained from cells or viruses, or prepared with polynucleotide ligase. The highest superhelix density, 0·11 tertiary turn per 10 base pairs, is approximately 50% larger than in the most supercoiled DNA used in the development and calibration of equation (1). In order to test the applicability of equation (1), the superhelix density of this M-DNA was determined by an independent method, a viscometric ethidium bromide titration (Révet, Schmir & Vinograd, 1971). The concentration of free EthBr required to relax

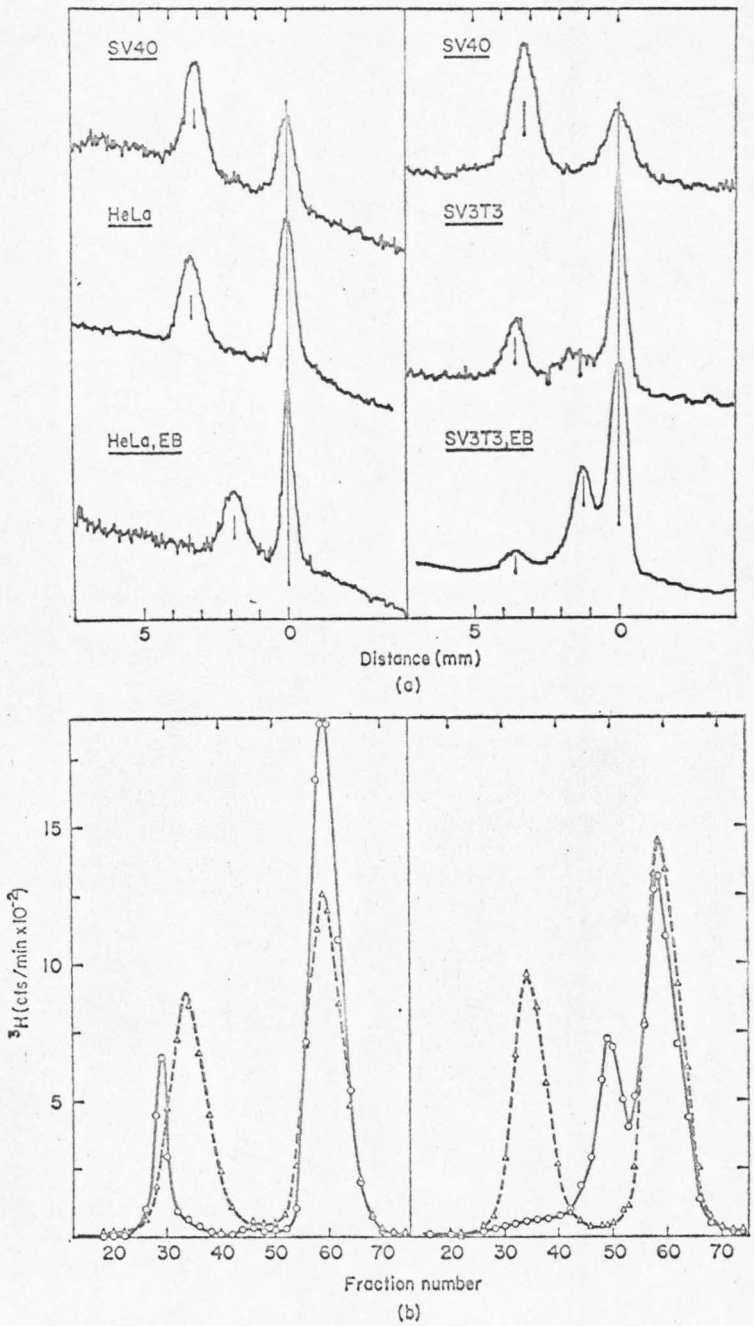

FIG. 1. The effect of the addition of 1·0 μg ethidium bromide/ml. to suspension cultures of HeLa and mouse SV3T3 cells on the superhelix density of mitochondrial DNA.

(a) Densitometer tracings of photographs of fluorescent bands in CsCl–EthBr density gradients. The tubes contained 3 ml. CsCl (1·57 g/ml.), and 330 μg EthBr/ml. They were centrifuged for 48 hr in an SW50.1 rotor at 40,000 rev./min at 20°C. Each panel presents the results of an experiment in which nicked and closed SV40 DNA and M-DNA from untreated and EthBr-treated cells were centrifuged in separate tubes. The HeLa and SV3T3 cells in log phase were exposed to 1 μg EthBr/ml. for 12 hr. The isolation and purification of the M-DNA's is described in Materials and Methods.

(b) Radioisotope distributions of SV3T3 M-DNA and SV40 DNA centrifuged in CsCl–EthBr gradients as described above. The M-DNA's were fractions of the samples used in (a). The left panel presents the results with the DNA from the untreated cells. The centrifugal field increases to the left.

—○—○—, M-DNA; -- △ -- △ --, SV40 DNA.

the closed DNA completely was determined at the maximum in the curve for flow time *versus* EthBr concentration in 2·85 M-CsCl at 20°C. These experiments, performed by Drs B. Révet and M. Schmir in this laboratory, showed that 40 and 1·7 μg EthBr/ml. were required to relax the M-DNA's isolated from treated and untreated mouse cells, respectively. The above results were used with the Scatchard equation and the binding parameters, $k = 2{\cdot}45 \times 10^{-4}$ M^{-1} and $\nu_m = 0{\cdot}241$, determined by Upholt, Gray & Vinograd (manuscript in preparation), to calculate the molar binding ratios and the superhelix densities (Bauer & Vinograd, 1971). The values of $|\sigma_0|$, 0·114 and 0·017, compared well with the results for the same samples, 0·115 and 0·017, obtained by the buoyant separation method.

(a) *Does the change in superhelix density occur during isolation of mitochondrial DNA?*

We have so far assumed that effects of ethidium bromide on the superhelix density occur in the living cell and are not incurred during the isolation of mitochondrial DNA from cells containing EthBr. The latter is improbable, because no change in superhelix density occurs in short treatment times (Fig. 3). It could, however, be argued that closure of an EthBr–DNA complex occurs during the isolation of DNA. Hershberger, Mickel & Rownd (1969) have reported that the circular drug resistance factor in *Proteus mirabilis* appears to be closed in the course of lysis and isolation of the DNA. This action could be prevented by lysing the cells in alkali or by heating the cells to 70°C.

We have used the latter procedure to determine whether or not closed M-DNA is present in the growing EthBr-treated cells. Four one-liter [^{3}H]thymidine-labeled HeLa cultures treated with 1 μg EthBr/ml. for 12 hours were harvested and the cell pellet suspended in 30 ml. of medium, all at 37°C. One-half was pipetted into 120 ml. of stirred medium at 75°C, maintained at 70°C for five minutes, and quick chilled with frozen buffer IV. The other half was chilled directly. The closed M-DNA's were isolated by method (v) (see Materials and Methods, section (a)). As indicated by the radioactivity in the closed bands, approximately 2×10^4 cts/min, the recovery of M-DNA from the heated sample was 115% of that from the unheated sample. The superhelix density was not measurably affected by the heat treatment, and the absolute value, 0·094 and 0·095, was similar to the result listed in Table 1. If we now assume that the mitochondrial ligase is inactivated at 70°C, we may conclude that the closed M-DNA in the growing EthBr-treated cells has a high superhelix density.

(b) *Structure and physical properties of mitochondrial DNA from ethidium bromide-treated cells*

Ethidium bromide treatment has been shown to yield petite mutants in yeast (Slonimski *et al.*, 1968). The mitochondrial DNA's in petite mutants generally have altered base compositions (Mounolou, Jakob & Slonimski, 1966). As judged by buoyant-density procedures, this is not the case for the M-DNA isolated from EthBr-treated animal cells. The purified DNA's from the lower bands from the treated HeLa and mouse cells had buoyant densities, as measured in the analytical ultracentrifuge, that were indistinguishable from the buoyant densities of the M-DNA's from untreated cells (Table 1), indicating that no change in the guanine plus cytosine content occurred at a level of detection of one mole per cent. The buoyant density differences between the well-separated complementary single strands in alkaline CsCl were also unchanged,

TABLE 1

Sedimentation properties of closed mitochondrial DNA from HeLa and SV3T3 cells treated with 1 μg ethidium bromide/ml.

Treatment time (hr)	Superhelix density $-\sigma_0 \times 10^2$ (turns/10 base pairs)	Sedimentation velocity pH 7·0 $S^0_{20,w}$ [a] (sec × 10^{13})	Sedimentation velocity pH 12·5 S^0 [b] (sec × 10^{13})	Buoyant density pH 7·0 θ (g/ml.)	Buoyant density pH 12·5 $\Delta\theta$ [d] g/ml.)
HeLa					
0	2·8 ± 0·4	37·4 ± 0·3	80·5 ± 0·9	1·700	0·041[e]
1	6·2 [f]	36·3 ± 0·7			
5	7·4	42·3 ± 1·5			
12	8·8	47·7 ± 0·6	77·3 ± 1·9	1·700	0·043
SV3T3					
0	2·0 ± 0·3	37·2 ± 0·4	79·9 ± 0·6	1·695	0·031
12	11·4	58·9 ± 0·4	76·4 ± 1·2	1·696	0·031

[a] The sedimentation solvent was 2·85 M-CsCl, 0·1 M-Tris, pH 8·0. The observed sedimentation coefficient was converted to a standard sedimentation coefficient for NaDNA (Bruner & Vinograd, 1965).

[b] Observed values in 2·85 M-CsCl, 0·1 M-KOH, 0·005 M-EDTA at 20°C.

[c] Relative to *Micrococcus lysodeikticus* DNA, 1·725 g/ml., or crab d(A,T), 1·670 g/ml., 25°C. The value of θ for *E. coli* DNA on this scale is 1·704 g/ml.

[d] In 1·76 g CsCl/ml., 0·1 M-KOH, 0·05 M-K_3PO_4 at 25°C.

[e] Result obtained by R. L. Hallberg in this laboratory.

[f] This material contained about 20% M-DNA with normal superhelix density.

indicating that there is no detectable change by this method in the composition of the complementary single strands.

The sedimentation velocity properties of the DNA's with high superhelix density (Table 1) are in accord with the observations of Upholt, Gray & Vinograd (manuscript in preparation), who have found that the sedimentation coefficient rises rapidly from a local minimum at $|\sigma_0| \sim 0{\cdot}05$ as the absolute value of the superhelix density increases further. The sedimentation coefficients of closed HeLa and SV3T3 M-DNA from cells treated for varying lengths of time with 1 μg EthBr/ml. are listed in Table 1 along with the superhelix densities. The highest sedimentation coefficient, obtained with mouse DNA, was 59 S, compared with 37 S for the M-DNA from untreated cells. The fully titrated forms of these two DNA's sediment in alkaline CsCl (1·35 g/ml., pH 12·5) with sedimentation coefficients of 76 S and 80 S, respectively. The 5% reduction in sedimentation coefficient is a measure of the change in conformation that occurs when the interwinding of the two catenated, denatured single strands (Vinograd *et al.*, 1965) is reduced by 9·4% from a value of about 1600.

The bands of the high superhelix density HeLa DNA spread considerably during the course of sedimentation, indicating a heterogeneity in the sedimentation coefficient. This result suggests that there is a heterogeneity in superhelix density among the sedimenting molecules. An EthBr-induced heterogeneity in molecular weight is unlikely because the nicked DNA derived from the 47 S HeLa sample sedimented without noticeable additional spreading over that normally seen with M-DNA.

Ethidium bromide has been reported to cause the formation of circular molecules in kinetoplast DNA having two, three, and four times the length of the circular DNA

TABLE 2

Complexity of mitochondrial DNA from HeLa and SV3T3 cells treated with 1 μg ethidium bromide/ml.

Treatment time (hr)	Frequency (%)					
	Monomer	Catenated dimer	Catenated trimer	Catenated higher oligomers	Circular dimer	Molecules classified
HeLa†						
0·1	88	8	1·8	2·5	0	1000
12	89	8	0·9	2·0	0	750
24	88	7	2·7	2·7	0	1000
Mouse						
0·0	72	16	5·6	2·8	3·0	889
12	72	18	4	2·5	2·7	828

† The DNA from the closed band was analyzed.

present in the untreated cells (Riou & Delain, 1969). In our experiments, we have not detected any significant change in the complexity of the M-DNA (Table 2). Neither the catenated nor the multiple-length forms of complex M-DNA increase in frequency as a result of the treatment with EthBr. Buoyant bands with reduced buoyant densities in CsCl–EthBr gradients might be formed upon aggregation of closed circular DNA with linear DNA. This could not have been the case in these studies, in which almost all of the DNA seen in the electron microscope was in the expected circular form.

(c) *Effects of ethidium bromide on the growth of HeLa and mouse cells*

A brief study of the growth of the HeLa and mouse cells was made in order to establish the range of time and dose suitable for the experiments. The cell density of HeLa cells was monitored approximately every 20 hours as described in the legend for Figure 2. In the absence of EthBr the generation time in log phase was 19 hours. At 0·1 μg EthBr/ml., the division time was 22 hours for the first 50 hours, and 40 hours for the next 50 hours. The cell count then became stationary and began to decline after another 40 hours. The changes in superhelix density described later occurred in cells during the first 24 hours after addition of EthBr. In this period, growth occurred at a substantially normal rate.

At 1 μg EthBr/ml., the mean division time was 33 hours in the first 24 hours, and 82 hours in the next 24 hours. The cells increased only twofold before decreasing in number, in contrast with a fourfold increase reported by Perlman & Penman (1970) with a single initial dose of 1·0 μg EthBr/ml. In our experiments we have added one-half volume of media containing fresh EthBr at each time of analysis. Since the division time is increasing during the first 24 hours, the mean division time in the first five hours, in which the effect on the superhelix density is almost complete, must be substantially shorter than 33 hours. Cell growth clearly continues after the superhelix density of all of the closed M-DNA has reached its maximum value at both EthBr concentrations, as is shown below.

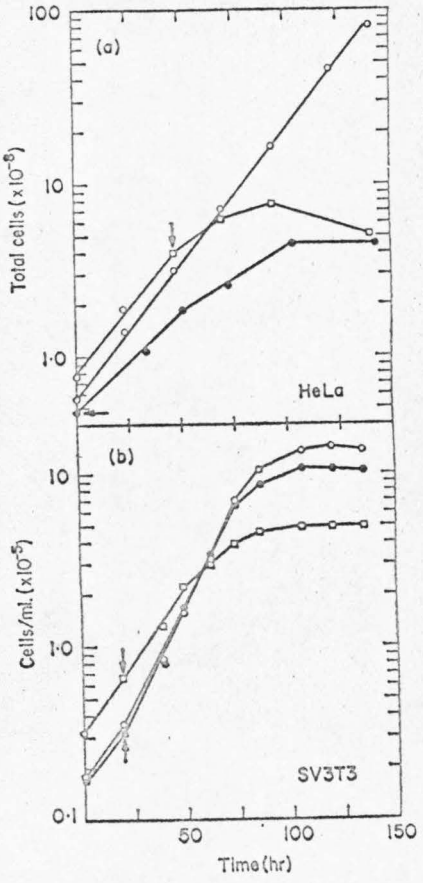

FIG. 2. (a) Effect of ethidium bromide on the growth of HeLa cells. Spinner cultures of HeLa cells in Dulbecco's modification of Eagle's phosphate medium (Grand Island Biological Co.) with 5% calf serum were grown to 1×10^5 cells/ml. at 37·4°C. At selected times in the experiment, indicated by the data points, one-half of each culture was removed for counting; the other half was diluted with an equal volume of fresh medium with or without EthBr. The cell density was maintained between 0·5 and 2×10^5 cells/ml. The cells were centrifuged at 1500 rev./min for 7 min in an IEC PR-6 centrifuge and resuspended in 5 ml. of 0·025% trypsin solution. A 0·5-ml. sample of this suspension was diluted to 25 ml. in a 0·05 M-Tris buffer (pH 7·4) and counted in a hemocytometer. The ordinate value represents the number of cells which would have accumulated in the absence of sampling. At the times indicated by the arrows, EthBr was added to the cultures. —○—○—, No EthBr; —◎—◎—, 0·1 μg EthBr/ml.; —□—□—, 1·0 μg EthBr/ml.

(b) Effect of ethidium bromide on the growth of SV3T3 cells. Suspension cultures of Dulbecco's modification of Eagle's medium with 5% calf serum were set up at the concentrations indicated at zero time. EthBr was added at the times indicated by the arrows. The cell concentrations in the cultures were determined by withdrawing a 5-ml. sample after shaking the spinner vessel and counting in a hemocytometer. —○—○—, No EthBr; —◎—◎—, 0·1 μg EthBr/ml.; —□—□—, 1·0 μg EthBr/ml.

The effect of EthBr on the growth of SV3T3 mouse cells was followed in suspension cultures which were sampled at regular intervals. The generation time in log phase of both the untreated cells and cells treated with 0·1 μg EthBr/ml. was 13 hours, and was 17 hours at 1·0 μg/ml. The cells increased in number between the time of addition and the saturation level by factors of 5·2, 5·0 and 2·9 as the EthBr concentration increased.

In the study of the time-dependence of the effect of EthBr on the superhelix density, the EthBr was always added when the cells were in log phase and between 2 and 3×10^5/ml.

(d) *Time- and dose-dependence of the effect of ethidium bromide on the superhelix density of mitochondrial DNA*

The results presented in Figure 1 show that the major part of the M-DNA appears in a discrete band with altered superhelix density after 12 hours in EthBr. If new M-DNA synthesis does not occur in the 12-hour treatment time, the results provide a maximum estimate for the time required for EthBr to reach the site of its action and for a nicking–closing cycle to occur. We have, therefore, investigated the time-dependence and dose-response of the superhelix density.

The positions of the M-DNA bands in standard CsCl–EthBr buoyant separation experiments are presented in Figure 3. These positions change in a similar way at

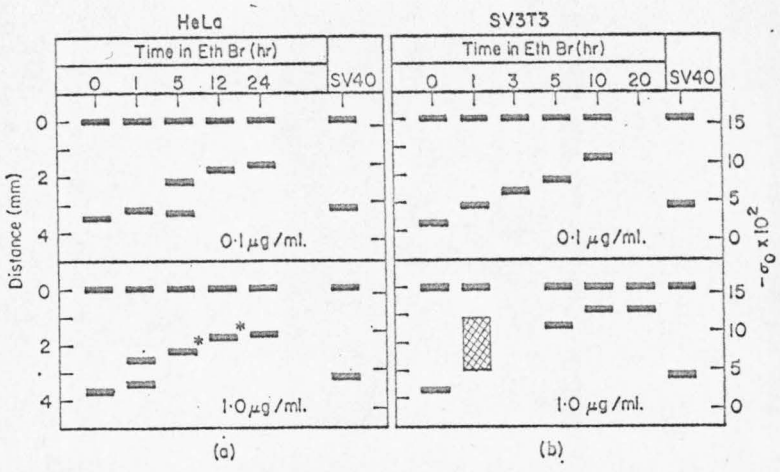

FIG. 3. The superhelix density and buoyant separations of mitochondrial DNA as a function of time of treatment of cells in log phase with two concentrations of ethidium bromide.

The superhelix densities were calculated with equation (1). The corrected buoyant separations were obtained by multiplying the quantity in parenthesis in equation (1) by 3·15 mm, so as to normalize these distances to a typical value of Δr for SV40 DNA. The bars with zero values on the left-hand ordinate represent the positions of the nicked DNA. The centrifugation conditions are described in the legend to Fig. 1. (a) HeLa cells; the asterisks represent the mean of two experiments. (b) SV3T3 mouse cells. A band containing less than 5% of the M-DNA in the tube was observed 1·0 mm below the upper band in the experiment at 0·1 μg EthBr/ml. for 3 hr. The significance of this band was not investigated.

both 0·1 and 1·0 μg EthBr/ml. After five hours at 0·1 μg/ml. and one hour at 1·0 μg/ml., two closed HeLa bands are observed, one of which has an altered value of σ. The major part of the closed DNA is in the altered form. At the next time, 12 hours at 0·1 μg/ml. and five hours at 1·0 μg/ml., the material with native superhelix density had disappeared and all of the closed DNA was in one band. The superhelix density of the altered M-DNA appears to level off by 12 hours at both concentrations. Similar results were obtained with the mouse cells (Fig. 3(b)). A very wide, diffuse band was observed at one hour in 1 μg EthBr/ml. Again at 10 hours at both EthBr concentra-

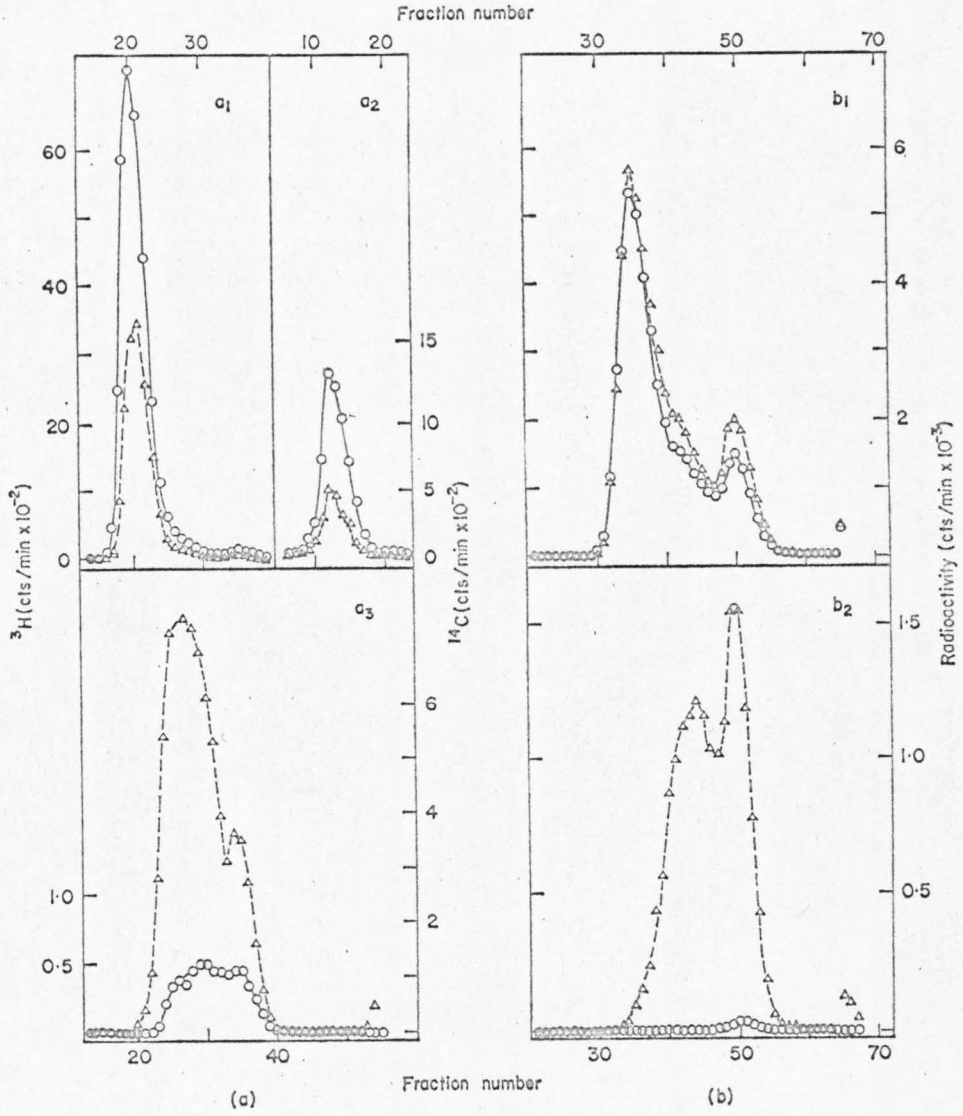

FIG. 4. The effect of 1 μg ethidium bromide/ml. on the incorporation of DNA precursors into HeLa mitochondrial DNA.

(a) 5-hr pulse with [^{3}H]thymidine. HeLa cells were grown in suspension culture for 40 hr in the presence of 0·04 μCi/ml. of [^{14}C]thymidine. When cells were at a concentration of about 2×10^5 cells/ml., EthBr was added to one-half of the spinner cultures to 1 μg/ml. 30 min later, [^{3}H]-thymidine, 1 μCi/ml., was added and the cells were harvested after 5 hr. Light was excluded after addition of EthBr. M-DNA was prepared as described by method (ii) (see Materials and Methods, section (a)). CsCl–propidium di-iodide gradients were fractionated visually. The separated samples of closed and open DNA from the untreated cells and the total M-DNA from the EthBr-treated cells were dialyzed to remove CsCl. The three samples were freed of low molecular weight materials by velocity centrifugation (method (iii)), and recentrifuged to equilibrium in 1·56g CsCl/ml. containing about 300 μg propidium di-iodide/ml. The fractions were collected directly onto glass filter papers which were dried and counted in a Packard Tri-Carb scintillation spectrometer. (a_1) Closed M-DNA (lower band) from untreated cells; (a_2) nicked M-DNA (upper band)

tions, only one band with high superhelix density was observed, indicating that the major part of the M-DNA has now changed in superhelix density. It is shown below that the M-DNA bands in Figure 3 consist almost entirely of M-DNA present in the cells prior to EthBr addition.

A part of the M-DNA in HeLa cells treated with 1·0 μg EthBr/ml. for one hour was unchanged in superhelix density. Either a fraction of the mitochondria or a fraction of the cells has not yet experienced the EthBr-induced effect on σ. This could be due to differential uptake of the EthBr or to a slower action of the nicking–closing cycle in some of the mitochondria or in some of the cells. The M-DNA has changed again to a still higher superhelix density, and has participated in at least one further nicking–closing cycle in the next four hours. Beyond five hours there must have been still another nicking–closing cycle with a still larger EthBr effect. The results at 0·1 μg EthBr/ml. are comparable, except that longer times are required to achieve similar effects. At 10 μg/ml., one hour is sufficient to change the superhelix density of all of the M-DNA to −0·07. No further change was observed at five hours, presumably because of toxic effects on the cells.

As indicated previously, the mouse cells behave qualitatively like the HeLa cells. The maximum superhelix density reached after 20 hours in 1·0 μg EthBr/ml. is 30% higher than in the HeLa cells under comparable conditions. The diffuse band at 1 hour and 0·1 μg EthBr/ml. is regarded as an expression of a heterogeneous EthBr effect among the cells, as already noted in HeLa cells under comparable conditions.

(e) *Is the DNA of altered superhelix density old or new?*

Since a large fraction of the closed DNA could be altered within a short time compared to the doubling time of the cells, it seemed improbable that the M-DNA of altered σ was newly formed after addition of EthBr. The experiments described below confirm that old DNA is involved.

Two double-label experiments were carried out with HeLa cells to determine the amount of DNA precursor incorporated into DNA after addition of EthBr. The cells were first labeled for approximately two generations with [^{14}C]thymidine and then pulsed in the presence or absence of EthBr for five hours with [^{3}H]thymidine (Fig. 4(a)). The incorporation of pulse label as measured by the ^{3}H/^{14}C ratio (Table 3) in the closed M-DNA was 2% of that observed in the untreated cells. A similar result was obtained with the upper band material. The ^{3}H/^{14}C ratio in the nuclear DNA shows that the pulse label was incorporated in the nucleus of EthBr-treated cells.

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from untreated cells; (a_3) total M-DNA (upper and lower bands) from EthBr-treated cells. The fraction size in (a_1) and (a_2) was 1·25 times that in (a_3).

—○—○—, ^{3}H; – – △ – – △ – –, ^{14}C.

(b) 12-hr pulse with ^{32}P-labeled inorganic orthophosphate. HeLa cells were grown in suspension culture for 50 hr in the presence of 0·2 μCi [^{3}H]thymidine/ml. Cells were harvested without chilling, washed, and resuspended in non-radioactive medium containing a reduced concentration (0·001 M) of phosphate. EthBr(1 μg/ml.) was added to one-half of the cultures. 0·5 hr later, [^{32}P]orthophosphate was added to 5 μCi/ml. Cultures were kept in the dark. After a further 12 hr, the cells were chilled, M-DNA prepared, and radioactivity assayed as described above. The fraction size is the same as in (a_3). (b_1) Total M-DNA from untreated cells; (b_2) total M-DNA from treated cells.

—○—○–, ^{32}P; – – △ – – △ – –, ^{3}H.

TABLE 3

Effect of ethidium bromide on the incorporation of [^{3}H]thymidine and [^{32}P]orthophosphate in mitochondrial and nuclear DNA of HeLa cells

EthBr (μg/ml.)	DNA	Isotopes incorporated†			
		$^{3}H/^{14}C$	R_{EthBr}/R_0‡	$^{32}P/^{3}H$	R_{EthBr}/R_0
0·0	Lower band	4·19	—	0·95	—
1·0	Lower band	0·06	0·02	0·00	0·00
0·0	Upper band	5·69	—	0·75	—
1·0	Upper band	0·12	0·02	0·02	0·03
0·0	Nuclear		—	0·36	
1·0	Nuclear	3·19		0·11	0·31

See the legend to Figure 4 for protocol of experiments.

† The primary radioisotope data are shown in Figure 4.

‡ Incorporation of pulse label per unit of long-term label in the presence of EthBr relative to the incorporation in the experiment without EthBr.

The experiment above does not rule out the possibility that the EthBr prevents incorporation of exogenously added precursor without affecting the endogenous precursor. To test this possibility, the experiment was repeated with [^{32}P]phosphate as the pulse label (Fig. 4(b)). [^{3}H]Thymidine was used to pre-label the M-DNA. The inhibition in EthBr-treated cells was now even more striking. There were no ^{32}P counts detected in the closed band. The incorporation of isotope in the upper band material was only 3% of that observed in the upper band material from the untreated cells. The isotope ratio in nuclear DNA was 0·11; a nuclear DNA contamination of 25% in the upper band would, therefore, account for the isotope there. Nuclear synthesis appears to be inhibited to about 70%. We conclude that 1 μg EthBr/ml. inhibits new M-DNA synthesis in HeLa cells to at least 98% and that the HeLa M-DNA with altered superhelix density was present in the cells with a normal superhelix density prior to addition of EthBr.

A similar result was obtained with SV3T3 cells incubated with 0·1 and 1·0 μg EthBr/ml. for 12 hours. The [^{3}H]thymidine was added one hour after the EthBr was added. The specific activity of the mixture of open and closed M-DNA and of the nuclear DNA was determined (Table 4). The incorporation of isotope into M-DNA at the two EthBr levels was 10 and 5% of that in the untreated cells. The residual incorporation can only in small part be accounted for by contamination with nuclear DNA, which we would have detected at the 10% level in the alkaline buoyant analyses which were performed in the analytical ultracentrifuge.

It is shown below that several acridine derivatives are effective in increasing the absolute value of the superhelix density of SV3T3 M-DNA. One of these, acriflavin, was examined for its effect on DNA synthesis by the procedure described above for HeLa cells (Table 5). This drug inhibits mitochondrial DNA synthesis by 99%, and nuclear DNA synthesis by 97%. It should be noted that molar concentration of drug in these experiments was approximately twice that used in the experiments with EthBr (see Table 4).

TABLE 4

Effect of ethidium bromide on the incorporation of [^3H]thymidine into mitochondrial and nuclear DNA in SV3T3 cells

EthBr (μg/ml.)	DNA (μg)	cts/min/μg $\times 10^{-3}$
	Mitochondrial DNA	
0·0	4·9	31·1
0·1	5·7	3·0
1·0	9·8	1·6
	Nuclear DNA	
0·0	17·5	15·1
0·1	74·7	7·8
1·0	60·0	7·0

Cells in log phase were harvested and resuspended in fresh medium at $2{\cdot}5 \times 10^5$ cells/ml., all at 37°C. After 1·5 hr and 2·5 hr, the EthBr and [^{3}H]thymidine (0·6 μCi/ml.) were successively added. The cells were incubated in suspension for 5 hr, and the DNA isolated by method (iv) (see Materials and Methods, section (a)). The purified M-DNA was assayed fluorimetrically. The nuclear DNA concentration was determined spectrophotometrically.

TABLE 5

Effect of acriflavin on the incorporation of [^{32}P]orthophosphate into mitochondrial and nuclear DNA in SV3T3 mouse cells

Acriflavin (μg/ml.)	^{32}P (cts/min $\times 10^{-3}$)	Isotope incorporated $^{32}P/^3H$	R_{EthBr}/R_0†
		Mitochondrial DNA	
0·0	15·6	1·17	—
2·2	1·74	0·0015	0·013
		Nuclear DNA	
0·0	32·3	1·12	—
2·2	4·80	0·038	0·033

The cells were pre-labeled with [^{3}H]thymidine (0·1 μCi/ml.) for 3 generations. The cells were washed and transferred to fresh medium containing $5{\cdot}0 \times 10^{-4}$ M-phosphate. After 1 hr and 2 hr, the acriflavin and the [^{32}P]orthophosphate were successively added. The cells were incubated in suspension for 12 hr. The DNA's were isolated by method (iv), see Materials and Methods, section (a).

† Incorporation of ^{32}P per unit amount of DNA in the presence of drug relative to the incorporation in the experiment without drug.

(f) In vivo *effects of other drugs on the superhelix density of mitochondrial DNA*

In order to examine the hypothesis that the direct intercalative action of EthBr is responsible for the changed superhelix density of mitochondrial DNA, we have tested the effects of a series of drugs, several of which are known to intercalate into DNA. These compounds (nos 1 through 5, 7 and 8) are listed in Table 6, along with

TABLE 6

In vivo *effects of drugs on the superhelix density of SV3T3 mitochondrial DNA*

Compound	Concentration (μM)	($\Delta r/\Delta r^*$)	$\frac{\sigma_0 \text{ (drug)}}{\sigma_0 \text{ (control)}}$
(No drug)	—	1·15	1·0
1. Ethidium bromide†	5·1	0·41	5·1
2. Acriflavin†	6·5	0·40	5·2
3. Chrysanaline‡	5·0	0·41	5·1
4. Acridine orange‡	8·4	0·72	3·4
5. Acranil†	4·4	0·73	3·4
6. Riboflavin†	5·3	1·05	1·5
7. Actinomycin D†	—	1·13	1·0
8. Propidium di-iodide §, ‖	7·5‖	1·1	1·0

The compounds were added at the indicated concentration to cells in log phase. The cells were harvested after 12 hr.

† These compounds are listed in *The Merck Index*, 8th ed. (1968), ed. by P. G. Stecher, M. Windholz & D. S. Leahy, Rahway, N. J.: Merck & Co., Inc.

‡ These compounds are listed in Acheson (1956).

§ See Hudson *et al.* (1969).

‖ This experiment was performed with HeLa cells.

their effects on the superhelix density. The binding properties of compounds 3 and 5 have not previously been studied. They do, however, exhibit maxima in viscometric dye titrations of PM2 DNA in 0·01 M-Tris·HCl, 0·001 M-EDTA(pH 8·0) and therefore may be regarded to be intercalators. Acriflavin and chrysanaline increase the superhelix density by a factor of about 5, and are as effective as EthBr. Acridine orange and Acranil are about two-thirds as effective as EthBr. Actinomycin D is without effect. Propidium di-iodide, as analogue of EthBr (Hudson *et al.*, 1969), has no effect in HeLa cells. The effects of compound 6, an analog of atebrin, and of riboflavin (which when absent from the diet appears to affect mitochondrial division in mice (Tandler, Erlandson, Smith & Wynder, 1969)) are marginal and possibly within the variation of the method of analysis.

Actinomycin D is known to have little effect on mitochondrial RNA synthesis in HeLa cells (Zylber *et al.*, 1969), even though it has profound effects on nuclear RNA and cytoplasmic protein synthesis. Propidium di-iodide, a salt of a doubly-charged cation, is apparently not taken up by HeLa cells. The washed cell pellets are only faintly colored, as are the purified mitochondria. The latter are pink when EthBr is used in the treatment. Cells treated with 1·0 μg EthBr/ml. for five hours fluoresced strongly, while those treated with 5 μg propidium di-iodide/ml. for 24 hours did not fluoresce at all under a microscope provided with ultraviolet illumination. In summary, of the seven intercalating drugs that we have examined, five have similar effects on the superhelix density of the mitochondrial DNA, while two are ineffective. One appears not to penetrate the cell, while the other may be excluded from the mitochondria.

4. Discussion

The apparent analogy between the *in vitro* method of formation of closed DNA with high superhelix density and the *in vivo* effects reported here suggests a "direct mechanism" for the action of ethidium bromide *in vivo*. The ethidium bromide enters the mitochondria, binds to the mitochondrial DNA, and thereby reduces the rotation angle of the duplex. The EthBr–DNA complex is then nicked and reclosed to yield a DNA with a low topological winding number. Upon isolation and purification, the rotation angle increases as the regular duplex structure is assumed and a highly supercoiled molecule is formed. This mechanism might account for the action of the five intercalating dyes described in this study.

The above proposal may be examined in terms of our knowledge of the effects of ethidium bromide on the rotation angle of DNA. We assume that each EthBr molecule bound unwinds the duplex by 12° (Fuller & Waring, 1964). It may then be calculated that 0·18 mole EthBr per nucleotide was bound in order to form a closed DNA with an absolute superhelix density of 0·12. The Scatchard equation, which adequately describes the isotherm for the strong binding of EthBr to DNA in salt solutions can then be written

$$kc = 0{\cdot}18/(\nu_m - 0{\cdot}18) \qquad (2)$$

where c is the free dye concentration, and k and ν_m are the intrinsic binding constant and number of independent binding sites per nucleotide, respectively. The free electrolyte concentration in rabbit liver mitochondria, according to Gamble & Hess (1966), is approximately 100 mequiv./l. LePecq & Paoletti (1967) have measured k and ν_m at 37°C as a function of NaCl concentration. With their parameters for 0·1 M-NaCl, 3×10^5 M^{-1} and 0·20, respectively, the free dye concentration at the site of the M-DNA and the closing enzyme would have to be 12 μg/ml., 12 times larger than the total dye concentration. Surely, a large but unknown fraction of the EthBr is bound to the 0·2 mg bovine albumin/ml. in the medium and to the cell constituents other than M-DNA. It is safe to conclude that the free EthBr is, according to this simple concept, too low by a factor of at least 10 to 100. The direct mechanism could account for the results, if there were a compensating active uptake of EthBr to increase the free EthBr concentration in the aqueous space around the DNA, or if the binding constant were elevated by a factor of 10 to 100. It is possible that the mixture of K^+ and Mg^{2+} salts of ATP, ADP, phosphoenolpyruvate, and pyruvate, reported to exist in the mitochondria, could substantially enhance the binding of EthBr to DNA. This possibility can be tested experimentally, as can the possibility that active uptake occurs. Since we do not know the phenomena responsible for the supercoiling or the reduced rotation angle of the DNA in the mitochondria in the absence of EthBr, indirect mechanisms for the effect of EthBr, while not improbable, cannot be usefully discussed.

We have demonstrated in this study that non-replicating DNA undergoes repeated nicking–closing cycles in cultured cells treated with drugs that bind to DNA. It is inappropriate to infer from these results that a similar action does or does not occur in the absence of the drugs.

Finally, we note that the superhelix density of SV3T3 mitochondrial DNA in the absence of ethidium bromide, $-0{\cdot}020 \pm 0{\cdot}003$, is the lowest so far observed in any *in vivo* closed DNA. Wang (1969) and Upholt, Gray & Vinograd (manuscript in preparation) have shown that changing the solvent from dilute NaCl to 2·85 M-CsCl

and changing the temperature from 37°C to 20°C causes a change in rotation angle that would account for a change of about −0·006 in the superhelix density. The superhelix density of this mitochondrial DNA, if measured in 0·1 M-NaCl, would, therefore, have been about −0·014 ± 0·003, only three- to five-tenths as large as the value for viral SV40 DNA.

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Note added in proof: After this paper had been submitted, Nass (1970. *Proc. Nat. Acad. Sci., Wash.* 67, 1926.) reported a study of the effects of treatment of mouse L cells with ethidium bromide at 1 μg/ml. for 45 hours. She found no mitochondrial DNA in the normal lower band position in a CsCl–EthBr gradient. The absence of DNA in this region could be a consequence of nicking or degradation of the mitochondrial DNA during the long exposure to the drug, as suggested by the author. Alternatively, an increase in the superhelix density could have occurred and resulted in an overlap of the bands formed by the closed and open DNA's.

ADDENDUM

Further Studies of the Effects of Ethidium Bromide on HeLa Cells

The preceding publication demonstrated that ethidium bromide effectively inhibits new mtDNA synthesis and causes a gradual increase in the magnitude of the superhelix density of the pre-existing mtDNA. It was postulated that the EthBr is imported into the mitochondrion and binds to the DNA. A nicking-closing cycle operates on the DNA, causing an increased superhelix density with increased EthBr bound to the DNA. Additional studies with HeLa cells were performed to determine if the effect is reversible, to determine the fate of the DNA in the absence of new synthesis, and to obtain a better estimate of the rapidity of the nicking-closing cycle.

(a) Co-treatment with cycloheximide

Cycloheximide (B grade, Calbiochem, Los Angeles, Calif.) was added to cells to a concentration of 50 μg/ml one hour prior to the addition of EthBr. In this concentration, cytoplasmic protein synthesis is rapidly and effectively inhibited. The cycloheximide pre-treatment was found to have no effect on the alteration of superhelix density caused by the EthBr. Thus, any proteins necessary for EthBr transport and the nicking-closing cycle are either mitochondrial in origin or are stable for periods exceeding this time.

(b) Recovery from EthBr treatment

Experiments at both 1.0 and 0.1 μg EthBr/ml were performed. Cells were treated at 1 μg/ml for 5 hours, harvested by centrifugation at 37° C, washed, and resuspended in fresh medium not containing EthBr. Cells were then harvested at 5 and 24 hours after the resuspension, the mtDNA prepared and centrifuged to equilibrium in a CsCl-EthBr density gradient. Qualitative examination of these gradients showed that the banding position of the closed mtDNA from cells resuspended for 5 hours was the same as that of DNA prepared at the end of the EthBr treatment period. However, all the DNA seen from cells incubated in fresh medium for 24 hours was seen at or near the position of DNA with normal superhelix density.

Cells were treated for 12 hours with 0.1 μg EthBr/ml and then transferred to medium not containing the drug and allowed to grow for 24 hours. The superhelix density of the mtDNA from cells treated for 12 hours was -0.090, as expected. The superhelix density of the mtDNA from the cells allowed to grow in medium without EthBr was -0.062, indicating that the superhelix density was returning to its normal position, but had not yet reached it. It appears that the effects of EthBr on superhelix density are reversible.

(c) Fate of the DNA with altered superhelix density

It has been established that EthBr causes the gradual degradation of the closed circular kinetoplast DNA of trypanosomes (1). Nass (2) has suggested that degradation of mtDNA of mouse L cells occurs

when such cells are treated for 48 hours with 1 μg EthBr/ml. Her data were not, however, conclusive (see Note Added in Proof, preceding publication). Attempts to determine the fate of the DNA are subject to the general problems of quantitation of mtDNA, as well as to some special ones, due to the drug treatment. Because of the alteration of the superhelix density induced by the drug, the closed DNA bands in EthBr gradients very close to the position of linear and nicked DNA. It is possible that with long treatments the linear and closed DNA may co-band, especially if a wide band of contaminating nuclear DNA is present. Effective removal of contaminating nuclear DNA necessitates the use of DNase to digest such DNA. However, with long treatment times, the effects of the drug on mitochondrial morphology (2) suggest that some or all of the mitochondria may become permeable to DNase. Such alteration may also cause a change in organelle density sufficient to alter the position at which mitochondria are found in the sucrose step gradient used for their purification. Thus, an apparant loss of mtDNA recovered could reflect factors other than actual degradation of the DNA. With these provisos, the results of some experiments are described below.

In an experiment in which DNase was not used in preparation of the mtDNA, cells were treated with 0.1 μg EthBr/ml for 17, 46, and 71 hours. The mass of cells recovered at each point indicated that they were growing with an average doubling time of 30 hours. At each time point, one third of the culture was harvested and mtDNA prepared. When the DNA recovered was banded in CsCl-EthBr, the expected band

of high superhelix density DNA was seen in the 17-hr sample. Much less DNA was seen at this position in the 46-hr sample and no DNA was visible as a lower band in the 71-hr sample. Thus, the DNA was either being nicked or selectively lost, or further increases in superhelix density caused it to band at the same position as the nuclear DNA. Due to the large amount of contaminating nuclear DNA, quantitation of mtDNA in these samples was not possible.

In an experiment in which DNase was used, cells were treated for 17 and 48 hours with 0.1 μg EthBr/ml, and all the mtDNA recovered was analyzed by centrifugation to equilibrium in neutral CsCl in the analytical ultracentrifuge. Under these conditions, mtDNA may be distinguished from nuclear DNA due to their buoyant density difference. It was found that the ratio of mtDNA in the two samples corresponded roughly to the ratio of cells in the two cultures at the time of drug addition. Thus, DNA of mitochondrial buoyant density is maintained for at least 48 hours in this concentration of EthBr. The experiment was repeated using treatment for 9 and 58 hours, and preparative velocity sedimentation was used to remove DNA with a molecular weight less than about 5×10^6. The amount of DNA with mitochondrial buoyant density in the 58-hr treated sample was only one sixth that expected if no loss had occurred.

These results suggest that with increasing treatment times, the amount of mtDNA recoverable in HeLa cells as closed or undegraded circles decreases. Like the results of Nass, they are not conclusive; further experiments would be necessary to reach a firm conclusion.

(d) EthBr treatment at low temperatures

The possibility that the rate of the nicking-closing process might be studied independently of the rate of entry of the drug into its active site was investigated by treating cells at lower temperatures, at which it was hoped the two processes might be differentially affected. Temperature control at 4° C or 22° C was achieved by placing spinners in plastic tubs containing water at these temperatures, either in a cold room (4° C) or in the laboratory (22° C). The tubs were placed on the same 4-place stirring devices used in the 37° C room. To lower temperatures from 37° C, spinners were placed in an ice-water bath and spun as above, along with a spinner containing water at 37° C used to monitor temperature. When 4° C or 22° C was reached, spinners were transferred to the appropriate bath. When the temperature was to be raised, spinners were placed in water at 70° C until the temperature was 37° C, then transferred to a 37° C room. The chilling and warming took about 10 to 15 minutes. Cells were maintained at 4° C for 5 hours in the presence and absence of 1 μg EthBr/ml. The superhelix density of the mtDNA of both samples was determined to be -0.024, the normal value. Thus, one or both processes mentioned above are inhibited at this temperature. When the 5-hr incubation at 4° C was followed by a 30-min incubation at 37° C, the superhelix density of the mtDNA from a culture treated with EthBr was identical to that from a culture treated in a similar way without EthBr. This suggests that the transport of EthBr into the mitochondria is inhibited at 4° C.

The mtDNA from cells incubated at 22° C for 5 hours in the presence of 1 μg EthBr/ml had a superhelix density of -0.053. This represents an increase in magnitude of about half that found under these conditions at 37° C, where the change is from -0.024 to -0.074. When the 5-hour incubation at 22° C was followed by a 30-minute incubation at 37° C, the superhelix density was found to be -0.068, a value very close to that seen with the 5-hour incubation at 37° C. Only a single closed band was seen in each case. The increase in the superhelix density found when the 37° C incubation was added indicates that all the mtDNA underwent at least one nicking-closing event in that 30 minutes. The increased magnitude of the superhelix density would indicate more EthBr was bound to the DNA at closure. This may be due to a rapid transport of additional EthBr into the mitochondria at 37° C, or to an increased intra-mitochondrial concentration of EthBr available for binding, perhaps due to changes in EthBr binding to other cell substrates. The effect is apparently not due to the difference in the binding constant of EthBr to DNA, as this should be higher at the lower temperature (3).

Effects of Ethidium Bromide on the Superhelix Density of mtDNA of Swiss Mice

The nicking-closing cycle demonstrated by the action of EthBr upon rapidly growing cells in culture could be a part of the active replication systems in the mitochondria of such cells. It was decided to

study the effects of EthBr on the mtDNA superhelix density of animal tissue in situ, where such replication is not so active.

MATERIALS AND METHODS

Mice: White, full-grown Swiss mice of both sexes were obtained from Mission Lab Supply, Rosemead, Calif.

EthBr treatment: EthBr was administered at a concentration of 10 mg/ml by intraperitoneal injection. The solution was sterilized by Millipore filtration.

MtDNA preparation: Mice were killed and the organs chosen were quickly removed and washed in TD (4) at 4° C several times. The organs were then minced into pieces approximately 0.5 cm^3 in cold RSB (0.01 M-NaCl, 0.0015 M-$MgCl_2$, 0.01 M-Tris, pH 7.5), and homogenized with 2 to 3 strokes in a glass Dounce homogenizer fitted with a loose-fitting motor-driven nylon pestle. This homogenate was allowed to stand 5 minutes and then homogenized with 2 to 4 strokes in a glass Dounce homogenizer fitted with a tight-fitting glass pestle. Sucrose was added to a concentration of 0.25 M and the homogenate was centrifuged for 7 minutes at 2500 rev./min in an International PR-6 centrifuge. The supernatant was recentrifuged and the resulting supernatant was spun 15 minutes at 12,000 rev./min in a Sorvall SS34 rotor to pellet crude mitochondria. Further purification of mitochondria and preparation of mtDNA was similar to the methods of the preceding publication. No DNase treatments were used.

Measurement of superhelix density was as described in the preceding publication.

RESULTS

(a) Single dose

A single dose of 1 mg EthBr was administered per mouse and 10 hours later the mtDNA was prepared from livers and from a mixture of spleens and kidneys. Preparations were also made from these organs in untreated mice. In the first EthBr-CsCl density gradients, all preparations showed normal separations between the closed and nicked DNA. The closed DNA was isolated and nicked to provide the form II DNA necessary for superhelix density measurement in later experiments.

(b) Multiple dose schedule

After preliminary experiments at various doses, a dose schedule was found which resulted in a high rate of survival and an effect on superhelix density. Some mice died shortly after injection, probably due to internal injuries sustained by a poor injection, as the mice are small and the investigator's technique is not perfect. The schedule was 2 mg at 0, 11, and 22 hours. Mitochondrial DNA was prepared 4.5 hours after the last injection. The clearance of EthBr at these doses is quite rapid, the urine being almost the color of the solution injected. The total clearance of EthBr from mice has been reported to have a half-time of about 24 hours, using a dose of 100 μg. About 75% of the EthBr found in spleen, kidney, and liver at 15 minutes post-injection was found to be removed by 9 hours (5).

(c) Gross effects

At the time of mtDNA preparation, about 80% of the mice had survived. Under ultraviolet light the fluorescence of circulating EthBr was easily seen in areas where the circulation is close to the skin, such as in the ears, feet, and tails. The mice generally became very lethargic after the second injection, and seemed to have trouble breathing. They huddled together even when in the light, and their body temperature seemed to be lower than normal, as judged simply by holding them in the palm of the hand. Four days after the last injection, only 50% had survived. Some adhesion of the internal organs occurred; this was quite marked in the mice surviving 4 days after injection.

(d) Effects on the mtDNA superhelix density

Two experiments using the dose schedule mentioned above were performed (Table 1). In one experiment the superhelix density of mtDNA from livers and from a mixture of spleens and kidneys was measured. In a second experiment, only liver tissue was studied, and the mtDNA of liver was also prepared from the mice which had survived 4 days after the last injection.

The effect on the superhelix density of the mtDNA is clearly apparent in all tissue investigated, although the magnitude of the change is larger in the liver, where it equals that found for mouse cells in culture at high EthBr concentrations. The effect is reversible, as demonstrated by the fact that the mtDNA from the livers of mice surviving the treatment had a superhelix density equal to that of the mtDNA from the livers of untreated mice.

Table 1

Sample	Superhelix density	
	Experiment 1	Experiment 2
Untreated liver	-0.025	
Treated (spleens + kidneys)	-0.056	
Treated liver	-0.091*	-0.101
Liver, 4 days after injection		-0.025

* Less than 10% of the DNA banded just below the main closed band.

DISCUSSION

These experiments demonstrate that the effects of ethidium bromide on the superhelix density of mitochondrial DNA of cultured cells can also be seen in mouse liver, kidney, and spleen. This indicates that under the conditions used, a nicking-closing cycle is operative in these organs. The difference in density reached after treatment may represent differences in permeability of various tissue to ethidium bromide.

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Chapter 7.

Eighteen-hour Isotopic Labeling of HeLa Cell Mitochondrial DNA

INTRODUCTION

In 1969, Koch (1) reported the results of isotopic labeling studies with a line of cultured human liver cells, the Chang liver cells. He observed that when ^{3}H-thymidine was used to label DNA in growing cells, the bulk of the label in DNA isolated from the mitochondrial fraction appeared in a DNA which was linear and had a buoyant density 12 mg/ml lighter than nuclear DNA. The light DNA has properties considerably different from human mitochondrial DNA, which has a buoyant density 10 mg/ml heavier than nuclear DNA, and most of which is closed circular. Moreover, when a 20-hour labeling period was followed by a 3-day growth in cold medium, the light DNA was no longer labeled and 40% of the label appeared in closed circular DNA. The same sequence of events was seen when a single culture was labeled, grown in the absence of label, and then relabeled with a different isotope. Koch postulated that the light DNA might be a metabolic precursor to the heavy closed circular DNA in the mitochondria.

Although no evidence for such a light satellite had been seen in this laboratory, we had routinely incubated the mitochondria with DNase in those cases in which total mtDNA was recovered, and it was possible that the light satellite reported was destroyed by this treatment. Because of the important implications of Koch's postulate on mtDNA replication, similar experiments were performed with another human cell line, HeLa cells. No evidence for such a precursor DNA was found.

MATERIALS AND METHODS

(a) Cells and media

HeLa S3 cells have been grown in this laboratory for several years. They were grown in suspension culture in Eagle's phosphate medium supplemented with 5% calf serum.

(b) Isotopic labeling

^{3}H-thymidine (Schwarz/Mann, 17 Ci/mmole) was added to a concentration of 0.5 μCi/ml to cells growing exponentially (1×10^{5} cells/ml), 18 hours before harvest. For preparation of marker nuclear DNA, ^{32}P-labeled inorganic orthophosphate (International Chemical and Nuclear Corp., carrier free) was added to a concentration of 5 μCi/ml.

(c) Preparation of nuclear DNA

Cells were harvested by centrifugation and washed with TD (0.14 M-NaCl, 0.005 M-KCl, 0.007 M-Na_2HPO_4, 0.025 M-Tris, pH 7.5). They were resuspended in 10 times their packed volume in RSB (0.01 M-NaCl, 0.0015 M-$MgCl_2$, 0.01 M-Tris, pH 8), allowed to swell for 10 minutes and disrupted with 2 strokes in a glass Dounce homogenizer. The homogenate was centrifuged at 1000 rev./min for 1 minute in an International PR-6 to remove unbroken cells. The supernatant was spun at 2500 rev./min for 5 minutes. The pellet was resuspended in RSB and made to a concentration of 0.8% SDS. After incubation at 20° C for 1 hour, the solution was chilled and made to 1 M-CsCl, then centrifuged at 12,000 rev./min for 20 minutes in a Sorvall SS34 rotor.

The supernatant was made to contain 300 μg EthBr/ml, the density was adjusted to 1.55 g/ml with CsCl, and the solution centrifuged at 38,000 rev./min for 24 hours in an SW50 rotor. The fluorescent band was removed with a pasteur pipet.

(d) Preparation of mtDNA

Because no DNase treatment was used, extreme care was taken to keep nuclear contamination low by resuspending mitochondria with a small tight-fitting glass Dounce homogenizer, keeping the amount of mitochondria sedimented through each sucrose gradient low, and employing more low speed centrifugation than usual. Cells were harvested, washed and homogenized as above, sucrose was added to a concentration of 0.25 M and the mixture centrifuged at 2500 rev./min for 6 minutes in the International PR-6 centrifuge (low speed spin). This spin was repeated on the supernatant, and the resulting supernatant centrifuged at 12,000 rev./min for 15 minutes in a Sorvall SS34 rotor (high speed spin). The pellet was resuspended in MS (2); the solution was spun at low speed and the supernatant at high speed, and the pellet resuspended in MS. This solution was layered onto a step gradient in sucrose consisting of layers of 1.5 and 1.0 M-sucrose, 0.01 M-Tris (pH 8), 0.001 M-EDTA, and spun for 45 minutes at 25,000 rev./min in an SW27 rotor. One gradient was used for every 2×10^8 cells. The mitochondria at the interface between the layers were removed, diluted with 5 volumes of 0.01 M-Tris, 0.001 M-EDTA, and spun at high speed. The pellet was resuspended in MS and the suspension spun at low speed; the supernatant was spun at high speed. This final mitochondrial pellet was resuspended in

0.1 M-NaCl, 0.01 M-Tris, 0.001 M-EDTA, and lysed with the addition of SDS to 0.8%. DNA was recovered and banded as described above. Both fluorescent bands were isolated by drop collection.

(e) Purification of DNA

The DNA samples were dialyzed against the buffer last mentioned to remove CsCl and layered on top of a step gradient in CsCl consisting of 2.5 ml CsCl, 1.3 g/ml, 50 μg EthBr/ml and 0.8 ml CsCl, 1.7 g/ml, and centrifuged for 3 hours at 40,000 rev./min in an SW50.1 rotor. The lower third of the ube was removed by drop collection. This procedure was used to remove radioactivity not in high molecular weight DNA. The samples were freed of EthBr by dialysis against Dowex-50 resin.

RESULTS

(a) Characterization of the ^{3}H-labeled DNA

An aliquot of the DNA from cells labeled for 18 hours was centrifuged to equilibrium in a CsCl-EthBr density gradient. The tube was dripped directly onto glass filters, which were dried and assayed for radioactivity by liquid scintillation spectrometry (Fig. 1). The closed circular DNA contained 42% of the radioactivity, a result which is in marked contrast to that of Koch, who found less than 10% of the radioactivity in closed DNA in a similar experiment.

Another aliquot was centrifuged to equilibrium in neutral CsCl in the analytical ultracentrifuge. The photoelectric scanner trace showed no detectable DNA banded at a position lighter than nuclear DNA, and roughly 65% of the mass of the DNA was seen at the density of mitochondrial DNA.

(b) The density of the ^{3}H label

An aliquot of the ^{3}H-labeled DNA was mixed with ^{32}P-labeled nuclear DNA from HeLa cells. The solution was made to a density of 1.7 g/ml with CsCl and centrifuged for 44 hours at 40,000 rev./min in a Spinco fixed-angle titanium 50 rotor at 20° C. The tube was assayed for radioactivity as before (Fig. 2). No tritium label was seen in a species lighter in density than the marker nuclear DNA. Between 60 and 70% of the counts were in the region heavier than nuclear DNA, which is the position of mtDNA, and the remaining counts were at the position corresponding to nuclear DNA, as judged by the added marker.

FIG. 1. Radioisotope distribution of the DNA centrifuged in a CsCl-EthBr gradient. The DNA was isolated from purified mitochondria of HeLa cells labeled as described in the text. The band of closed circular DNA is to the left. The fractions above and below the bands, which are not shown, did not contain radioactivity above background.

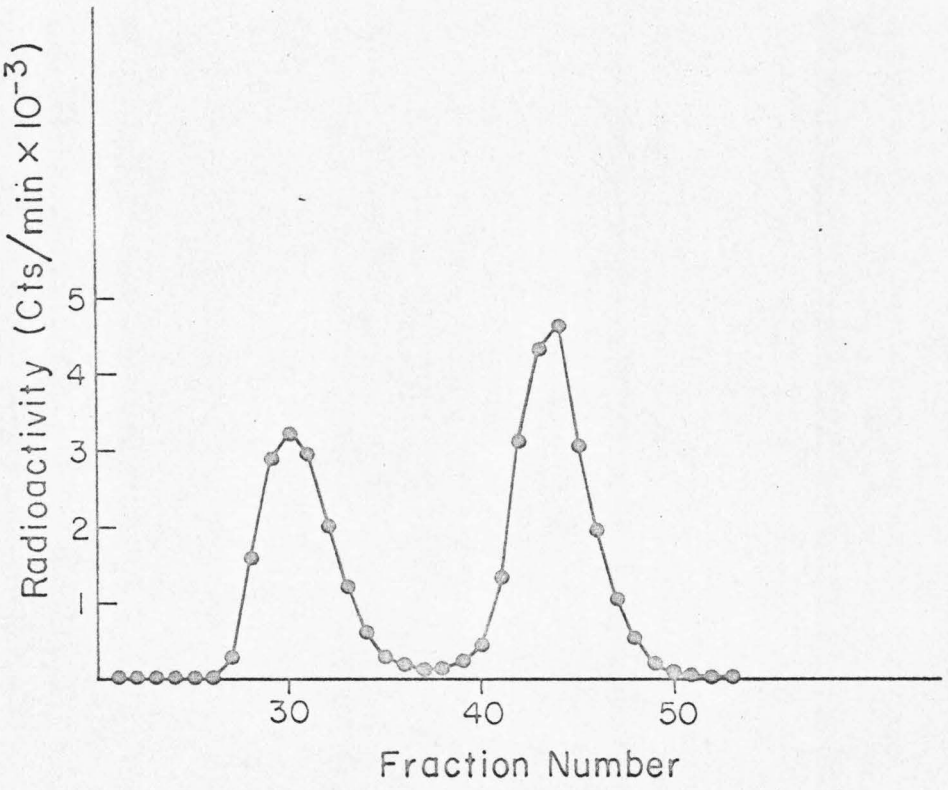
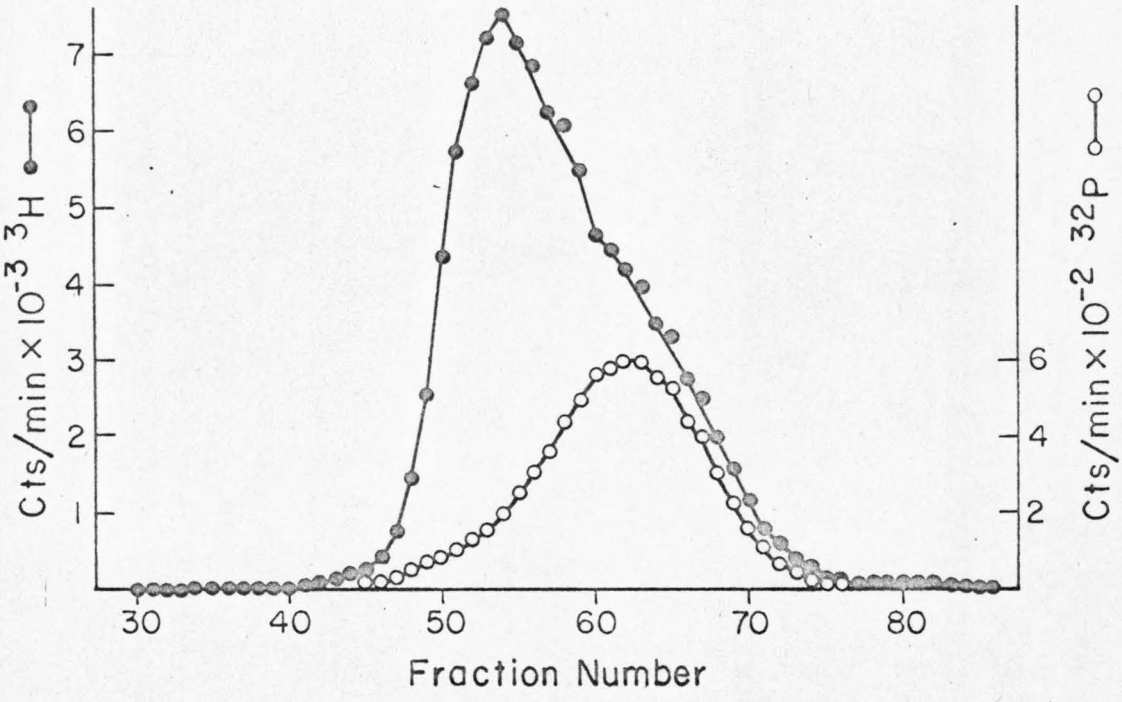

Radioactivity (Cts/min × 10⁻³)
1
2
3
4
5
30
40
50
Fraction Number

FIG. 2. Radioisotope distribution of the DNA from HeLa cell mitochondria and purified HeLa nuclear DNA centrifuged in the same tube in neutral CsCl. Density increases to the left. The fractions not shown did not contain radioactivity above background.


Cts/min × 10⁻³ ³H
Cts/min × 10⁻² ³²P
Fraction Number
30
40
50
60
70
80
1
2
3
4
5
6
7
2
4
6

DISCUSSION

These experiments were designed to duplicate as closely as possible those reported by Koch. No evidence was found for a light satellite, either by label or by mass. The fraction of label which bands at the mitochondrial DNA density corresponds to the fraction of mass banding at that position.

Unpublished results (C. A. Smith) have shown that with pulses as short as 0.5 hour, label appears in DNA with the buoyant density of mitochondrial DNA.

Although Koch reported several experiments designed to rule out the possibility that his results were due to a mycoplasma contaminant, either mycoplasma or some bacterial contamination is the most likely explanation for his results.

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PART II

SMALL POLYDISPERSE CIRCULAR DNA OF HELA CELLS

The following paper has been prepared for submission to the Journal of Molecular Biology.

Small Polydisperse Circular DNA of HeLa Cells

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Running Title: SMALL POLYDISPERSE CIRCULAR DNA

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Summary

Small, polydisperse closed circular DNA has been studied in HeLa cells and has also been found in several other animal cells in culture. The DNA is recovered from ethidium bromide-CsCl gradients from whole cell extracts or from homogenates from which nuclei and mitochondria have been removed; it is absent from purified nuclei and mitochondria.

Exponentially growing cells contain 50 to 200 circles. Cycloheximide treatment results in a 20- to 30-fold increase. A 10- to 20-fold increase is seen in cells which remain at saturation density for two to five days.

The size distribution obtained by electron microscopy ranges from 0.1 μ to more than 2 μ, with a mean length of 0.32 μ and a weight average molecular weight ($\overline{M}_w$) of 1.0×10^6. The analytical band sedimentation patterns were consistent with the electron microscopy size distribution. The weight average sedimentation coefficients, 14.8 s at pH 8 and 26.5 s at pH 12.5, also indicate an $\overline{M}_w$ of 1.0×10^6. The neutral buoyant density, 1.692 g/ml., indicates a G + C content of 38 mole %. At pH 12.5, the closed DNA exhibits the expected buoyant shift, and is resistant to the first alkali-induced nick for at least 24 hours. The complementary strands are not detectably different in buoyant density at pH 12.5. The average superhelix density of spc-DNA†

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† Abbreviation used: spc-DNA, small polydisperse closed circular DNA.

is slightly higher than that of mitochondrial DNA of these cells. The genetic complexity of spc-DNA from cycloheximide-treated cells rules out the possibility that circles are composed of subunits having the size of the smaller circles in the population.

Cycloheximide was used to increase the amount of spc-DNA in cells first labeled with [^{3}H]thymidine and then allowed to grow one generation in unlabeled medium. The specific activities of spc-DNA and nuclear DNA were identical, suggesting that spc-DNA arises from preexisting nuclear DNA. A possible mechanism for the formation of spc-DNA is discussed.

1. Introduction

New species of closed circular DNA have been reported regularly since the discovery of this structure in the DNA of polyoma virus. The list of well-characterized closed DNA's now includes bacteriophage DNA, extrachromosomal bacterial DNA (sex factors, drug-resistance factors, colicinogenic factors, and defective phage genomes), animal virus DNA's, animal mitochondrial DNA, and kinetoplast DNA (Helinski & Clewell, 1971). All of these above DNA's appear to be homogeneous in size, except for those that contain deletions or consist of multiples of a monomer unit. In some cases a cell may harbor two or more species of different size. Closed circular DNA's which appear to be polydisperse, or heterogeneous in size have been reported to occur in Bacillus megaterium (Carlton & Helinski, 1969), unfractionated boar sperm (Hotta & Bassel, 1965) (no evidence that the circles are covalently closed), yeast cells (Sinclair, Stevens, Sanghavi & Rabinowitz, 1967; Shaprio, Grossman, Marmur & Kleinschmidt, 1968; Guernineau, Grandchamp, Yotsuyanagi & Slonimski, 1968), and in two animal cell lines, BSC-1 (African green monkey) (Rush, Eason & Vinograd, 1971) and HeLa (Radloff, Bauer & Vinograd, 1967).

A circular DNA species, ranging from 0.2 to 2 microns and heterogeneous in size, was first seen in the electron microscope examination of the closed DNA from unfractionated HeLa cells (Radloff, Bauer & Vinograd, 1967), and will be referred to here as small, polydisperse circular DNA (spc-DNA). This report presents the results of a more complete study of this species in HeLa cells, as well as some

results with other tissue culture cells. A method has been found for obtaining enough spc-DNA for physical studies. The DNA displays properties consistent with a closed circular DNA, and is not found in mitochondria or nuclei. Reannealing studies indicate that the spc-DNA is not an oligomeric series of the smallest size in the population.

2. Materials and Methods

(a) Cells

HeLa S3 cells have been grown in this laboratory for several years. BSC-1 cells were obtained from Flow Laboratories. LA9 cells were obtained from L. Crawford, Imperial Cancer Research Fund, London. WI38 cells were obtained from H. Koprowski, Wistar Institute, Philadelphia. L929 cells were obtained from the American Type Tissue Culture Collection. L_D cells were obtained from C. Schildkraut, Albert Einstein College of Medicine, New York.

(b) Cell growth

Cells were grown in suspension culture in Eagle's phosphate medium (Grand Island Biological Co., Berkeley, Calif.), supplemented with 5% calf serum (HeLa cells) or 10% calf serum (all others). Eagle's medium supplemented with calf serum in the same amount as for suspension culture was used for growth on petri plates. Mouse myeloma cells were a gift from W. J. Dreyer, California Institute of Technology, and were propagated in the peritonea of Balb/C mice at approximately 10-day intervals. Except where noted, the cells were growing exponentially when harvested.

(c) Phenol extraction of cytoplasm of HeLa cells

Cells were harvested by centrifugation and chilled to 4° C. They were washed twice in cold buffer (0.14 M-NaCl, 0.005 M-KCl, 0.007 M-Na_2HPO_4, 0.025 M-Tris, pH 7.5) and homogenized in 7 times their

packed cell volume in MS buffer (0.07 M-sucrose, 0.21 M-mannitol, 0.01 M-Tris, pH 7.5, 0.001 M-EDTA) with a glass homogenizer fitted with a motor-driven teflon pestle. The homogenates were spun at 2500 rev./min in an International PR-2 centrifuge for 10 min to remove nuclei and unbroken cells. The supernatant was recentrifuged in the same manner and the resulting supernatant was centrifuged at 12,000 rev./min in a Sorvall SS34 rotor for 15 min. The supernatant was generally frozen at -70° C until used. The supernatant was thawed and the volume reduced to approximately 250 ml. by flash evaporation in a bath at 20° C. The concentrate was extracted with an equal volume of freshly distilled phenol, saturated with a 0.01 M-Tris (pH 7.5), 0.001 M-EDTA, 0.1 M-NaTCA solution. The phases were separated by centrifugation. The aqueous layer was removed and re-extracted. The phenol layer and interface were re-extracted with the buffer mentioned above. In some cases a third extraction was performed. The final aqueous layers were pooled and extracted several times with 2 vol. of ethyl ether to remove phenol. This solution was then concentrated by several cycles of flash evaporation followed by dialysis versus 0.01 M-Tris (pH 7.5), 0.001 M-EDTA, until the volume was approximately 15 ml. The closed DNA in this solution was recovered by one or more density gradient centrifugations employing CsCl and ethidium bromide (Radloff, Bauer & Vinograd, 1967). Final closed DNA was isolated by drop collection.

(d) Whole cell extracts for closed DNA

A modification of the Hirt procedure (1966) was used to extract

DNA from whole cells. The procedure has been described previously (Smith, Jordan & Vinograd, 1971).

(e) Preparative sedimentation velocity

Spc-DNA was often separated from mtDNA† by sedimentation through neutral CsCl containing 100 μg EthBr†/ml. Linear gradients were made with CsCl solutions having densities of 1.3 g/ml. and 1.4 g/ml. Sedimentation was generally for 3 hr at 40,000 rev./min in an SW50.1 rotor at 20° C. Self-generating gradients were also used. 0.5 ml. of the sample having a density of 1.1 to 1.2 g/ml. was layered on to 4 ml. of CsCl, 1.4 g/ml., containing 100 μg EthBr/ml., and run as above. The tubes were fractionated by drop collection or by removal of the upper portions with a pasteur pipet. Linear gradients of sucrose (5 to 20%) containing 0.5 M-NaCl, 0.01 M-Tris (pH 8), 0.001 M-EDTA were used to analyze radioactive samples.

(f) Preparation of DNA fragments

The DNA solution in 2 M-CsCl containing 100 μg EthBr/ml. was placed in a 1 × 1 × 3-cm glass cuvette and exposed to the visible light beam 10 cm from the lens of a slide projector equipped with a 500-watt lamp for 180 min. An aluminum-foil reflector was placed behind the cuvette, which was cooled with a small fan, and the solution was stirred with a magnetic stirrer. The closed DNA remaining in the spc-DNA

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† Abbreviations used: mtDNA, mitochondrial DNA; EthBr, ethidium bromide.

sample was isolated and exposed again. A single exposure was used for SV40 DNA.

(g) Electron microscopy

Procedures for the preparation of specimen grids for electron microscopy have been described for both the aqueous and formamide mounting techniques (Davis, Simon & Davidson, 1971). Two procedures were used for measurements: photographs were traced at a magnification of 50 on a Nikon 6F projection microcomparator, or tracings on transparent mylar film were made directly with a special television monitoring device attached to the Phillips EM300. All tracings were measured with a map measurer.

(h) Analytical ultracentrifugation

Buoyant density experiments were performed at 25° C for 24 hr at either 44, 000 or 44, 770 rev./min in a Beckman model E ultracentrifuge equipped with a photoelectric scanner. Alkaline buoyant densities were measured in 0.05 M-K_3PO_4, 0.05 M-KOH (pH 12.5). The buoyant density gradient (Vinograd & Hearst, 1962) was used in the calculation of the results in both the neutral and alkaline experiments. The relative amounts of DNA in different peaks were determined by ordinate summation, either on an enlargement of the trace from the Offner recorder in the scanner or directly from a scan recorded on a Moseley 7001 AM recorder. Correction was made for the sector shape of the centrifuge cell.

The sedimentation velocity experiments were performed by the

band procedure (Vinograd, Bruner, Kent & Weigle, 1963) in a Beckman model E ultracentrifuge equipped with a photoelectric scanner. Sedimentation was through 2.85 M-CsCl buffered with 0.01 M-Tris (pH 8), or containing 0.1 M-KOH, 0.005 M-EDTA. Correction of observed sedimentation coefficients at neutral pH to standard sedimentation coefficients has been described (Bruner & Vinograd, 1965).

(i) Measurement of superhelix density

The EthBr banding method for superhelix density determination has been described (Gray, Upholt & Vinograd, 1971).

(j) Chemicals

Cycloheximide (B grade) was purchased from Calbiochem, Los Angeles, Calif. Ethidium bromide was a gift from Boots Pure Drug Co. Ltd., Nottingham, England. Optical grade cesium chloride was obtained from the Harshaw Chemical Company, Solon, Ohio. Cytochrome c was purchased from Calbiochem. Crab d(A-T) was a gift from R. Hyman. Sodium dodecyl sulfate was obtained from the Matheson Company, as was 99% formamide. All other chemicals were reagent grade.

3. Results

(a) Physical properties of small polydisperse DNA

The small amounts of spc-DNA obtainable from exponentially growing cells were inadequate for certain physical studies. Accordingly, some of the physical properties described below have been studied using the increased amounts available when cells are treated with cycloheximide.

(i) Size

The spc-DNA's range in size from 0.1 to 2 μ (Plate I). The size distribution is very sensitive to adventitious nicking during preparation because larger molecules will be preferentially lost. The length and mass distribution of a sample of spc-DNA prepared by phenol extraction of HeLa cell cytoplasm is shown in Figure 1. The lengths were determined using an external standard and, therefore, molecular weights may be in error by as much as 10%. The distribution ranges from about 0.05 μ (the smallest circle which can reliably be distinguished from small holes in the parlodion film) to 2 μ, and peaks sharply in the region of 0.2 μ. The mean length is 0.32 μ; the weight average molecular weight is 1.0×10^6 daltons. A value of 2×10^6 daltons per micron was used in this calculation. Approximately 86% of the molecules are 0.5 μ or shorter; 65% of the mass is accounted for by molecules less than 1 million daltons in molecular weight.

The heterogeneity of the size distribution of the spc-DNA is apparent in the tailing of the distribution at longer lengths and in analysis

PLATE I. Electron micrographs of closed DNA obtained from HeLa cells.

Upper panel, spc-DNA in the supercoiled form; center panel, relaxed spc-DNA; lower panel, mitochondrial DNA, 4.8 μm.

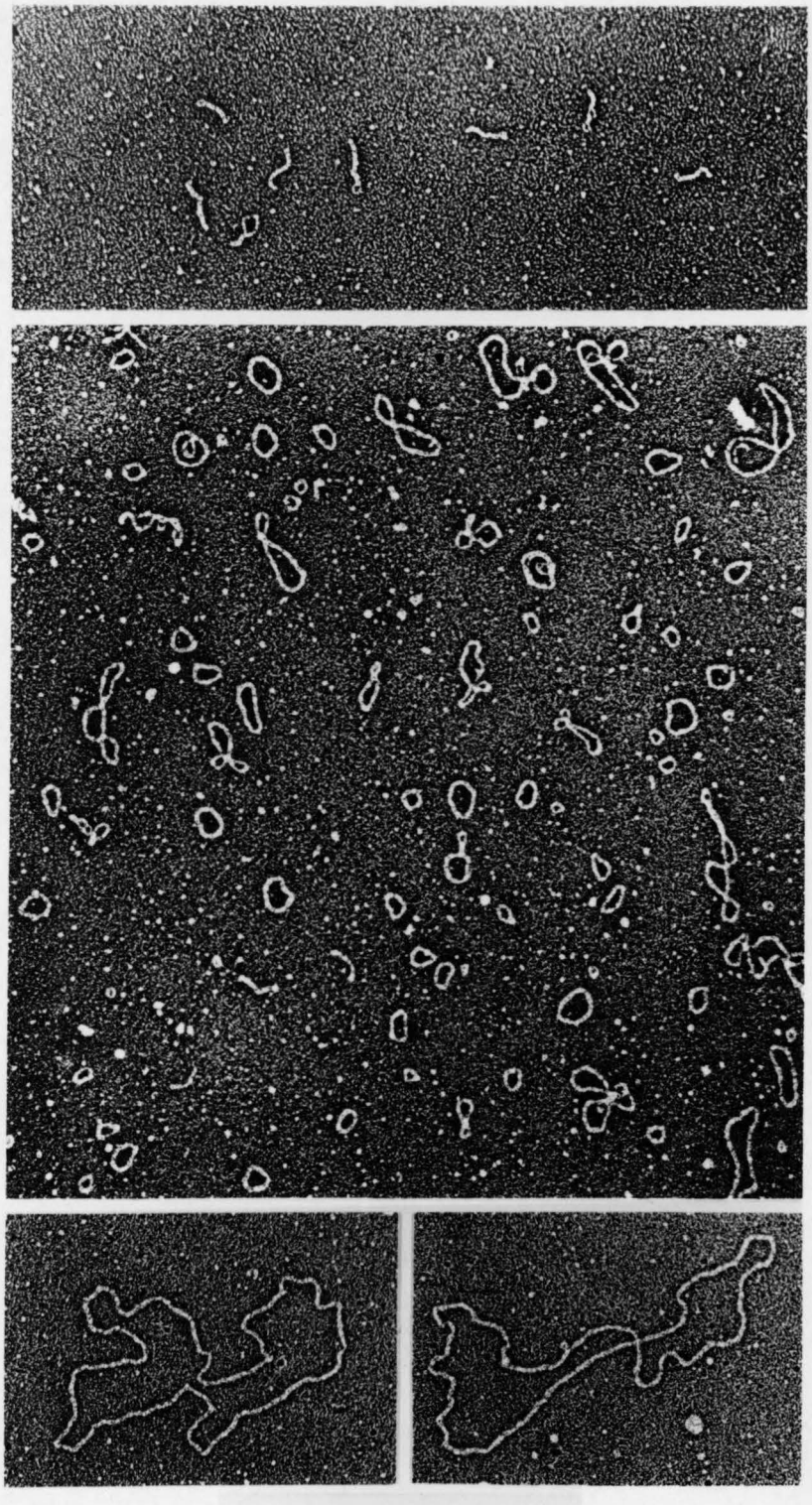
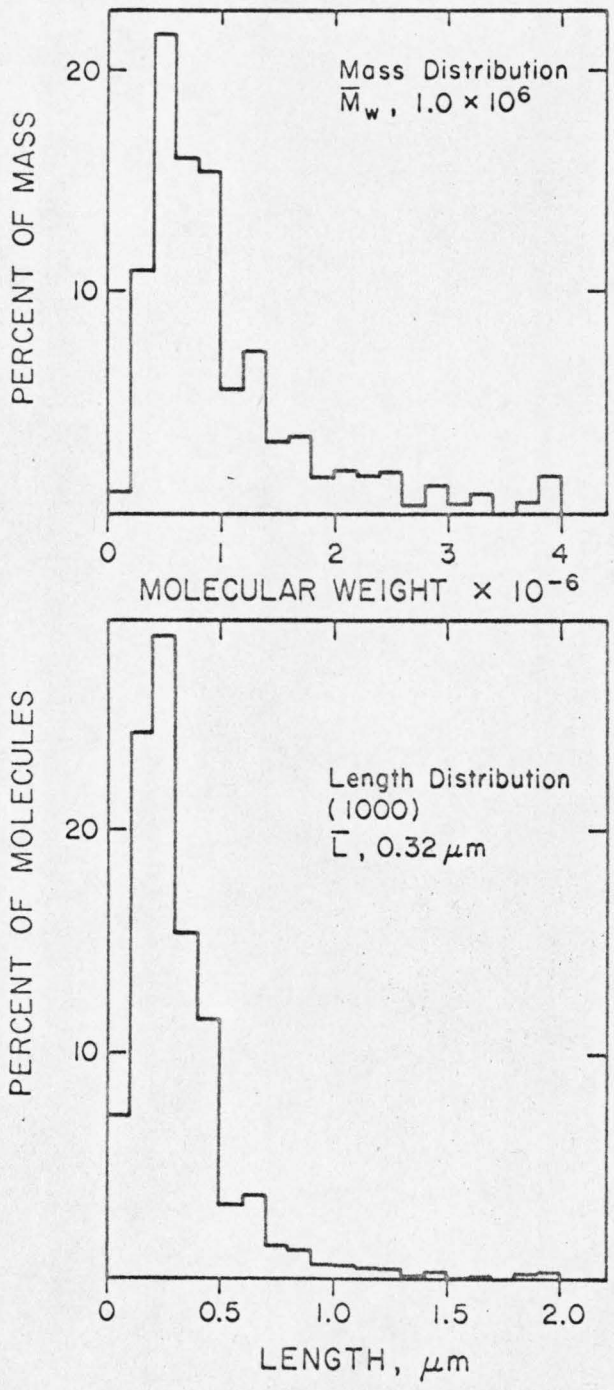

1 μm

FIG. 1. Length and mass distribution of spc-DNA obtained by phenol extraction of cytoplasm.


Mass Distribution
$\bar{M}_w$, 1.0×10^6
PERCENT OF MASS
20
10
0
1
2
3
4
MOLECULAR WEIGHT $\times 10^{-6}$
Length Distribution
(1000)
$\bar{L}$, 0.32 μm
PERCENT OF MOLECULES
20
10
0
0.5
1.0
1.5
2.0
LENGTH, μm

of the expected standard deviations for measurement of molecules of this size. A homogeneous DNA of this size class, the plasmid DNA of Escherichia coli-15, had a mean length of 0.67 ± 0.03 μ (Lee & Davidson, 1970). Davis, Simon & Davidson (1971) have derived an empirical relation between the size and the standard deviation of a distribution of a monodisperse duplex DNA as measured in the electron microscope. This relation predicts a standard deviation of 0.02 μ for a monodisperse DNA with a size of 0.3 μ. The width of the distribution below 0.5 μ, approximately ±0.13 μ, makes it clear that we are not dealing with a DNA homogeneous in size. Since the interval of the histogram in Figure 1 is three to five times the standard deviation expected for monodisperse DNA species in these size classes, the distribution is compatible with a minimum of 20 evenly spaced size classes. The distribution plotted with intervals half the size of that in Figure 1 did not change in appearance. In the region less than 1 μ, we are unable to distinguish between a paucidisperse distribution containing 10 or more evenly spaced size classes and a polydisperse distribution.

The sensitivity of the form of the distribution to nicking was shown in a distribution of the DNA from the upper band of a sample of spc-DNA which had been extensively, but not completely, nicked. The average length was 0.6 μ; the weight average molecular weight was 1.7×10^6 daltons.

(ii) Sedimentation velocity

To prepare adequate quantities of spc-DNA for analytical band sedimentation velocity, cells were treated with cycloheximide and total

closed DNA was extracted. After two CsCl-EthBr density gradients, the DNA was fractionated using preparative velocity sedimentation through CsCl. The DNA moving slower than mtDNA was rebanded in CsCl-EthBr. The closed DNA from a further rebanding was examined by electron microscopy and estimated to contain 1 to 2% small linear DNA.

Analytical band sedimentation through CsCl, 1.35 g/ml., was performed on this sample at both pH 8 and pH 12.5 (Plate II). The material forms a very heterogeneous sedimentation pattern with extensive spreading. This confirms the heterogeneity observed in the size distribution. The spreading is greater at alkaline pH, as expected from the values of α in the relation between sedimentation velocity, S, and molecular weight, M;

$$S = kM^{\alpha}$$

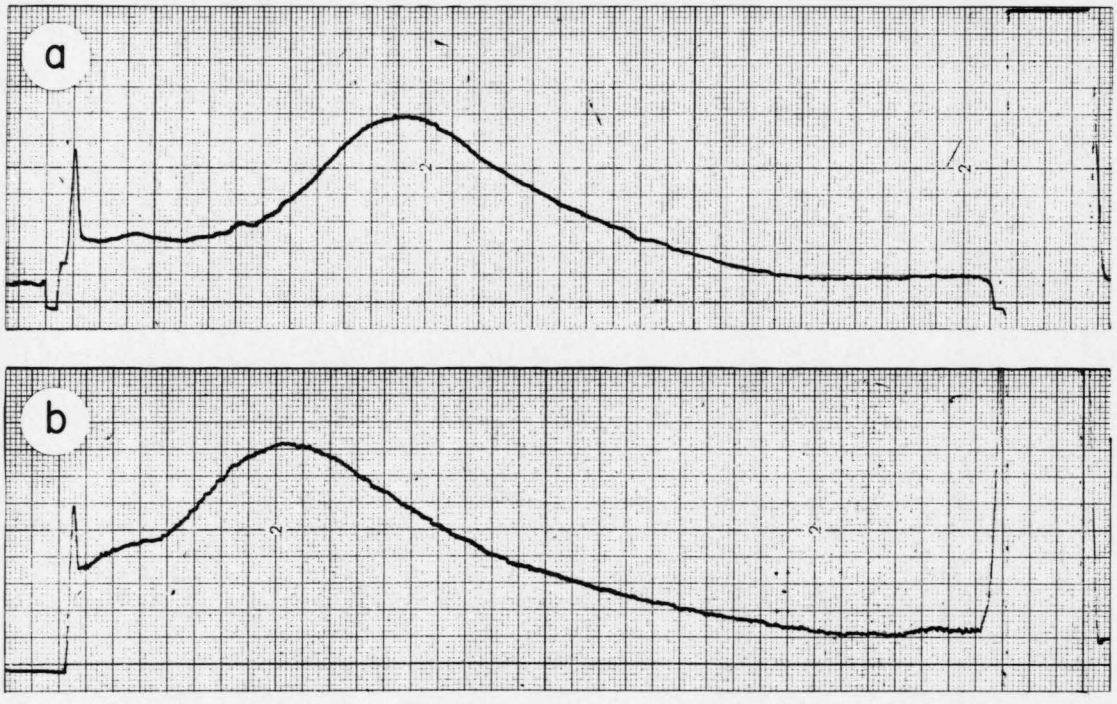
where k is a constant. At neutral pH, α is 0.39, whereas at alkaline pH it is 0.49 (Clayton & Vinograd, 1967).

The sedimentation coefficient obtained from the motion of the maximum in the concentration distribution gives an underestimate of the weight average molecular weight. For a better estimate, the approximate center of gravity of the mass, $\langle r \rangle$, was determined at several times during each run, from the relation

$$\langle r \rangle = \frac{\Sigma r^2 c}{\Sigma r c} \quad .$$

Here, r is a radial position in the cell and c is the concentration of

PLATE II. Band sedimentation velocity patterns of closed circular spc-DNA in (a) neutral and (b) alkaline CsCl solutions. Photoelectric scans of the liquid column showing the meniscus on the left and the bottom of the column on the right. The scan in (a) was taken approximately 40 min after reaching 52, 640 rev./min. The scan in (b) was taken approximately 16 min after reaching 39, 460 rev./min.


a
b

DNA at that position. Six to 12 different radial positions were used over each band. The observed sedimentation coefficient was determined from a least-squares analysis of a $\ln\langle r\rangle$ versus t plot. This coefficient was corrected to give a standard sedimentation coefficient of 14.8 s. The latter value corresponds to a weight average molecular weight of 1.0×10^6 daltons (Hudson & Vinograd, 1969).

The sedimentation coefficient at alkaline pH, uncorrected except for deviations from a density of 1.35 g/ml., is 26.5 s. An extrapolation of the least-squares line for sedimentation coefficients between 3 to 65×10^6 daltons (Clayton & Vinograd, 1967) gives a value of 1.1×10^6 daltons for the weight average molecular weight of spc-DNA. The results clearly indicate that the DNA is closed. The weight average molecular weights derived from these analyses are in agreement with the values obtained from the measurements made using electron microscopy.

(iii) Buoyant properties

The neutral buoyant density of spc-DNA was obtained with a sample which still contained some mtDNA. In addition to the sharp band of mtDNA, a broad band lighter in density was observed. Using the value of 1.700 g/ml. for mtDNA, the spc-DNA had a density of 1.692 g/ml. This buoyant density is very close to that reported for human nuclear DNA (Clayton & Vinograd, 1967), and indicates a G + C content of 38 mole % (Schildkraut, Marmur & Doty, 1962).

Further buoyant characterization was done using spc-DNA from cycloheximide-treated cells. The total closed DNA from such cells was

subjected to preparative velocity sedimentation through CsCl. The tube was fractionated into five 1-ml. fractions and each was analyzed by electron microscopy and analytical buoyant sedimentation equilibrium. The fraction near the top of the tube containing the highest proportion of spc-DNA also contained the highest proportion of the wide band with a density 0.008 g/ml. lighter than mtDNA. Another sample of spc-DNA from cycloheximide-treated cells containing only 5% mtDNA was analyzed using crab d(A-T) as a marker, with a density of 1.670 g/ml. The value of 1.692 g/ml. for spc-DNA was confirmed.

Spc-DNA, virtually free of contaminating mtDNA, was subjected to buoyant equilibrium sedimentation at pH 12.5, using crab d(A-T) as a density marker. The closed DNA showed a single peak which was 0.045 g/ml. heavier than the marker. This difference is 0.023 g/ml. greater than the difference in neutral pH and is expected of a closed circular DNA (Weil & Vinograd, 1963). When this experiment was repeated using nicked spc-DNA, a single band was seen banding 0.022 g/ml. heavier than the marker, the same density difference seen at neutral pH. The presence of only one band indicates that the complementary strands do not differ greatly in their amount of G + T, as do the animal mtDNA's which separate into two bands under these conditions. The alkali stability of spc-DNA is shown by the fact that after 48 hours at pH 12.5, less than 10% of the DNA bands at the position of single-stranded DNA.

(iv) Superhelix density

A characteristic property of a closed circular DNA is its super-

helix density, σ_0 (the number of superhelical turns per ten base pairs). The value of σ_0 of spc-DNA was determined by the buoyant separation method, in which the separation between the closed and nicked forms in an EthBr-CsCl equilibrium gradient under defined conditions is used as an index of the superhelix density (Gray, Upholt & Vinograd, 1971). All measurements are made relative to a DNA of known superhelix density run at the same time. SV40 viral DNA with a σ_0 of -0.039, was used as a standard.

Two different determinations of the superhelix density of spc-DNA were made using this method. DNA prepared from cycloheximide-treated cells was banded and the separations were determined on photographs of the tubes containing the fluorescent bands. In this case the SV40 standard was in another tube in the same rotor. A measurement of the superhelix density of ^{3}H-labeled spc-DNA from exponentially growing cells was made by centrifuging this DNA with added ^{32}P-labeled SV40 DNA. The sample was assayed for radioactivity by dripping the tube directly onto glass filter papers and counting in a liquid scintillation spectrometer. The centers of mass of each of the four bands in the tube were calculated and a measurement of the relative separations was obtained. The superhelix density of the mtDNA from these cells was also determined at the same time as a check on the procedure.

The result of the optical determination from cycloheximide-treated cells was -0.045 ± 0.002. The result for the untreated cells was -0.039 for spc-DNA and -0.025 for the mtDNA. The estimate of the error in radioactive experiments has been given as ± 0.003 (Eason &

Vinograd, 1971). Thus, the spc-DNA has a superhelix density significantly higher than that of the mtDNA of the cell.

(b) Amount of spc-DNA per cell

(i) Exponentially growing cells

The amount of spc-DNA in exponentially growing cells was determined from the ratio of spc-DNA to closed circular mtDNA in extracts of whole cells. The recovery of closed mtDNA was approximately 25% of that obtained when mtDNA is extracted from isolated mitochondria, presumably because of the size and location of mtDNA. A minimum estimate of the amount of spc-DNA per cell is obtained by assuming that the spc-DNA is recovered quantitatively.

The closed DNA from 3.5×10^8 cells was prepared by the modified Hirt procedure and examined in the electron microscope. The ratio of small circles to mitochondrial monomer units was 0.64 among 1500 molecules examined. The amount of mtDNA recovered was determined by simultaneously banding a known fraction of the DNA and a known amount of PM2 viral DNA is separate cells in the analytical ultracentrifuge. Comparison of the areas under the bands allowed the calculation that the original sample contained 0.5 μg of mtDNA or 0.14 μg of DNA per 10^8 cells. When mtDNA is prepared from purified mitochondria of HeLa cells, 0.5 to 0.6 μg of closed DNA is recovered per 10^8 cells. This corresponds to 300 to 360 closed mitochondrial molecules per cell. The yield of mtDNA in the Hirt extract was, therefore, about 25%. The minimum estimate of spc-DNA is thus about 50

molecules per cell, 1 % of the mtDNA mass, or about 6×10^{-3} μg per 10^8 cells. It should be noted that the above are average values. The variability of the amount of spc-DNA per cell among the cells is not known.

(ii) Cells in stationary phase

When HeLa cells are allowed to reach saturation density in a spinner culture and allowed to stay at this density for one to three days, the total closed DNA from the cells contains a higher amount of spc-DNA as judged by qualitative electron microscopy. A buoyant analysis on spc-DNA isolated from cells in stationary phase for two days indicated that the spc-DNA increased ten- to twenty-fold over the yield from exponentially growing cells.

(iii) Cycloheximide-treated cells

Cycloheximide was used routinely to recover increased amounts of spc-DNA in cells grown in suspension culture. Qualitative estimates of the increase over the normal amount indicated little effect of varying the concentration between 10 and 60 μg/ml. for 15 hours. Conditions usually used to prepare samples of spc-DNA were treatment for 12 to 16 hours at 50 μg/ml. The amount of spc-DNA recovered was estimated by analytical ultracentrifugation and varied between 0.1 and 0.2 μg per 10^8 cells. Increased yields of spc-DNA were also obtained upon cycloheximide treatment of cells grown on petri plates.

(c) Intracellular location

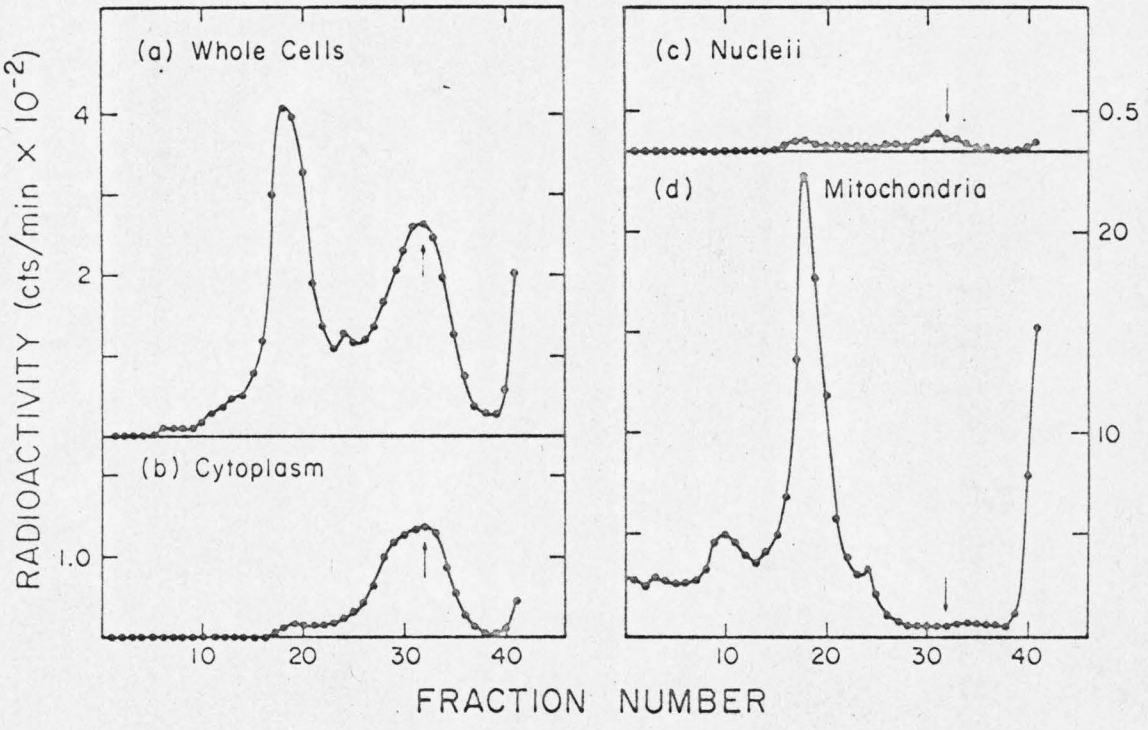
An intra-mitochondrial location for spc-DNA appeared to be unlikely in that this DNA has never been reported as a constituent of the closed circular DNA isolated from purified mitochondria. Spc-DNA can be prepared from supernatants after sedimenting nuclei and mitochondria from cell homogenates. An experiment was performed to determine the amount of spc-DNA that could be isolated from nuclei, mitochondria, and cytoplasm.

Cells were labeled with [^{3}H]thymidine while in exponential growth. Five doses of 100 μCi were added to a one-liter culture at approximately 10-hour intervals. Six hours after the last addition, the cells were harvested and divided into two samples of 2×10^8 cells each. The closed DNA was extracted by the Hirt procedure from one sample. Cells in the other sample were taken up in a hypotonic buffer (0.01 M-NaCl, 0.001 M-EDTA, 0.01 M-Tris, pH 8) and homogenized with a glass Dounce homogenizer. The homogenate, adjusted to 0.25 M-sucrose, was layered on to a step sucrose gradient consisting of layers of 1.0, 1.5, and 2.5 M-sucrose containing the above buffer, in an SW27 tube. The gradient was centrifuged at 25,000 rev./min for one hour at 4° C. A volume equal to that layered on was carefully removed from the top of the tube. Mitochondria banding at the interface between 1.0 and 1.5 M-sucrose were removed and the DNA extracted. Nuclei banding at the interface between 1.5 and 2.5 M-sucrose were removed, diluted, pelleted and treated as were whole cells in the Hirt extraction for closed DNA. The top layer, designated as the cytoplasmic fraction, was made

to 1% sodium dodecyl sulfate and processed as described in the Hirt extraction procedure. All four DNA samples were banded in CsCl-EthBr. The region containing the entire lower band and a little of the heavy side of the upper band was rebanded, collected, and aliquots of the fractions assayed for radioactivity. The lower bands were rebanded with the addition of differentially labeled nicked SV40 DNA as a position marker. The last rebanding was performed to remove the last traces of small nuclear DNA from the lower band region. The final gradients contained less than 10% of the ^{3}H-counts in the upper band. Equal-sized aliquots of the lower band fractions were freed of EthBr and dialyzed versus 0.5 M-NaCl, 0.001 M-EDTA, 0.01 M-Tris (pH 8). One third of the mitochondrial sample and all of each of the other samples were sedimented in neutral, 5 to 20% sucrose gradients containing the above buffer (Fig. 2). Care was taken to keep the DNA from exposure to low ionic strength and the loss of labeled 7 s DNA fragments from mtDNA (Kasamatsu, Robberson & Vinograd, 1971).

The pattern from the whole cell extract contains a band with the sedimentation coefficient of mtDNA and a slower band of spc-DNA with a sedimentation coefficient of about 14 s. The pattern from the mitochondrial extract is characteristic of HeLa mtDNA and contains no band of 14 s DNA, although the background in this region is considerably higher than in other gradients. The remaining two thirds of the mitochondrial sample was sedimented and the 14 s region was collected and resedimented. The counts appeared only in the mitochondrial peak. It is concluded that the background in the 14 s region of the first gradient is not spc-DNA. As judged by the label in the 14 s bands, the

FIG. 2. Sedimentation velocity profiles of ^{3}H-labeled DNA extracted from different fractions of HeLa cells. Sedimentation is from right to left. DNA samples were layered on 5 to 20% sucrose gradients containing 0.5 M-NaCl, 0.001 M-EDTA, 0.01 M-Tris (pH 8). The tubes were centrifuged in an SW50.1 rotor for 110 min at 36,000 rev./min at 20° C. Drops were collected directly onto glass filter papers which were dried and counted in a Beckman model LS-250 scintillation spectrometer.


(a) Whole Cells
(b) Cytoplasm
(c) Nucleii
(d) Mitochondria
RADIOACTIVITY (cts/min × 10⁻²)
FRACTION NUMBER
4
2
1.0
0.5
20
10
10
20
30
40

cytoplasmic fraction contained 60% as much spc-DNA as the whole cell extract. A part of this difference can be accounted for by the failure to recover the cytoplasmic components completely from the SW27 tube. Radioactivity in the 14 s region of the nuclear extract amounted to only 6% of that in the same region of the whole cell extract.

(d) Formation of spc-DNA in cycloheximide-treated cells

Whether the spc-DNA seen in exponentially growing cells is replicating or is produced from some pre-existing DNA was not studied. The origin of the spc-DNA recoverable from cycloheximide-treated cells was investigated. In a preliminary experiment, it was found that when [^{3}H]thymidine was added shortly after the drug, the specific activity of the spc-DNA recovered after 20 hours was the same as that for the mtDNA. This result suggested that the increased spc-DNA was not due to a burst of replication of pre-existing spc-DNA. In this experiment, the cycloheximide may have inhibited the entry of the label into the precursor pools. In the experiment described below, cycloheximide was added to cells first labeled and then grown in cold medium.

Three doses of [^{3}H]thymidine were added to exponentially growing cells (150 μCi/l. at zero and 8 hours; 100 μCi/l. at 18 hours). Seven hours after the last addition, the cells were harvested, washed and resuspended in fresh medium, free of label. At this time an aliquot of cells was taken and nuclear DNA (N1) prepared. Thirty hours later, 50 μg cycloheximide/ml. was added to two thirds of the cultures, and nuclear DNA (N2) was again prepared from a small aliquot of cells. After a further 12 hours, closed DNA was prepared from both treated

and untreated cells. The mtDNA and spc-DNA were separated by velocity sedimentation on a 5 to 20% linear sucrose gradient containing 0.5 M-NaCl. Treated cells contained ten times the radioactivity in the 14 s region than did the untreated cells. The relative specific activities of the 14 s DNA and mtDNA from treated cells, the 14 s DNA of untreated cells, and the second nuclear DNA sample were determined (Table 1). Aliquots of each were sedimented to equilibrium in CsCl and the relative amount determined from photoelectric scans taken with a Moseley XY recorder. After the run, the solutions in the analytical cells were thoroughly mixed and aliquots of each sample were counted by liquid scintillation spectrophotometry using a solubilizer. The buoyant experiments provided an assay for the purity of the preparations. The spc-DNA from the treated cells was contaminated by less than 10% DNA banding at the position of mtDNA. However, almost all the DNA from the 14 s region from the untreated cells formed a band characteristic of mtDNA. Thus, the increase in spc-DNA in the cycloheximide-treated cells was considerably larger than the factor of 10 indicated by the radioactivity ratio.

The specific activity of the nuclear DNA after 30 hours of growth in cold medium (N2) is about half that of the DNA just after labeling (N1) (Table 1). The specific activity of the spc-DNA recovered after cycloheximide treatment is indistinguishable from the specific activity of nuclear DNA at the time of addition of the drug. This argues strongly that the increase in amount of spc-DNA is not the result of replication, and that the new spc-DNA must have been formed from pre-existing DNA or nucleotides derived from pre-existing DNA. To insure that the

TABLE 1

Specific activities of DNA from cells labeled with [^{3}H] thymidine before treatment with cycloheximide

Sample	Specific activity† (cts/min/μg)	Specific activity relative to N2
Nuclear DNA at end of labeling period (N1)	5.4×10^4	2.2
Nuclear DNA at start of drug treatment (N2)	2.4×10^4	1.0
Spc-DNA from treated cells		1.0
MtDNA from treated cells		1.0

† Determined spectrophotometrically.

species called spc-DNA in this experiment was not contaminating nuclear DNA, a sample was rebanded in CsCl-EthBr. Sixty-six percent of the counts appeared in the closed band.

(e) Complexity

The apparent polydisperseness of spc-DNA could be accounted for if this DNA contains varying numbers of a single sequence. Such a sequence would have a genetic complexity no greater than the smallest circles observed. These circles contained approximately 1500 base pairs. The possibility was tested by allowing fragmented denatured spc-DNA to renature for varying lengths of time. The extent of renaturation was assessed from the buoyant pattern of the product in the analytical ultracentrifuge. The buoyant density of denatured DNA is approximately 15 mg/ml. greater than that of the duplex DNA. Also, under the conditions used, the renatured product is expected to form aggregates of higher molecular weight than the starting material, and to form sharper bands.

Spc-DNA was prepared from cycloheximide-treated cells and purified by velocity sedimentation. The DNA was nicked using the visible light-EthBr method, freed of EthBr, and dialyzed exhaustively against a phosphate buffer (0.01 M in Na^+, pH 7.5). Approximately 2 μg of DNA was heated to 95°C for 7 minutes and quickly quenched to 0°C. An aliquot of this DNA was examined in the electron microscope using the formamide mounting procedure, which permits visualization of single-stranded DNA. The length distribution obtained by measuring 266 molecules corresponded to a weight average molecular length of

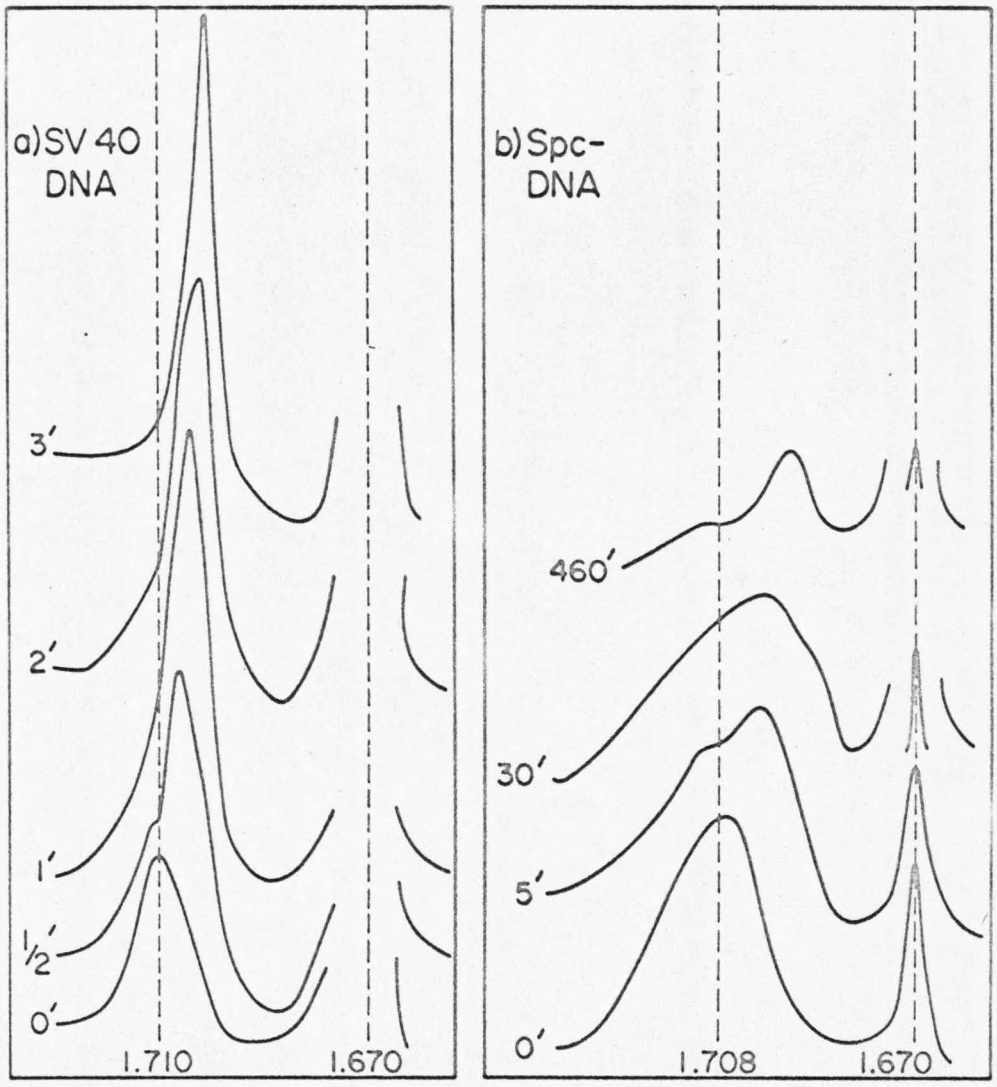
900 ± 50 nucleotides. Since duplex DNA was absent, it is reasoned that complete denaturation occurred and that renaturation did not occur during quenching. The DNA formed a single broad band in the analytical ultracentrifuge.

Aliquots of the DNA sample in 1 M-CsCl were incubated at 76° C for 5, 30, and 460 minutes. Closed circular SV40 viral DNA, which has a complexity of about 3×10^6, was fragmented in the same manner for comparison. Analytical band velocity sedimentation at pH 12.5 indicated a molecular weight of 5.7×10^5, corresponding to a length of about 1700 nucleotides (Studier, 1965). A 2 μg/ml. sample was denatured and renatured for 0.5, 1, 2, and 3 minutes in 1 M-CsCl. After addition of CsCl, the renatured materials were sedimented to equilibrium in the analytical ultracentrifuge. Scans were taken, and crab d(A-T) was added to each as a density marker and the solutions brought to equilibrium a second time. The photoelectric scans of these latter solutions are superimposed in Figure 3.

Denatured SV40 DNA formed a broad band at a density of 1.710 g/ml.; the native DNA has a buoyant density of 1.694 g/ml. As the renaturation time increased, the band became sharper and less dense. After a three-minute reannealing, the buoyant density was 1.700 g/ml. Material renatured to 99% completion (5 μg/ml. for 60 minutes) formed a very sharp band at a density of 1.698, which is taken as the density of extensively renatured material. The buoyant density shift for renaturation under these conditions is thus 12 mg/ml.

The results obtained with spc-DNA rule out a complexity of 1500 base pairs. Thirty to 40% of the DNA renatured for five minutes banded

FIG. 3. Traces of photoelectric scans of buoyant patterns obtained with renatured SV40 and spc-DNA. The traces, obtained with the Moseley AM 7000 SY plotter, were aligned with the crab d(A-T) marker. The maxima of the marker bands have been omitted in some cases for simplicity. (a) SV40 DNA and (b) spc-DNA, fragmented as described in the text, were renatured for the times indicated. The buoyant density of crab d(A-T) was taken to be 1.670 g/ml.


a) SV 40 DNA
3′
2′
1′
½′
0′
1.710
1.670
b) Spc- DNA
460′
30′
5′
0′
1.708
1.670

at a density about 9 mg/ml. lighter than the denatured DNA. After 30 minutes' renaturation, 40 to 60% of the DNA remained at the denatured density. In neither case was appreciable band sharpening seen in the DNA at the renatured density. Even after 460 minutes of renaturation, 20 to 30% of the DNA banded at the denatured density; the remaining DNA formed a band at a buoyant density close to that of native spc-DNA. Calculations (Wetmur & Davidson, 1968) indicate that a DNA with a complexity of 1500 base pairs would have completely renatured under these conditions after five minutes. Had 30 different sequences been involved (a complexity of 3×10^6), the patterns should have resembled the SV40 results; almost complete renaturation would have occurred after 30 minutes. The fact that some DNA remained denatured after 460 minutes indicates that the spc-DNA contains classes of different complexities, for if the DNA were all of a complexity to account for the result at 460 minutes, almost no renaturation would have taken place at five and 30 minutes.

(f) Spc-DNA in other cultured cells

Rush, Eason & Vinograd (1971) noted the presence of the spc-DNA in both SV40-infected and non-infected BSC-1 cells, a permanent line of African green monkey cells. We have confirmed the species to be present in exponentially growing BSC-1 cells by whole cell extracts of closed DNA. As with HeLa cells, when these cells are allowed to remain at stationary phase for two to three days, the level of spc-DNA recovered is markedly increased as judged by electron microscope examination.

Spc-DNA has been found in whole cell extracts of mouse multiple myeloma cells propagated in the peritonea of mice. The length distribution obtained with 50 molecules is similar to that seen in HeLa cells.

Spc-DNA has also been seen in cycloheximide-treated mouse L cells, both in L929, a clone of the original L cell, and in a variety of L cell we designate L_D (Kasamatsu, Robberson & Vinograd, 1971). Spc-DNA has also been seen in whole cell extracts of mouse 3T12 cells.

In order to determine if the presence of the spc-DNA is related to the aneuploid state of the cells, an investigation of diploid WI38 cells was undertaken. Cells at transfer number 38 were processed as described for HeLa cells, and total closed DNA was examined. The presence of spc-DNA was confirmed, but the frequency appeared to be smaller than in HeLa Cells. These cells did not respond to cycloheximide treatment at 100 μg/ml.

4. Discussion

Although many different species of closed circular DNA are known, most are of a single size or are made up of oligomers of a single monomer unit, the oligomers rarely being more than five or six monomer units. Some bacteria contain two or more size classes of closed circular DNA which do not appear to be multiples of a monomer unit. Shigella dysenteriae Y6R has been reported to contain closed circles of different lengths (Rush, Gordon & Warner, 1969; Yanze, Zandenberg, Van de Pol & Van Bruggen, 1969) which fall into a small number of discrete length categories. At least two drug-resistance factors seem to consist of three separate species, the largest of which may represent the combination of the other two (Nisioka, Mitani & Clowes, 1969; Cohen & Miller, 1970). Heterogeneously sized closed circles have been reported in two strains of Bacillus megaterium with lengths ranging up to 34 μ, but with most of the lengths between 1 and 5 μ (Carlton & Helinski, 1969). Electron microscope examination of unfractionated boar sperm DNA revealed a few circles varying from 0.5 to 10 μ in length (Hotta & Bassel, 1965). It is not known whether the circles are closed.

Attempts to define the nature of the mitochondrial DNA of yeast have led to several reports of heterogeneously sized closed DNA circles. It now seems clear that yeast harbor an oligomeric series based on a monomer unit of 2.2 μ (Guerineau, Grandchamp, Paoletti & Slonimski, 1971). This closed circular species has a buoyant density corresponding to nuclear DNA, and can be extracted from a fraction

containing membranes and mitochondrial fragments. It is not clear whether this species is mitochondrial in origin. It readily contaminates mitochondrial DNA prepared from isolated mitochondria and from buoyant fractionations of total yeast DNA. It accounts for some of the circles seen in such mtDNA preparations (Sinclair, Stevens, Sanghavi & Rabinowitz, 1967; Shapiro, Grossman, Marmur & Kleinschmidt, 1968; Guerineau, Grandchamp, Yotsuyanagi & Slonimski, 1968). Heterogeneously sized circles less than one micron in length have been seen in mtDNA preparations from petite mutations and from the preparations of mitochondria osmotically shocked directly onto electron microscope grids from such cells (Avers, Billheimer, Hoffmann & Pauli, 1968; Billheimer & Avers, 1969). These circles are reported to be present in large amount in certain petite mutations induced by ethidium bromide (Hollenberg, Borst, Thuring & Van Bruggen, 1969). It has been suggested that the heterogeneously sized circles from normal yeast cells are associated with the petite mutants which arise spontaneously (Hollenberg, Borst & Van Bruggen, 1970).

The spc-DNA in HeLa cells is not associated with the mitochondria or with nuclei which sediment through 1.5 M-sucrose. On this basis the spc-DNA is regarded as "cytoplasmic." Cytoplasmic DNA's have recently been described as free in the cytoplasm or associated with particles (I-DNA) (Bell, 1969), as associated with microsomes (Bond, Cooper, Courington & Wood, 1969), and as associated with the plasma membrane (Lerner, Meinke & Goldstein, 1971). It has been demonstrated that the first two instances are most likely artifacts of the extraction process used (Fromson & Nemur, 1970). The HeLa cell

spc-DNA is differentiated from the plasma membrane-associated cytoplasmic DNA which is reported to be linear. The sedimentation coefficient, 16 s, of this latter DNA corresponds to a molecular weight of about 3×10^6 daltons.

It is possible that spc-DNA is produced in damaged or dying cells in which the nuclear membrane may have broken down. Cycloheximide-treated cells, which contain greatly increased amounts of spc-DNA, do not seem to have lost nuclei as judged by their appearance in the light microscope and by the presence of particles which behave as nuclei in cell fractionation procedures. Nevertheless, since the possibility remains that spc-DNA originates in damaged nuclei, we regard the cytoplasmic designation as tentative.

Thomas, Hamkalo, Misra & Lee (1970) have reported the formation of circular molecules in large quantity from animal cell nuclear DNA. Circles were produced from fragmented DNA which was denatured and reannealed or treated with exonuclease prior to annealing. The first method resulted in the formation of circles of the same size class as the spc-DNA. Circles formed after exonuclease treatment were generally larger than spc-DNA, but did include some in this size class. The sizes of the DNA fragments used to made the circles will, in this case, place lower limits on the sizes of circles which can be produced. Whichever model of chromosome organization will ultimately explain the above results, the experiments demonstrate that homologous regions are spaced along animal cell chromosomes, and that some of the spacing corresponds to the sizes of spc-DNA.

The spc-DNA formed after treatment of the cells with cycloheximide has been shown to arise from some pre-existing DNA. Unless another cytoplasmic DNA is postulated, it seems most likely that this spc-DNA is excised from nuclear DNA perhaps by recombination events between these homologous regions. The spacing of these regions could account for the polydisperseness of the circles excised. The size distribution could reflect the lengths of DNA which participate in such recombination, and the minimum requirements of a recombination system can account for closed circular excision products.

The fact that up to 60% of some animal nuclear DNA can be found as circular molecules in the circularization experiments employing exonuclease indicates that a large amount of nuclear DNA would be subject to possible recombination events which could lead to loss of genetic material. Such recombination might be suppressed by the action of the elements which control gene activity, especially if the homologous regions represent the receptors for such controlling elements (Britten & Davidson, 1969). It is possible that the inhibition of protein synthesis by cycloheximide interferes with this regulation and allows recombination to occur in 0.02% of the nuclear DNA, which is adequate to produce the amount of spc-DNA formed.

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PART III

SEQUENCE HETEROGENEITY IN CLOSED SIMIAN VIRUS 40 DEOXYRIBONUCLEIC ACID

The following paper has been submitted to the Journal of Virology.

Sequence Heterogeneity in Closed Simian Virus 40 Deoxyribonucleic Acid

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Abstract

The heteroduplex molecules formed by self-annealing of denatured, singly nicked simian virus 40 (SV40) deoxyribonucleic acid (DNA) prepared from closed viral DNA were examined by formamide-protein film electron microscopy to test the DNA for sequence homogeneity. Sequence inhomogeneity appears in the heteroduplexes as single-strand loops. These result from sequence deletion or from sequence substitution, if regions greater than 50 nucleotides are involved. The undenatured DNA from viruses passaged twice at multiplicities of infection much less than one p. f. u. per cell appeared to be homogeneous in size. The heteroduplexes formed by this DNA indicated that approximately 2% of the molecules carried deletions, but that substitutions were below the level of detection. In contrast, undenatured DNA from viruses grown by passaging undiluted lysates 7 times or by infection with stock virus at a multiplicity of infection of 5 p. f. u. per cell contained a large frequency of molecules shorter than the full length. The heteroduplex samples indicated that 12 and 7% of the undenatured material contained base substitutions and 13 and 11% contained deletions. The deletions and substitutions appear to occur in separate molecules.

Length measurements on heteroduplexes displaying the loop characteristic of substitutions have established that these molecules are from true sequence substitutions, and not from adjacent or overlapping deletions. More than 80% of the molecules carrying substitutions are shorter than the native SV40 length. On the average, the substituted sequence is about 20% of the length of SV40, but it replaces

a sequence about 30% of the native length.

The substituted sequences may be host cell nuclear DNA, possibly arising from integration of SV40 into the chromosome followed by excision of the SV40 DNA together with the chromosomal DNA.

Introduction

Simian virus 40 (SV40) is a small deoxyribonucleic acid (DNA)-containing virus which has been used extensively for the study of virus-cell interactions and as a model system for viral oncogenesis in animal cells. Infection of permissive host cells results in viral replication and culminates in cell death and lysis, releasing the newly made virus. Infection of nonpermissive host cells results in the alteration of cellular morphology and growth characteristics, a process termed transformation. That the viral DNA persists in the transformed cell has been shown by the production of whole virus after fusion of virus-free transformed cells and permissive cells (1, 2, 3), by the detection of viral DNA sequences in the transformed cell by nucleic acid hybridization (4, 5), and by the detection of viral-specific RNA in such cells (6, 7, 8). Other experiments have suggested that the viral DNA persisting in the transformed cells is covalently linked with host cell nuclear DNA (5).

In the lytic infection with polyoma virus, a DNA virus very similar to SV40, it has been shown that some linear host cell DNA is encapsidated into virus particles (9, 10). These so-called "pseudovirions" have also been seen in SV40 infection of primary African green monkey kidney cells, but not with infection of BSC-1 cells, a permanent cell line derived from African green monkey cells (11).

Aloni et al. (12), investigating interactions between SV40 and the permissive BSC-1 cells, found hybridization between closed circular SV40 DNA and host cell nuclear DNA. They suggested that host cell DNA sequences might be incorporated into SV40 viral DNA. Gelb, Kohne,

and Martin (13) were unable to detect such hybridization using SV40 DNA from viruses grown by serial passage. However, the conditions used for passage were such that each infection was at low multiplicity (personal communication).

Yoshiike (14) has found that passage at high multiplicity produces noninfective particles containing closed circular DNA, heterogeneous in size and somewhat shorter than the DNA of infective particles. The foregoing results raise the possibility that host cell sequences are incorporated into DNA molecules heterogeneous in size.

To investigate this possiblity we have used electron microscopy to examine heteroduplex molecules artificially constructed from SV40 viral DNA recovered from viruses grown at low and high multiplicities. We have found that SV40 DNA from viruses grown at low multiplicity exhibits both length and sequence homogeneity. Like Yoshiike (13), we found that DNA from viruses grown at high multiplicity exhibits length heterogeneity. In addition, heteroduplexes constructed from such DNA contain both deletion loops and substitution loops. Measurements of molecules with substitution loops demonstrate that these loops are not the result of renaturation of single strands carrying overlapping or adjacent deletions. In most cases the substituted sequence is somewhat shorter than the sequence it replaces.

In a preliminary examination of heteroduplex molecules formed from laboratory stock SV40 DNA, R. Davis (Ph. D. dissertation, California Institute of Technology, 1970) observed molecules which contained deletion loops and apparent substitution loops.

MATERIALS AND METHODS

Cells. African green monkey kidney (BSC-1) cells were obtained from Flow Laboratories, Los Angeles, Calif. The cells were grown in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Berkeley, Calif.), supplemented with 10% calf serum.

Growth and isolation of low multiplicity SV40 virus. A laboratory stock of strain RH911 SV40 virus was banded in CsCl. About the first 10% of the drops containing virus were collected and dialysed against TD (0.14 M NaCl, 7 mM KCl, 7 mM Na_2HPO_4, 25 mM Tris, pH 7.5). The dialysed virus suspension was then titered using the agar overlay procedure (15). BSC-1 monolayer cultures were infected at a multiplicity of infection of 10^{-1} plaque-forming units (p.f.u.)/cell. After a full cytopathic effect (CPE) was observed, usually after 10 days, the viruses were harvested and purified as described below. The virus band in CsCl was collected, dialysed, and titered. This virus was used to infect BSC-1 cells at a multiplicity of 10^{-2} p.f.u./cell, and after a full CPE the virus was purified and the DNA extracted.

Growth and isolation of high multiplicity SV40 virus. Two independent stocks of high multiplicity virus were prepared. The undiluted lysate obtained with the low multiplicity virus (see above) was used to make the subsequent infection; this procedure was repeated 6 times to form high multiplicity stock (HM-A). In the other method, laboratory stock SV40 lysate was used to infect BSC-1 cells at a multiplicity of 5 p.f.u./cell and the cells were harvested after 3 days (HM-B).

Purification of virus and preparation and purification of closed circular viral DNA. The infected cells were scraped from the plates and the entire suspension was frozen and thawed 3 times in plastic tubes. The suspension was homogenized with 10 strokes in a loose-fitting glass Dounce homogenizer. Sodium deoxycholate was added to a concentration of 1% and the solution incubated at room temperature for 10 min. Crude DNase I and RNase A were added to concentrations of 0.4 and 1.0 mg/ml, respectively, and the solution incubated at room temperature for another 40 min. This mixture was centrifuged for 10 min at 10,000 rev/min in a Sorvall SS34 rotor, and 25-ml portions of the supernatant were layered over 15 ml of saturated KBr in Spinco SW27 cellulose nitrate tubes. These were centrifuged for 3 hr at 22,000 rev/min at 20 C. The opalescent virus bands recovered by drop collection were dialysed against TD. The solution was made to 10 mM $MgCl_2$ and crude DNase I was added to a final concentration of 5 mg/ml. After incubation at 37 C for 30 min, EDTA was added to a concentration of 50 mM and CsCl was added to a density of 1.3 gm/ml. The solution was centrifuged for 20 hr at 36,000 rev/min in an SW50 rotor at 20 C. The virus band was recovered by drop collection. In some cases the CsCl banding of the virus was repeated after another DNase treatment. After dialysis to remove CsCl, sodium dodecyl sulphate (SDS) was added to the virus suspension to a concentration of 1% and the solution incubated at 37 C for 10 min. The solution was chilled to 0 C and CsCl added to a concentration of 1 M. The mixture was maintained at 0 C for 10 min and then centrifuged for 10 min at 10,000 rev/min in an SS34 rotor at 4 C. The supernatant was adjusted to

contain 300 μg/ml ethidium bromide (EthBr) or propidium di-iodide, and CsCl was added to a density of 1.55 g/ml. The solution was centrifuged at 38,000 rev/min for 24 hr in an SW50 rotor at 20 C. The lower fluorescent band, which contains the closed circular DNA, was recovered by drop collection (16).

Preparation of singly nicked viral DNA. Three methods were employed. (a) DNase. The nicking activity of a solution of DNase I with SV40 DNA as a substrate was assayed by analytical band velocity centrifugation through alkaline CsCl. Conditions were chosen such that 30% of the form I DNA was converted to form II. After nicking, the DNA was banded in CsCl-EthBr and the two components were separated by drop collection. (b) X-irradiation. The DNA in 1 M CsCl, 1 mM EDTA, 2 mM histidine, 10 mM Tris, pH 8, was subjected to X-rays (100 roentgen/min) for 20 min. The form II DNA was separated from form I as above. (c) Visible light-ethidium bromide. The DNA in 1 M CsCl, 1 mM EDTA, 10 mM Tris, pH 8, 70 μg/ml EthBr, was placed 8 cm from the lens of a slide projector containing a 500 W bulb and illuminated for 7 to 10 min. This converted 25% of the form I to form II. The components were separated as above and the process repeated on the form I two times.

Formation of the heteroduplexes for electron microscopy. Approximately 0.2 μg of singly nicked SV40 DNA was denatured in 0.3 M NaOH for 10 min, neutralized with 2 M Tris-HCl, and diluted to a total volume of 0.2 ml with 99% formamide. This sample was dialysed for 2 hr against 50 ml of 99% formamide at room temperature. Unrenatured control samples were prepared by mixing 25 μl of the above

dialysed solution with an equal volume of 0.2 M Tris, 0.02 M EDTA, pH 8.5, immediately prior to the spreading for preparation of the specimen grids. Heteroduplexes were formed by incubating a similar mixture for 5 hr at 25 C just prior to specimen grid preparation.

Electron microscopy. Both the aqueous and the formamide procedures for preparing samples for electron microscope examination have been described (17). Grids were stained and rotary-shadowed, and examined in a Phillips 300 electron microscope. Photographs on 35 mm film were traced on a Nikon 6F projection comparator. Lengths of molecules were determined with a map measurer with an accuracy of ±0.25 cm. Absolute lengths were determined by photographing a diffraction grating with each series of photographs.

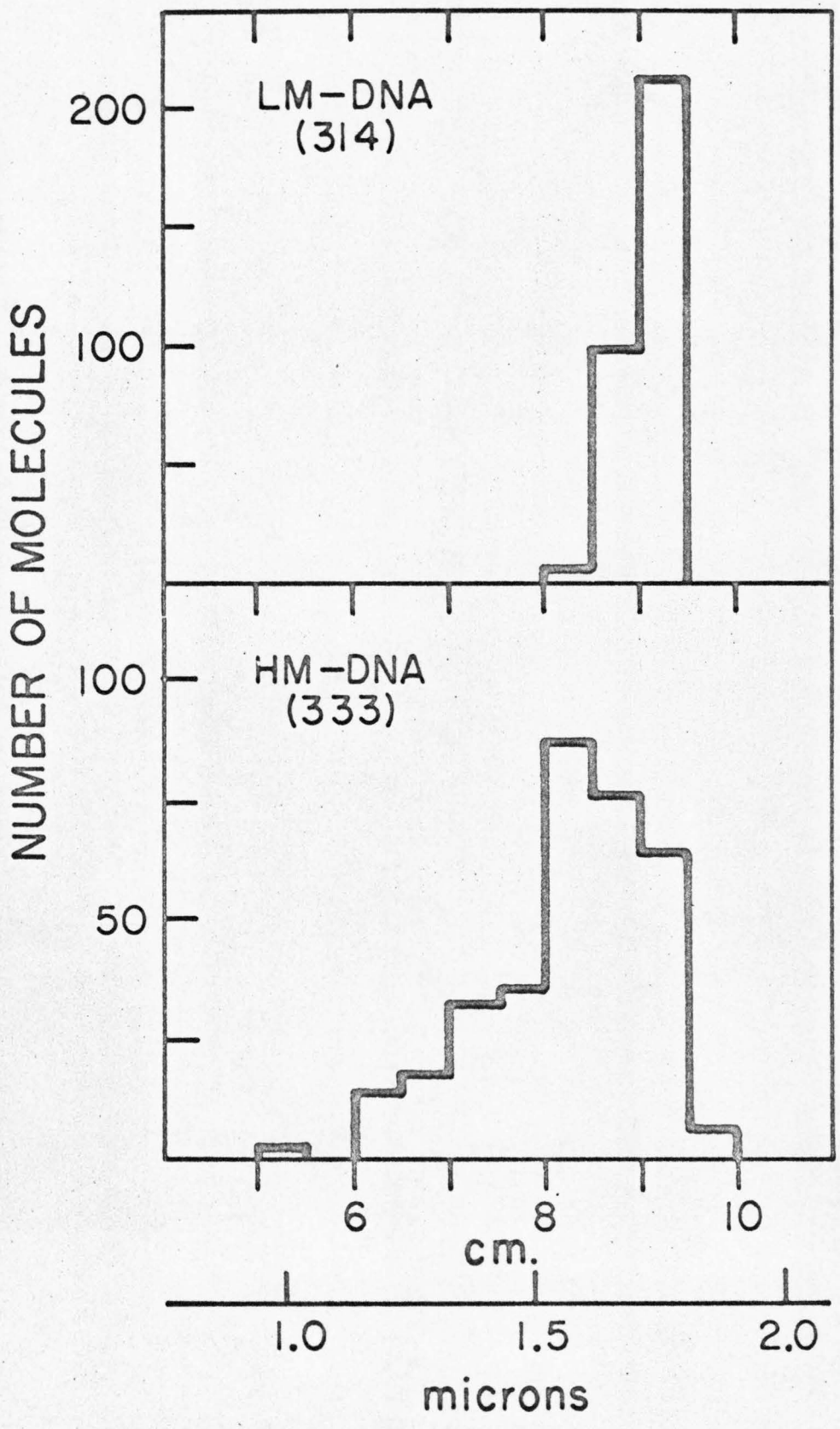
Reagents. Ethidium bromide was a gift of the Boots Pure Drug Co. Ltd., Nottingham, England. Propidium di-iodide was a gift of Calbiochem, Los Angeles, Calif. Optical grade cesium chloride was obtained from the Harshaw Chemical Company, Cleveland, Ohio. DNase I and RNase A were obtained from Sigma Chemical Co., St. Louis, Mo. Cytochrome c was obtained from Calbiochem. Formamide and sodium dodecyl sulfate were obtained from Matheson, Coleman and Bell, Cincinnati, Ohio. All other chemicals were reagent grade.

RESULTS

Lengths of DNA from viruses grown at high and low multiplicities of infection. In order to compare our results with those of Yoshiike (13), length distributions of the DNA extracted from viruses grown at low multiplicity of infection (LM DNA) and at high multiplicity of infection (HM-A DNA) were determined (Fig. 1). The LM DNA was obtained as described earlier from infection at a multiplicity of 0.01 p.f.u./cell. The HM-A DNA was derived from serial passaging of undiluted lysates 6 times, starting with the low multiplicity virus stock. LM DNA exhibits a narrow length distribution with a mean of 1.7 μ. The length distribution of HM-A DNA is skewed toward the short lengths. Both distributions cut off sharply at about the same upper length, presumably because of a limit on the size that can be encapsidated.

Molecular weight of LM DNA. The molecular weight of LM DNA, which has a narrow length distribution, was determined. This DNA was mixed with lambda-c_{26} DNA and electron microscope grids were prepared by the aqueous technique. The lengths of 139 SV40 molecules and 15 lambda molecules were measured on photographs obtained with a single grid. The SV40 DNA had a mean length of 29.8 ± 1.3 cm; the lambda DNA had a mean length of 255.5 ± 7.4 cm. Based on the molecular weight of lambda, 30.8×10^6 daltons [N. Davidson and W. Szybalski. In A. D. Hershey and N. F. Dove (eds.), The Bacteriophage Lambda, Cold Spring Harbor Laboratory, New York, in press], the molecular weight of the SV40 is $3.6 \pm 0.2 \times 10^6$ daltons. The uncertainty in the value for lambda brings our value to $3.6 \pm 0.3 \times 10^6$ daltons. The 5.5%

FIG. 1. Length distributions of SV40 DNA from viruses grown at low multiplicity (LM DNA) and at high multiplicity (HM DNA). Electron micrographs were traced and measured with a map measurer in centimeters, as shown. The micron scale is approximate; an external standard was used to determine the magnification. The number of molecules measured is given in parentheses.


LM–DNA
(314)
HM–DNA
(333)
NUMBER OF MOLECULES
200
100
100
50
6
8
10
cm.
1.0
1.5
2.0
microns

standard deviation in SV40 length compares well to the 4% expected for a homogeneous DNA of this size (17).

Classification of the heteroduplexes. SV40 DNA, renatured so that about 35% of the DNA was in duplex form, was mounted for electron microscopy by the formamide technique and examined at a magnification of 60,000. The procedure allows both single- and double-stranded DNA to be visualized and distinguished. Both appear filamentous, but the single-stranded DNA is more irregular and thinner than duplex DNA, and has been described as "knobby," "lumpy," and "kinky." In addition, its contrast is usually somewhat less than that of duplex DNA. The differences are readily seen at the electron microscope, where magnification and focus can be manipulated.

All molecules seen while systematically scanning a grid were classified into one of seven categories. The types of molecules placed into each category are diagrammed in Fig. 2 and some photographs of typical molecules are shown in Fig. 3. The majority of the DNA strands were undegraded. Forty percent of the single strands were circular. The categories were: (a) linear single-stranded DNA; (b) circular single-stranded DNA; (c) heteroduplexes without single-stranded loops; (d) heteroduplexes with one or two deletion loops, a loop of single-strand DNA tangent at one point to a duplex region; (e) one or two substitution heteroduplexes with loops, regions where two single strands replace the duplex structure along a portion of its length; (f) heteroduplexes containing a forked structure at which the duplex separates into separate single-strands—these could arise from a single-strand scission in a substitution loop or in a deletion loop; (g) heteroduplexes

FIG. 2. Schematic representations of the categories of molecules used in classifying the DNA in the renatured samples. Solid lines represent duplex DNA; dashed lines, single-stranded DNA.

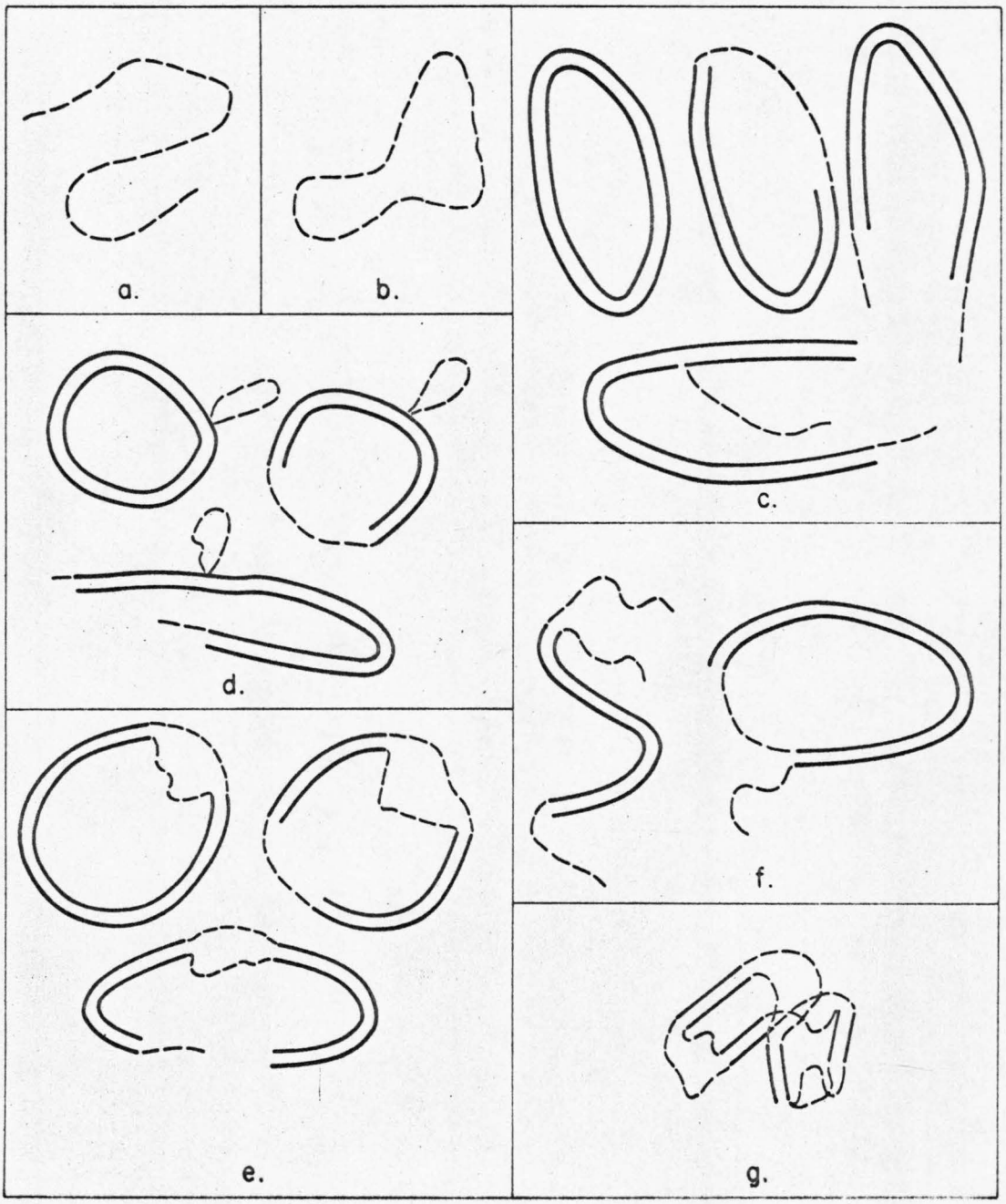
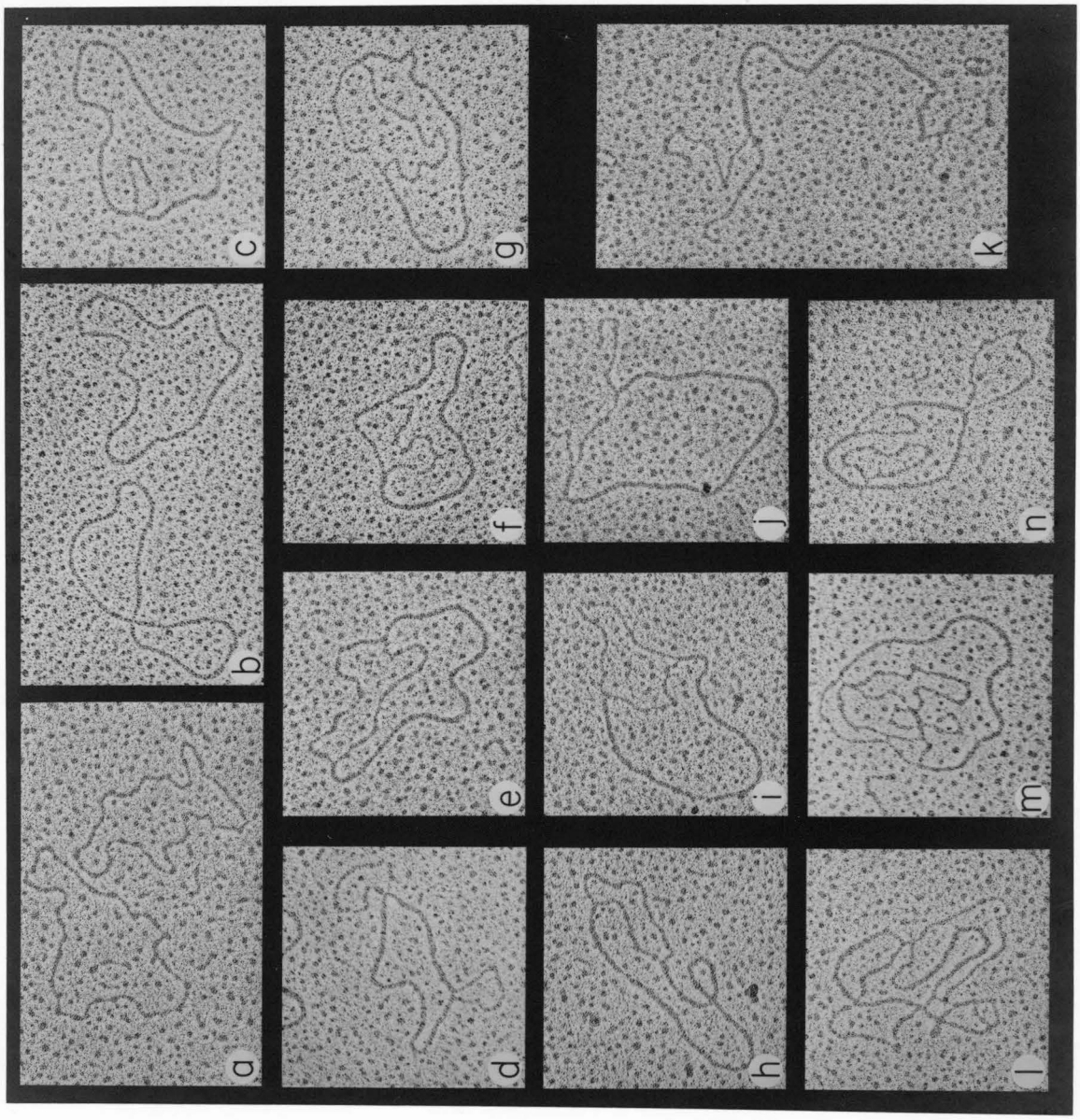

a.
b.
c.
d.
e.
f.
g.

FIG. 3. Electron micrographs of molecules in the renatured HM DNA sample. (a) Single-stranded circles. (b) Fully duplex circles. (c) Heteroduplex with a deletion loop. (d) Heteroduplex with two deletion loops. (e - i) Heteroduplexes with substitution loops. (j) A circular heteroduplex containing forks. (k) A linear heteroduplex with a fork. (l - m) Heteroduplexes with both single and double strands which are not interpretable. (n) A heteroduplex with two deletion loops and a substitution loop.


a
b
c
d
e
f
g
h
i
j
k
l
m
n

containing both single- and double-stranded regions, which are knotted, tangled, or so complex as to make assignment impossible. These occur in both LM and HM DNA samples, which vary widely in their sequence heterogeneity.

The data might be significantly influenced by the failure of all the DNA to denature before renaturation was allowed to occur. In some cases, specimen grids were prepared with the material obtained just prior to the last incubation step as described earlier. Almost all of the DNA appeared to be single-stranded and, therefore, denatured. Furthermore, the small amount of renaturation which did occur with the HM-A DNA sample led to some heteroduplexes containing non-homology regions. The numbers of molecules in each category in the renatured samples were corrected for the frequency of occurrence of these forms in the zero-time sample. These corrections were always small and well within the range of results found upon scoring different, renatured materials from the same sample. Nonrenatured samples contained 60% single-stranded linear and 40% single-stranded circular molecules, as expected for singly nicked DNA.

The frequencies of the various kinds of molecules containing duplex regions is presented in Table 1. Two separate denaturation-renaturations were carried out with LM DNA, and two spreadings were performed with each. The LM DNA heteroduplexes contained no detectable substitution loops in a total of 1000 molecules examined. About 4% of the heteroduplexes contained deletion loops and 1% contained forked structures. A molecule containing a deletion gives rise to two heteroduplexes, each with a deletion loop. The probability of annealing two

TABLE 1. Classification of heteroduplexes formed by self-annealing of high and low multiplicity SV40 viral DNA

Sample	Fully double-stranded	Deletions	Substitutions	Forks	Unscorable	No. of molecules scored
	%	%	%	%	%	
LM DNA						
1a	95	5	0	0	0	75
1b	93	4	0	0	3	311
2a	91	2	0	2	5	324
2b	84	4	0	1	11	460
HM DNA, Sample A						
3a	39	15	15	21	10	326
3b	41	18	17	13	11	306

(continued . . .)

(TABLE 1, continued)

HM DNA,						
Sample B						
4a	52	16	8	13	11	243
4b	57	17	6	12	8	299
4c	59	13	12	8	8	134

Each row in the table presents the results obtained with one spreading. Each number in the first column refers to one denaturation-renaturation experiment. Zero-time corrections were applied to experiments 1 and 4.

defective strands is small in this case. The above result indicates that 2.5% of the LM DNA molecules contain deletions.

Two samples of HM DNA were prepared, one by serial passage (Sample A) and one by a single infection (Sample B). The sequence heterogeneity in the high multiplicity samples, A and B, is shown by the high frequency of heteroduplexes containing deletions or substitutions. This is very apparent in comparison with the frequencies obtained with low multiplicity DNA. Disregarding the forked heteroduplexes, we calculate from the data in Table 1 that 8% of the HM-A molecules contained deletions and 8% contained substitutions. Eight percent of the HM-B molecules contained deletions and 4% contained substitutions. A better estimate is obtained by assigning half the forked molecules to deletions and half to substitutions. When this is done, HM-A DNA contains 13% deletions and 12% substitutions, and HM-B contains 11% deletions and 7% substitutions.

The nature of the substitution loops. A substitution loop seen in a heteroduplex molecule indicates that the two single-stranded regions unable to form duplex DNA have less than some critical amount of sequence homology. Such a structure could arise from a molecule which has undergone a deletion followed by an insertion of a segment of DNA with a different sequence. The two events need not be separate in time; for example, an inversion produced by an intramolecular recombination event would produce a substitution loop. A substitution loop could arise if each strand in the heteroduplex contains a deletion and the deletions are within a few base pairs of each other or overlap. The nature of the substitution loops encountered here was determined by

measuring lengths of circular molecules that were completely double-stranded except for the loop. If the loop were due to a substitution, one strand of the heteroduplex would be of the native length. Therefore, the sum of the duplex part of the molecule and one of the single-stranded segments should equal that length. However, if the heteroduplex is formed by renaturation of two strands, each containing a deletion, the sum of the duplex and either of the single-stranded segments must be shorter than the native length. Also for overlapping deletions, the sum of the duplex and both single-stranded segments can never exceed the native length, and the sum becomes smaller as the size of the deletion-overlap becomes larger. The comparable sum for a substitution will always be longer than the native length.

In order to compute these sums, the lengths of single-stranded circles, duplex circles, and circles containing substitution loops on a single grid prepared from HM-A DNA were measured. Similar measurements were made of the single-stranded and the duplex circles in the LM DNA sample for comparison.

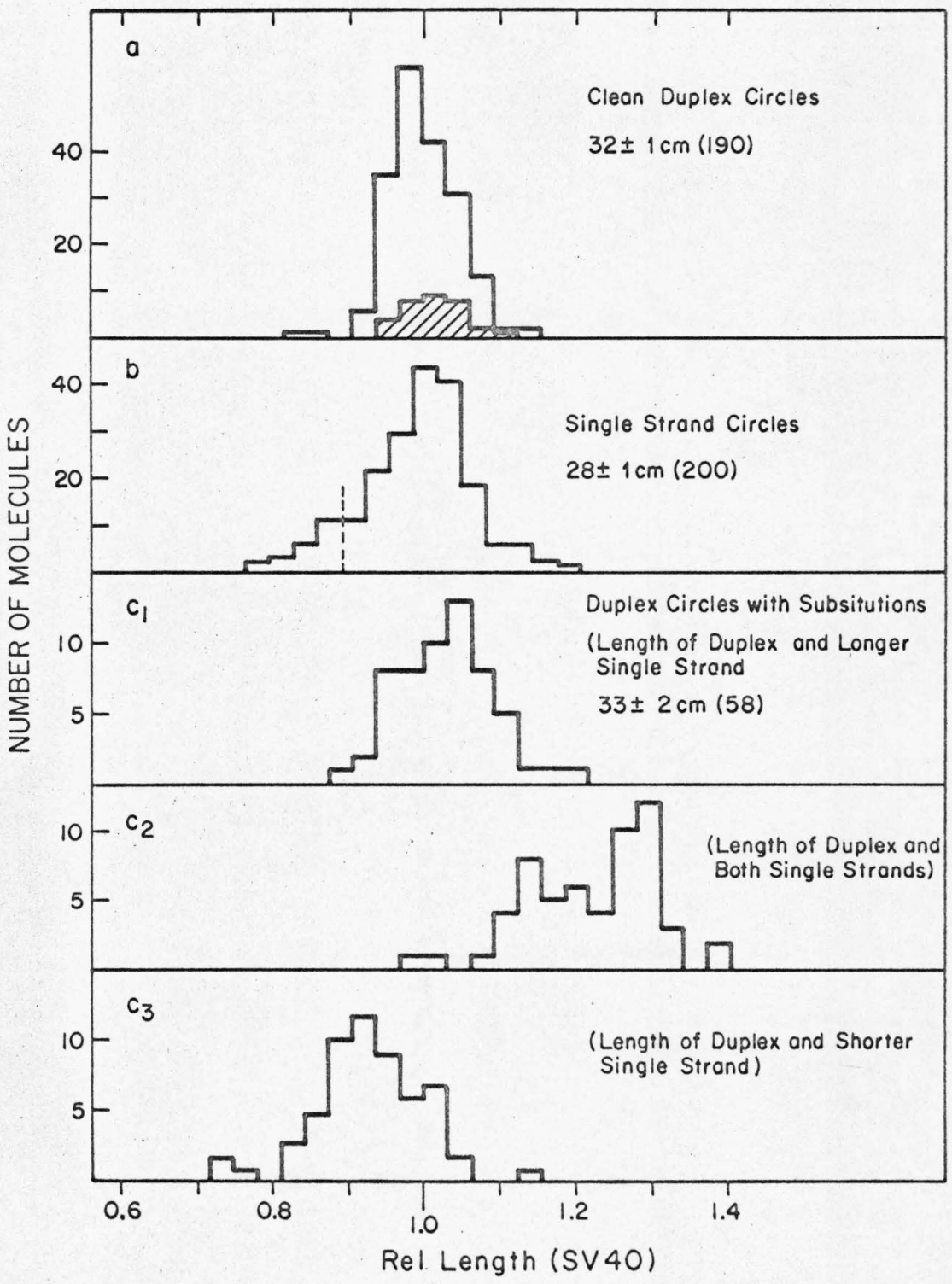
It would be expected that the complete duplex circles seen in the HM-A DNA would arise from the renaturation of two full-length native strands. That this is the case can be seen from the fact that the mean of the length distribution of these molecules (Fig. 4a) is very close to the mean of the distribution of lengths of complete duplex circles from the LM DNA. The small difference may be due to the fact that these measurements were necessarily made on grids from two separate spreading operations.

The distribution of lengths of single-strand circles (Fig. 4a) peaks at a slightly smaller length, due to a smaller length per unit mass, and tails off to the short side. The tailing is due to the defectiveness of the sample (see Fig. 1b). The mean length of molecules greater than 25 cm was taken as the mean single-stranded length. (It compares well with the mean of lengths of the single-stranded circles for the LM DNA). Single-stranded DNA lengths were multiplied by the ratio 32/28 to reduce the data to units of length per genome.

On a single specimen grid, the lengths of the duplex part and each single-stranded part of 58 heteroduplexes containing substitution loops were measured. The distribution for the sum of lengths of the duplex part and larger single-stranded part (Fig. 4c) is similar to that of the lengths of duplex circles (Fig. 4a). The means of the distributions were within one standard deviation. When the lengths of both single strands are added to the duplex length, the distribution is wide (Fig. 4e) and the lengths in the entire distribution are substantially longer than the mean value for the duplex lengths. This argues strongly that the loops are indicative of substitutions and not of overlapping deletions in the heteroduplexes.

Sequence lengths involved in the substitution process. Since the mean length of the longer strand in the heteroduplexes containing substitutions is the native length, we consider it to be native sequence, and the shorter strand to be the one containing the substituted sequence. The longer single-stranded segment length represents the size of the SV40 sequence removed from the substituted molecule prior to insertion of the new nonhomologous sequence. The shorter single-stranded

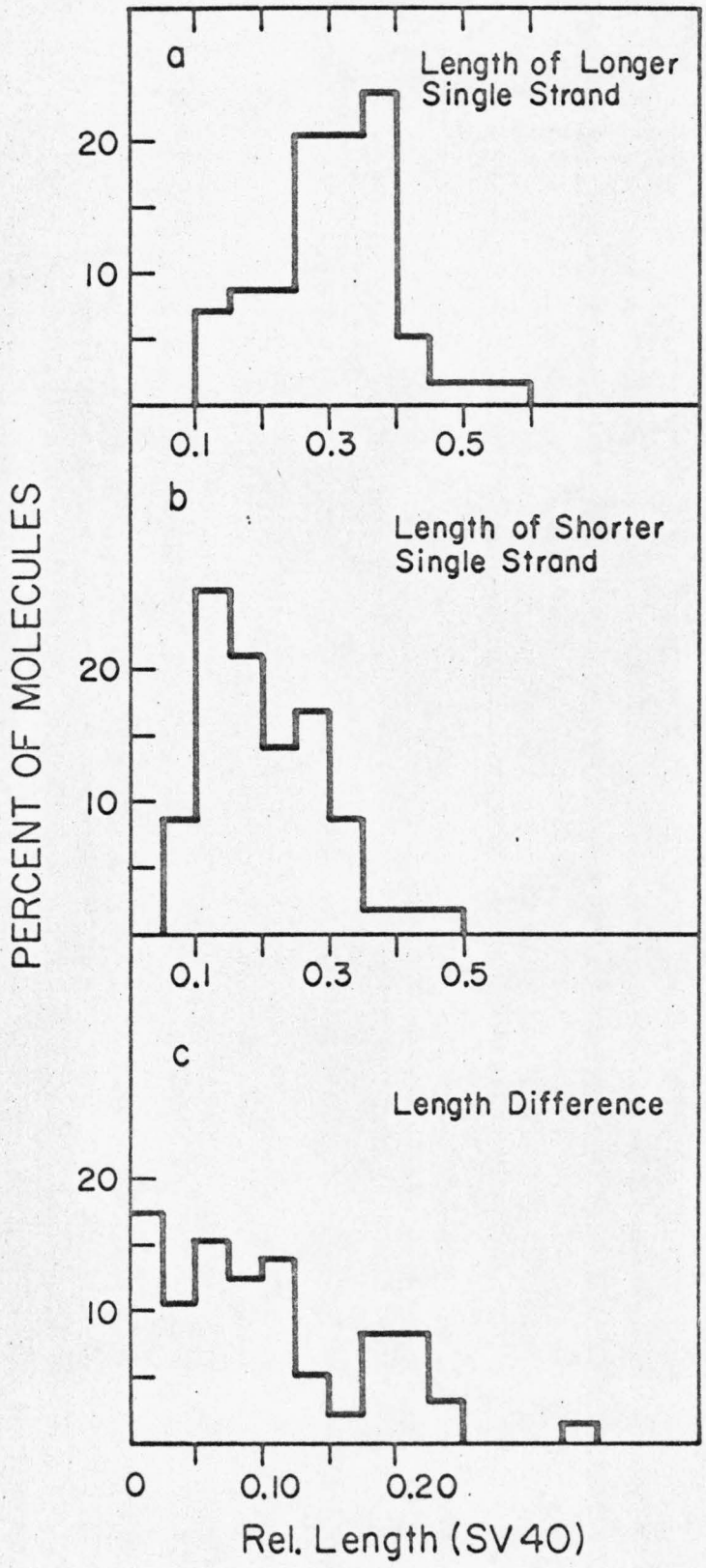
FIG. 4. Length distributions of molecules in the renatured HM-A DNA samples. Lengths are expressed as fractions of the full SV40 length. Mean lengths of the distributions and the standard deviations are given in units obtained with the map measurer together with the number of molecules measured. The distribution was truncated by the dashed line in (b) for the calculation of the mean. (a) Length distribution of fully duplex circles. The shaded histogram is for the LM DNA samples. (b) Length distribution of single-stranded circles. (c_1 - c_3) Heteroduplexes with clean substitution loops. Lengths of the duplex portions and single strands in the substitution loop were measured. After the single-strand lengths were corrected for the molecular weight per unit length, the indicated sums were computed. (c_1) Sum of duplex and longer single strand. (c_2) Sum of duplex and both single strands. (c_3) Sum of duplex and shorter single strand.


a
Clean Duplex Circles
32± 1cm (190)
40
20
b
Single Strand Circles
28± 1cm (200)
40
20
c1
Duplex Circles with Subsitutions
(Length of Duplex and Longer Single Strand
33± 2cm (58)
10
5
c2
(Length of Duplex and Both Single Strands)
10
5
c3
(Length of Duplex and Shorter Single Strand)
10
5
NUMBER OF MOLECULES
0.6
0.8
1.0
1.2
1.4
Rel. Length (SV40)

segment length represents the length of the inserted sequence. The length distribution of the molecules containing substitutions is shown in Fig. 4c. Here, the length of the duplex segment of the heteroduplex has been added to the length of the shorter single-stranded segment. The mean length of the substituted molecule is 11% shorter than native SV40.

The deleted sequence ranges from 10 to 60% of the SV40 length, with a mean of 31% (Fig. 5a). The inserted sequence ranges from 5 to 50% of the SV40 length, with a mean of 20% added (Fig. 5b). The distribution of the difference in lengths between the two single-stranded segments of each heteroduplex (Fig. 5c) shows that the difference is not constant, but varies widely: 83% of the molecules show some difference. Neither is the difference a constant fraction of the sequence deleted. The average difference in length between the sequence deleted and that inserted is 11% SV40. Thus, the reduced size of defective DNA is due both to simple deletions and to deletion-substitutions.

FIG. 5. Distributions of the lengths of the substitution loops obtained from the measurements used to prepare the distribution in Fig. $4c_1$-c_3. The lengths are expressed as fractions of the full SV40 length. (a) Distribution of the lengths of the longer single-strand portions of the loops. (b) Distribution of the length of the shorter single strands in the loops. (c) Distribution of the difference in length between the two single strands in each heteroduplex.


a
Length of Longer
Single Strand
20
10
0.1
0.3
0.5
b
Length of Shorter
Single Strand
20
10
0.1
0.3
0.5
c
Length Difference
20
10
0
0.10
0.20
PERCENT OF MOLECULES
Rel. Length (SV40)

DISCUSSION

We have demonstrated that 7 to 12% of the closed circular DNA molecules from SV40 viruses grown at high multiplicity of infection contain a region in which a new base sequence has replaced the native one. These new sequences are detected as nonhomology regions in heteroduplexes. These sequences could be SV40 sequences from the same molecule, SV40 sequences from other SV40 DNA molecules, or sequences from host cell DNA.

A substitution might be generated intramolecularly by a reciprocal recombination event involving two presumably homologous sites in the molecule. Depending upon which pairs of strands were rejoined, the sequence between the sites would either be deleted, or would be inverted. Such an inversion would give rise to an apparent substitution in a heteroduplex. This explanation seems unacceptable, since the molecules with substitutions are found to be somewhat shorter than the native molecules and because the size of the substitutions varies, making multiple recombination sites necessary.

Two recombination events could delete a sequence from one SV40 molecule and replace it with a sequence from another SV40 molecule. If the two sequences bounded by the recombination sites were different, a substitution would result. The lengths of the sequences need not be identical.

Host cell DNA might be incorporated into SV40 DNA by a mechanism similar to production of lambda-transducing phage. During the lytic infection, SV40 viral DNA would become integrated at one or more

sites into host cell DNA, as has been suggested for the transformation process. Subsequent excision would occur by recombination at a site within the viral DNA and a site somewhere on the cellular DNA. A molecule which has lost an SV40 sequence and gained a cellular DNA sequence results. The loss and gain need not be of the same size.

If integration of SV40 DNA into host cell chromosomes does exist and occurs at a common site on all SV40 DNA molecules, such a model predicts that the substitutions will all share a common end-point. Because SV40 DNA is circular, the position of the deletions or substitutions in the genome cannot be determined in the absence of a marker. We do not know whether the simple deletions that were seen are produced by a mechanism involving interaction with host cell DNA. Heteroduplexes have been observed which contain two non-overlapping deletion loops. If these heteroduplexes were formed from strands each containing a deletion, then at least two deletion sites are involved. We cannot rule out the possibility that these heteroduplexes are composed of one native strand and one strand containing both an inserted sequence and a deletion.

If we take the frequency of molecules containing substitution to be 12% for HM-A DNA and the average substituted sequence to be 0.2 genomes, then substituted sequences comprise 2.4% of the DNA. This value is in close agreement with the data of Lavi and Winocour (personal communication), who found that approximately 2% of highly sheared SV40 DNA from viruses grown at high multiplicity hybridized with BSC-1 nuclear DNA. This suggests that the substituted sequences are host DNA. It should be noted that our numbers are minimal estimates; molecules

which had long substitutions would not have renatured to form heteroduplexes as fast as those with smaller substitutions. Large substitutions would also have been more susceptible to nicking, which would place them into the forked category. The rare heteroduplexes which appeared to have only a very short duplex region were not included in the molecules measured. The fact that hybridization between SV40 DNA and host cell DNA is highly reduced for SV40 DNA from viruses passaged at low multiplicity (Lavi and Winocour, personal communication) is in agreement with this study of heteroduplexes.

Passaging the laboratory stock virus twice at low multiplicity effectively removed substituted molecules and substantially reduced the number of molecules containing deletions. This suggests that the substitutions, as well as the deletions, remove genes essential for a productive lytic infection. When the multiplicity of infection is low, any such defective molecules entering a cell are lost. The small number of deletions seen in the LM DNA may be due to deletions in nonessential regions of the genome or may represent the rate of production of deletions in the infection process.

The exact role of the high multiplicity infection in the production of substitutions and deletions is not yet established. Certainly, infection at high multiplicity with a stock of virus containing such molecules would allow them to replicate. Shorter molecules may even be selected for, if their replication time is shorter or if their encapsidation is more easily accomplished than full-length molecules. The rate of production of substituted and deleted molecules may be constant with each infection,

and be independent of the multiplicity of infection. Alternatively, the rate may be a function of the multiplicity. A threshold multiplicity is perhaps needed for the substitution process to occur at all.

The suggestion from this study that cellular DNA is integrated into the viral genome during the lytic cycle makes it tempting to suggest that there may be fewer differences between the lytic and transformational events induced by SV40 than once thought. Our studies point out the desirability of examining SV40 stocks for substitutions when studying virus-cell interactions and viral transformations of cells.

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