RENATURATION AND HYBRIDIZATION STUDIES OF MITOCHONDRIAL DNA

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ABSTRACT The products of the renaturation reaction of mitochondrial DNA from oocytes of *Xenopus laevis* have been studied by electron microscopy and CsCl equilibrium density gradient centrifugation. The reaction leads to the formation of intermediates containing single-stranded and double-stranded regions. Further reactions of these intermediates result in large complexes of interlinking double-stranded filaments. The formation of circular molecules of the same length as native circles of mitochondrial DNA was also observed. The formation of common high molecular weight complexes during joint reannealing of two DNA's with complementary sequences was used as a method to detect sequence homology in different DNA samples. Although this method does not produce quantitative data it offers several advantages in the present study. No homologies could be detected between the nuclear DNA and the mitochondrial DNA of *X. laevis* or of *Rana pipiens*. In interspecies comparisons homologies were found between the nuclear DNA's of *X. laevis* and the mouse and between the mitochondrial DNA's of *X. laevis* and the chick, but none between the mitochondrial DNA's of *X. laevis* and yeast. These results are interpreted as indicating the continuity of mitochondrial DNA during evolution.

INTRODUCTION

Mitochondrial DNA (M-DNA) from several animal species has been found to be a twisted circular molecule consisting of two covalently closed strands (Kroon, Borst, van Bruggen, and Ruttenberg, 1966; Dawid and Wolstenholme, 1967; Borst, van Bruggen, Ruttenberg, and Kroon, 1967). Continuing our studies we investigated the nature of the renaturation products of M-DNA. Similar information regarding other types of DNA has mostly been obtained by centrifugation studies (Kozinski and Beer, 1962; Britten and Waring, 1965; Subirana, 1966 *a* and *b*), while Sinclair, Stevens, Sanghavi, and Rabinowitz (1967) have presented electron micrographs of renatured yeast M-DNA. We have extended these observations using *Xenopus laevis* oocyte M-DNA, employing electron microscopy in conjunction with density gradient centrifugation. Sequence relations between M-DNA of different species and between M-DNA and nuclear DNA have also been investigated.

METHODS

Preparation of DNA

M-DNA from oocytes of *Xenopus laevis* and *Rana pipiens* was prepared as described previously (Dawid, 1966; Dawid and Wolstenholme, 1967). Mitochondria from adult chicken livers were prepared by differential centrifugation of the homogenate, treatment of the particle fraction with 25 μg/ml DNase for 30 min at 30°C, and banding of the mitochondria in a sucrose gradient (Rabinowitz, Sinclair, DeSalle, Haselkorn, and Swift, 1965). Cells of commercial bakers' yeast were disrupted by high-speed mixing with glass beads and mitochondria were isolated in the same way as the chicken liver particles. The DNA was then purified in the same way as DNA from frog mitochondria. Nuclear DNA was prepared from liver or erythrocytes as described previously (Dawid, 1965).

Renaturation

The M-DNA used in these studies consisted mainly of open circular molecules, so that reversible denaturation need not be considered (Dawid and Wolstenholme, 1967). Denaturation of DNA was achieved either by heating for 5 min to 100°C in solutions of ionic strength strength below 0.1, or by exposure to 0.1 N NaOH for 3 min at room temperature followed by neutralization. In the studies on the nature of the renaturation product, samples containing about 20 μg/ml of DNA were incubated under varying conditions which were designed to lead to different degrees of renaturation. In the hybridization experiments samples of the same concentrations were reannealed by incubation in the presence of 0.5 M CsCl at 60°C for 12 hr.

CsCl Density Gradient Centrifugation

CsCl density gradient centrifugation (Meselsohn, Stahl, and Vinograd, 1957) was carried out in a Beckman Model E centrifuge (Beckman Instruments, Inc., Fullerton, Calif.) at 20°C and 39,460 rpm. The tracings shown were made from photographs taken after 20 hr of centrifugation. Densities and base compositions were calculated according to Schildkraut, Marmur, and Doty (1962). Denatured DNA from *Pseudomonas aeruginosa* (density 1.739) was used as a reference, except in the experiments of Fig. 9, where native DNA of this organism was used (density 1.727). The reproducibility of density determination of native and renatured M-DNA from *X. laevis*, the material studied most extensively, is shown in Table I. The density indicates a base composition of 44% cytosine plus guanine (see also Dawid, 1966).

TABLE I

DENSITIES IN CsCl OF NATIVE AND RENATURED M-DNA OF *X. laevis*

Densities observed and number of observations in parenthesis	
Native	Renatured
1.702(1)	1.702(3)
1.703(2)	1.703(4)

Electron Microscopy

DNA was spread by the method of Freifelder and Kleinschmidt (1965). Details of further preparation and examination of specimens were the same as those described previously (Wolstenholme and Dawid, 1967; Dawid and Wolstenholme, 1967).

RESULTS

The Nature of the Renaturation Product

It has previously been reported (Corneo, Moore, Sanadi, Grossman, and Marmur, 1966; Borst and Ruttenberg, 1966) that denatured M-DNA could easily be renatured

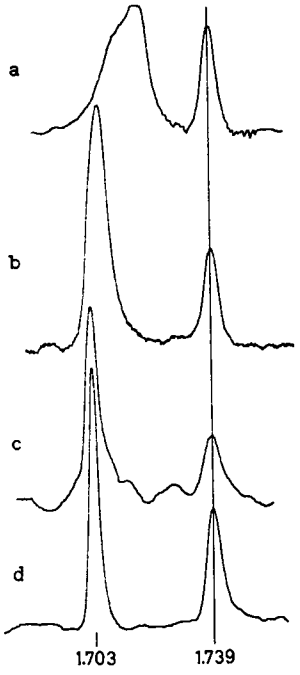

FIGURE 1. Tracings of the bands formed in CsCl by samples of M-DNA that were renatured to a different extent. Denatured unfragmented M-DNA was incubated in the heated spectrophotometer and samples were removed after the reduction in absorbancy indicated that reassociation was progressed to 25% (*a*), 75% (*b*), and 95% (*c*). Sample *d* was extensively reannealed (6 hr at 60°C in 0.5 M CsCl). The reference band to the right in this figure and in Figs. 6–8 is denatured DNA from *P. aeruginosa*.

to material banding in CsCl at the position characteristic for native DNA. We have studied the nature of the products during early and late stages of renaturation by CsCl density gradient centrifugation and by electron microscopy. The experiment shown in Fig. 1 was designed to follow renaturation by the return to native buoyant density in CsCl. Solutions of denatured M-DNA were incubated in a heated spectrophotometer and samples removed at different stages of reassociation as indicated by the degree of hypochromicity. Analysis in CsCl (Fig. 1) showed that a continuum of molecules of intermediate density was present during the earlier stages of the reaction. The degree of renaturation estimated at each time from the density agreed well with that obtained from the absorbancy measurements.

During the course of reannealing, M-DNA exhibited an extreme increase in molec-

ular weight. Similar observations have been made with viral DNA (Kozinski and Beer, 1962; Subirana, 1966 *a*), with animal nuclear DNA (Britten and Waring, 1965), and yeast M-DNA (Sinclair et al., 1967). Reannealed nuclear DNA (considering only material incubated for moderately long times like 12 hr) apparently consists of networks of largely single-stranded material, linked together by short double-stranded regions; reannealed viral DNA and M-DNA (see below) is essentially double-stranded. The large size of extensively reannealed M-DNA, i.e. material

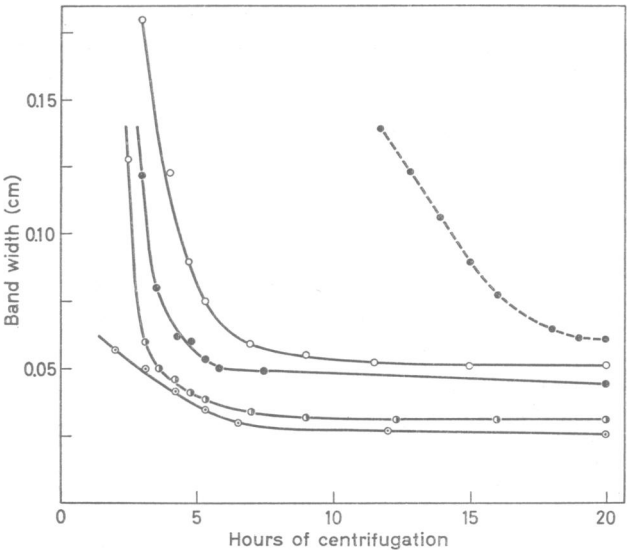

FIGURE 2. The band width at half height at different times of centrifugation in CsCl of samples of M-DNA that were reannealed in 0.5 M CsCl at 60°C for different periods. The reannealing times were: 30 min (open circles), 3 hr (filled circles, solid line), 6 hr (half filled circles), and 12 hr (open circles with dots). For comparison, the band width of native DNA from *P. aeruginosa* of a molecular weight of 3×10^7, centrifuged under the same conditions, is shown (dashed line). The width of this latter band did not change after an additional 10 hr of centrifugation.

incubated longer than needed to reach native density, is indicated by the narrow width of the bands formed in CsCl density gradients (Figs. 1 *d*, 2, 7–9). In order to follow the increase in molecular weight during annealing, denatured M-DNA was incubated in 0.5 M CsCl at 60°C and samples were removed at different times. Since this experiment was designed to study the later stages of the reaction, the first sample was removed after 30 min, at which time the density of the material was already indistinguishable from the native density. As a qualitative estimate of molecular weight, the band widths at half height after different times of centrifugation in CsCl were recorded (Fig. 2). The behavior of native DNA of *P. aeruginosa* under the same conditions has been included for comparison. It can be seen in Fig. 2 that the band width and the time needed for the formation of the band during centrif-

fugation decreases with increasing annealing time. Although native density is resumed by denatured M-DNA in less than 30 min of incubation under the present conditions, further reactions do take place during longer incubation times. The small band width and rapidity of band formation in the more extensively reannealed samples indicate that this DNA has a very high molecular weight. In boundary sedimentation of renatured M-DNA heterogeneous material with an average sedimentation coefficient of 3000S has been observed.

Three samples of M-DNA of *X. laevis* were examined in the electron microscope: DNA reassociated to an extent of 25 and 75% (Fig. 1) and DNA reannealed for 12 hr, which leads to apparently complete reassociation of the strands. During the early stages of reassociation, several types of structure were found. 1. "Perfect" double-stranded open circles (Fig. 3 *a*) with contour lengths (5.2–5.6μ) approximating those of the native open circles of frog M-DNA (Wolstenholme and Dawid, 1967). Such circles do not show a change of morphology along the length of their filament. 2. Double-stranded open circles of about the same length as native M-DNA but containing a region of fuzzy appearance, a "puddle" (arrow, Fig. 3 *c*). 3. A few "puddle"-containing open circles with contour lengths much less than those of native M-DNA (Fig. 3 *d*). 4. Single-stranded and double-stranded linear and looped molecules. (In electron micrographs, filaments of single-stranded DNA have less contrast, are thinner, and have a much more crinkled or wavy appearance than double-stranded DNA; see Freifelder and Kleinschmidt, 1965; Dawid and Wolstenholme, 1967.) Some linear molecules were branched and appeared to be either double-stranded (Fig. 3 *b*) or to be made up of both double- and single-stranded filaments. Frequently observed in these samples were structures (Fig. 3 *e*) consisting of double-stranded filaments which either radiate from or are joined at both ends to masses of material similar in appearance to the "puddle" regions of the circles described in structure 2.

In electron microscope preparations of extensively renatured M-DNA, the predominant type of structure was a mass of apparently interconnecting double-stranded filaments (Fig. 4) which have been found to reach dimensions of 21 $\times$ 8μ. The densest areas in Fig. 4 may arise from the superposition of filaments which are lying above each other. In some complexes of renatured M-DNA, amorphous dense areas occur which could represent much more extensive three-dimensional arrangements of DNA strands. Single linear double-stranded molecules of various lengths and "perfect" open circles were also found in the extensively renatured sample, but neither double-stranded "puddle"-containing circles nor masses of type 4 found in partially reassociated material were located. A few apparently "perfect" circles with contour lengths much less than those of native circles were seen.

A mechanism by which the majority of the observed forms of M-DNA could arise is presented schematically in Fig. 5. It is based essentially on the proposals of Subirana (1966 *a* and *b*) for the mechanism of reassociation of T2 DNA, which is a collection of circularly permuted molecules (Thomas and MacHattie, 1964;

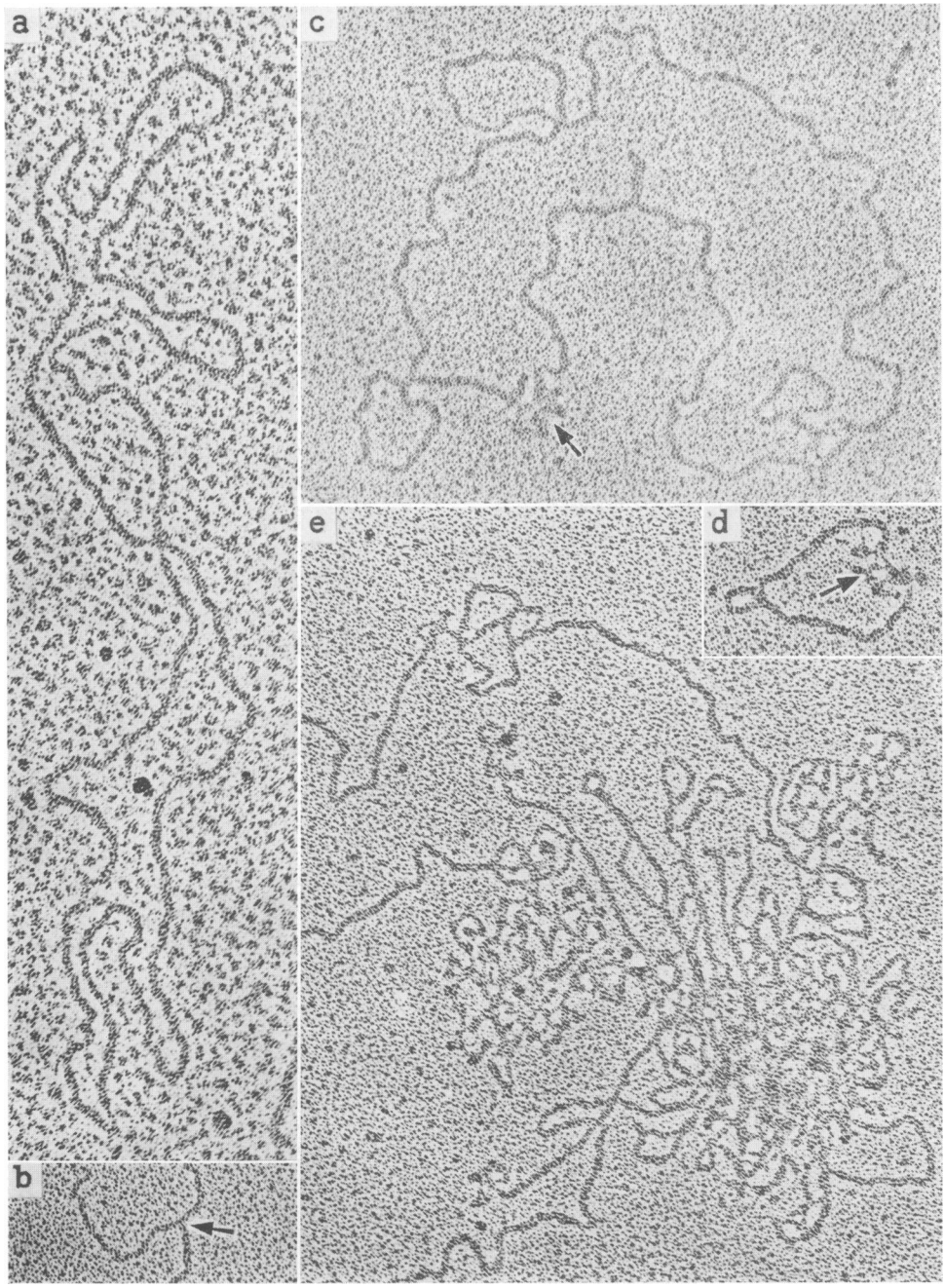

FIGURE 3. Electron micrographs of various forms of renaturation products of *X. laevis* oocyte mitochondrial DNA from samples which, by hypochromicity and density, showed 25% and apparently complete reassociation. *a*, a perfect circle (5.6μ) from a sample reannealed extensively (for 12 hr). *b–e*, from M-DNA reassociated to an extent of 25%. *b*, a double-stranded branched (arrow) molecule; *c*, a circle with a contour length of 5.4μ but containing a "puddle" (arrow); *d*, a circle with a contour length of only 0.8μ, and containing a "puddle" (arrow); *e*, a structure composed of double-stranded molecules continuous with "puddle"-like material which is considered to be single-stranded DNA (see text). All micrographs × 85,000.

Thomas and Rubenstein, 1964). The assumption was made that a molecule of M-DNA is a unique sequence, the native form of which is a circle. As about 96% of the circles in the sample used were of the open form, denaturation lead to separation of the strands into circularly permuted linear molecules and single-stranded circles (Dawid and Wolstenholme, 1967). Thermal agitation will cause breaks in both the circles and the linear forms. On reannealing, perfect circles can only be

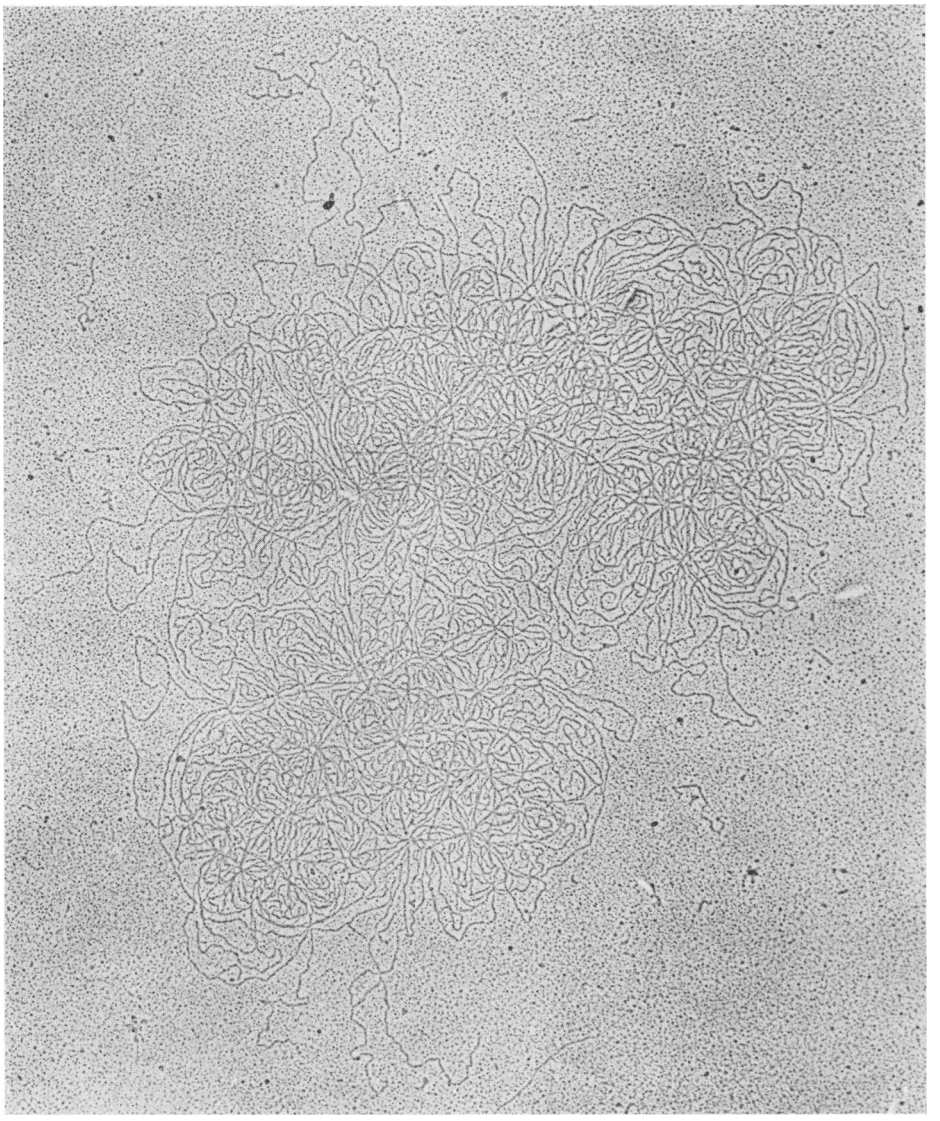

FIGURE 4. A mass of apparently interconnecting double-stranded filaments from the sample of extensively renatured M-DNA. × 24,000.

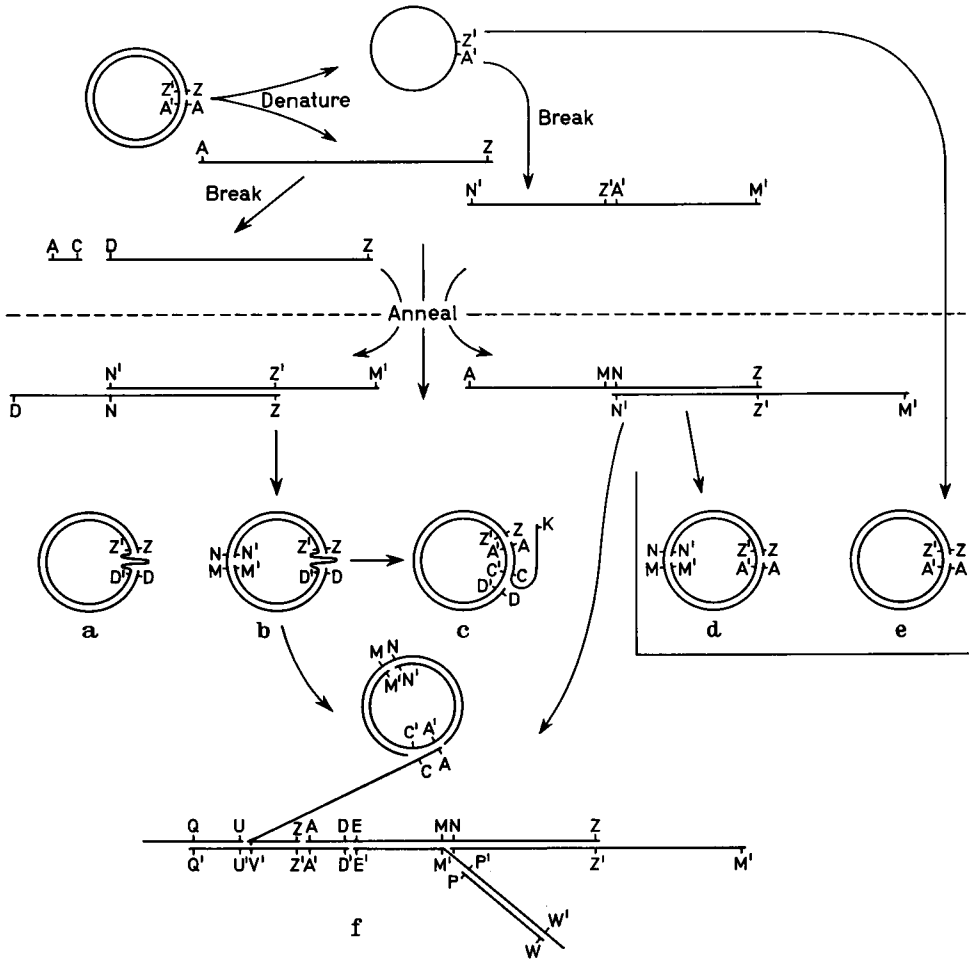

FIGURE 5 Schematic representation of the way in which the various structures observed in the electron microscope could be formed during reassociation of denatured M-DNA. It is assumed that a molecule of M-DNA is a unique sequence. Thermal breakage of circles leads to linear molecules with circularly permuted sequences. Complementary paired strands are symbolized by parallel lines; letters indicate sequences. Linear forms, either straight or branched (Fig. 3 *b*) result from the pairing of complementary single-stranded pieces of any size. Perfect circles (Fig. 3 *a*) can only be formed by molecules which either singly or in combination form two complete complementary sequences as shown in *d* and *e*. Such molecules can react no further. In the circular forms *a* and *b*, a small region of one strand is unpaired. This arrangement could be the basis of the "puddle"-containing circles found in 25 and 75% reassociated samples of M-DNA (Fig. 3 *c*). The appearance of a "puddle" in a circle might alternatively result from the coiling up of a piece of single-stranded DNA branching off from the circle as shown in part *c*. Circular forms, *a*, *b*, and *c*, could also react further with single-stranded regions of linear molecules as shown in *f*. The single-stranded regions of such a form would be the "puddle"-like regions shown in Fig. 3 *e*. Further pairing of single-stranded regions would eventually produce the large complex of double-stranded molecules found in extensively renatured M-DNA (Fig. 4).

formed by molecules which either singly or in combination form two complete complementary sequences (Fig. 5 *d* and *e*). The "puddle" regions, seen in some circles, as well as the "puddle"-like material of form 4 of partially renatured M-DNA, is considered to be single-stranded DNA since such material was not found in extensively renatured M-DNA. Material of similar appearance has been observed by MacHattie, Ritchie, Thomas, and Richardson (1967) in their work with T2 DNA and by Sinclair et al. (1967) in renatured yeast M-DNA; these authors have likewise interpreted such regions as single-stranded DNA. The "puddle" regions of circles may therefore, as shown in Fig. 5 (*a* and *b*), represent a region of the circle which is single-stranded. Alternatively, a piece of single-stranded DNA branching off a circle (Fig. 5 *c*) may appear as part of the circle. The presence of both single-stranded and double-stranded regions in the same molecules of partially reassociated M-DNA is also indicated by the appearance of a continuum of molecules of a density intermediate between that of denatured and native DNA (Fig. 1). During prolonged annealing, pairing of complementary single-stranded regions of the various molecules by a process called "concatenation" by Britten and Kohne (1966) would lead to the eventual disappearance of single strands and to the formation of the large complexes of interlocking double-stranded filaments (Fig. 4). Such complexes are undoubtedly the physical basis for the extremely high molecular weight of extensively reannealed M-DNA (see above). The rare occurrence of small circles cannot be satisfactorily explained by the mechanism proposed. Since only a very small proportion of the material occured in the form of small circles these could be the renaturation product of a different type of DNA which contaminated at a low level the M-DNA preparations.

Hybridization Studies

A method based on the formation of common complexes during the joint reannealing of related DNA samples has been used for studies on sequence homology. It has been shown in the work of Britten and Waring (1965) that the formation of common complexes is a valid test for homology, since unrelated DNA's, like plant and animal nuclear DNA, form separate complexes during coannealing. Similarly, viral and host cell DNA's have been reported to form separate networks during joint incubation (Kozinski and Beer, 1962; Jungwirth and Dawid, 1967). Therefore, unspecific aggregation of unrelated DNA's does not take place during reannealing. Distantly related DNA pairs, like the nuclear DNA's of calf and fish, formed common complexes, suggesting that a low level of sequence homology is sufficient to allow concatenation between two types of DNA (Britten and Waring, 1965). In such experiments the presence of common or of separate networks can be determined in CsCl gradients provided that the DNA samples studied have different densities initially. The sequence homology test based on such measurements is only qualitative, but has several advantages for the experiments to be described here.

Since it is difficult to prepare large quantities of M-DNA or pure, radioactively labeled M-DNA, it is important that the present method requires only small amounts of unlabeled material. Furthermore, in the comparison of M-DNA with nuclear DNA, cross-contamination would invalidate experiments with the agar or filter methods, while it would not present a problem with the present test.

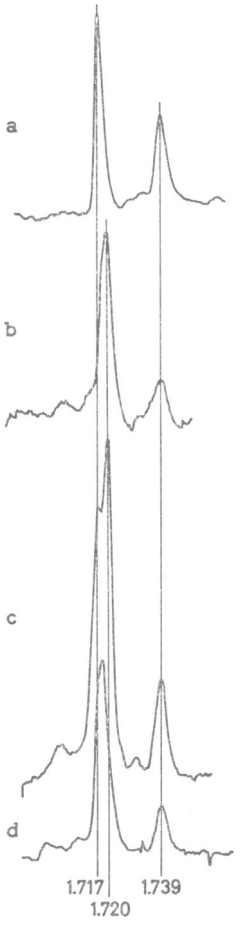

FIGURE 6. Hybridization of nuclear DNA. Tracings of photographs of CsCl gradients are shown. *a*, reannealed *X. laevis* DNA; *b*, reannealed mouse DNA; *c*, a mixture of separately reannealed *Xenopus* and mouse DNA's; *d*, jointly reannealed *Xenopus* and mouse DNA.

The method was tested with nuclear DNA of *X. laevis* and the mouse in an experiment analogous to that of Britten and Waring (1965). Fig. 6 *a* and *b* show the bands in CsCl formed by reannealed DNA of the two animals. The densities are identical with those of the denatured DNA, since most of the material remains single-stranded under these conditions. However, high molecular weight complex formation has occurred as shown by the narrow band width of both DNA preparations. Although the density difference between denatured mouse and frog nuclear DNA is small, the band width of the networks is so narrow that a mixture of separately annealed

DNA samples separated clearly (Fig. 6 *c*). After reannealing mouse and frog DNA together, a single symmetric band of intermediate density was observed (Fig. 6 *d*). The homology of 2–4% of the sequences in frog and mouse DNA (Denis, 1966) was sufficient to cause the formation of common complexes.

The application of this test to nuclear DNA and M-DNA of *X. laevis* is shown in Fig. 7. The top four tracings show the positions of the bands formed by the two

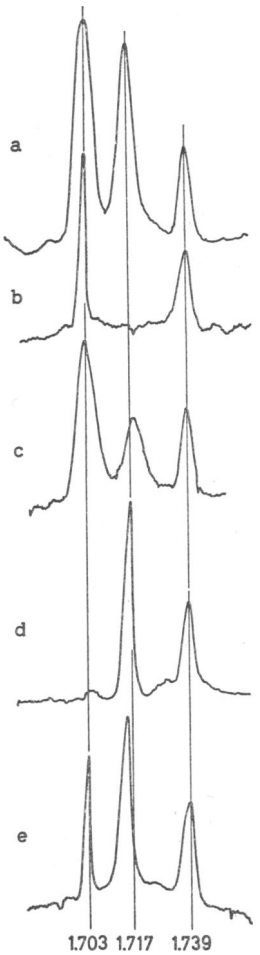

FIGURE 7. Absence of sequence homologies between nuclear and M-DNA of *X. laevis*. Bands in CsCl are shown of: *a*, native and denatured M-DNA; *b*, renatured M-DNA; *c*, native and denatured nuclear DNA; *d*, reannealed nuclear DNA; *e*, M-DNA and nuclear DNA reannealed in mixture (note the formation of two sharp bands at the positions of renatured M-DNA, 1.703, and reannealed nuclear DNA, 1.717).

types of DNA in different states. Denatured M-DNA is denser by 0.014 g/cm³ than the native material (Fig. 7 *a*); after renaturation it returns to native density and forms a narrow band (Fig. 7 *b*). Whereas native and denatured nuclear DNA (Fig. 7 *c*) have densities almost identical with those of the analogous forms of M-DNA, the reannealed nuclear DNA (Fig. 7 *d*) bands at a density close to the denatured material, at a considerable distance from renatured M-DNA. After reannealing nuclear DNA and M-DNA together, two bands appeared in the CsCl gradient (Fig. 7 *e*)

at positions corresponding to those assumed by separately reannealed nuclear DNA (Fig. 7 *b*) and M-DNA (Fig. 7 *d*). Both bands were very narrow, indicating that they were formed by material of a high molecular weight. It is assumed that the two bands arose by separate network formation of nuclear DNA, and separate renaturation of M-DNA. Linking of the two types of complexes did not take place. Since common complexes could be formed by DNA types which have a sequence homology of 2–4% (see Fig. 6 and text) the general homology between nuclear DNA and M-DNA of *X. laevis* is likely to be below this level, if any exists at all. Although nuclear and mitochondrial DNA differ greatly in their homogeneity, it is unlikely that this is the cause for their lack of interaction. The experiment shown in Fig. 2 demonstrated that M-DNA maintains single-stranded regions for long periods during reannealing, which allow an increase of network size up to at least 12 hr. This fact is explained in the model (Fig. 5) by considering incomplete overlaps which occur because of circular permutation of sequences and different length of fragments. Since concatenation of nuclear DNA leads to large networks in much shorter time there would be ample opportunity for reaction of the two DNA's if sufficient homologies were present. An experiment analogous to that shown in Fig. 7 was performed with nuclear and M-DNA of *R. pipiens* and again failed to give any indication for the presence of sequence homologies. A similar result has also been obtained by Sinclair et al. (1967), who reannealed a preparation of yeast M-DNA which contained about 50% nuclear DNA.

The reaction between the M-DNA's of *X. laevis* and the chicken is illustrated in Fig. 8. This pair was used because it exhibits a natural density difference. Native chicken M-DNA (Fig. 8 *a*) banded at a density of 1.709, which agrees with the finding of Rabinowitz et al. (1965). Renatured chicken M-DNA (Fig. 8 *b*) banded at 1.711, at almost but not exactly native density. The separation of individually renatured chick and *Xenopus* M-DNA is shown in Fig. 8 *c*; after corenaturation (Fig. 8 *d*) a single symmetrical band of intermediate density was found. No material remained at the positions of the individual materials, demonstrating the extensive concatenation which had occurred. It can be concluded that *X. laevis* M-DNA and chick M-DNA have nucleotide sequences in common despite the difference in their over-all cytosine plus guanine content.

The M-DNA's of *X. laevis* and of yeast were compared as an example of an evolutionary distant pair. Fig. 9 *a* shows the band of native yeast M-DNA; the density (1.683) is in agreement with the results of others (Tewari, Vötsch, Mahler, and Mackler, 1966; Corneo et al., 1966; Mounolou, Jacob, and Slonimski, 1966). Denatured yeast M-DNA bands at a density of 1.699 (Fig. 9 *b*), renatured M-DNA bands at 1.687 (Fig. 9 *c*), which is near to the position of native DNA. Fig. 9 *d* shows the result of corenaturation of yeast and *Xenopus* M-DNA: two bands at the densities of the individual components are seen. The experiment clearly indicates that it is not sufficient to reanneal two samples of M-DNA together in order to get common complexes. Since a variety of DNA's do not form common complexes when reannealed together, the cases in which such complexes are formed cannot be interpreted as being due to purely physical aggregation.

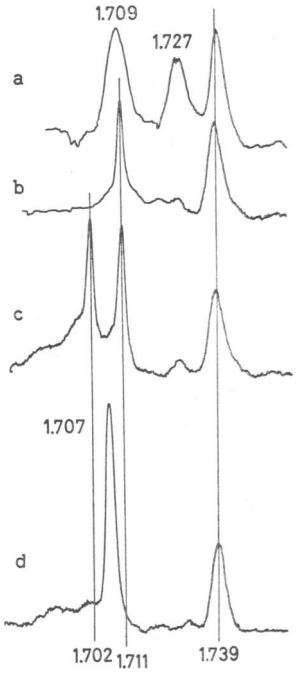

FIGURE 8. Hybridization of M-DNA of *X. laevis* and the chick. Bands in CsCl are shown of: *a*, native and denatured chick M-DNA; *b*, renatured chick M-DNA; *c*, separately renatured M-DNA's of *Xenopus* and chick; *d*, jointly renatured M-DNA of Xenopus and the chick. For the shape and position of native, denatured, and individually renatured *Xenopus* M-DNA, see Fig. 7.

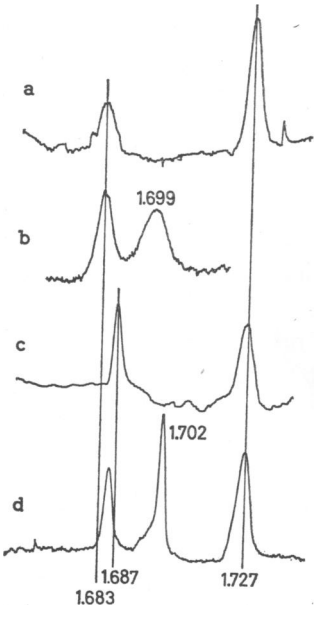

FIGURE 9. Absence of sequence homologies between the M-DNA's of *X. laevis* and bakers' yeast. Bands in CsCl are shown of: *a*, native yeast M-DNA; *b*, native and denatured yeast M-DNA; *c*, renatured yeast M-DNA; *d*, jointly renatured M-DNA's from *Xenopus* and yeast. For bands of *Xenopus* M-DNA, see Fig. 7. The reference band to the right is native DNA of *P. aeruginosa*.

DISCUSSION

Relatively high concentrations of DNA were chosen in the renaturation experiments since it was our aim to study the concatenation reaction which is favored under these conditions. We did not attempt to produce a maximum yield of "perfect" circles (Fig. 3 *a*) since the circular nature of the starting material was established. Therefore, the present results are compatible with the work of Thomas and MacHattie (1964) in which T2 DNA was reannealed at much lower concentrations to increase the yield of circles. The renaturation mechanism which is based on our observations on frog oocyte M-DNA is consistent with the expected reactions of a homogeneous DNA preparation with circularly permuted sequences. It might be postulated that other types of M-DNA could renature by the same mechanism. An important difference must be pointed out, though. Whereas frog M-DNA renatured to give a a product that banded in CsCl at exactly the native position (Table I and Figs. 1, 7–9), other renatured M-DNA's band at a slightly higher density. This has been reported by Corneo et al. (1966) for guinea pig M-DNA, by Sinclair et al. (1967) for yeast M-DNA, and in the present work for chick and yeast M-DNA (Figs. 8 and 9). The renaturation conditions used by different authors were similar but not identical. Our observations on frog and chick M-DNA were made under equal conditions, though. Because of the repeated observation, the small density differences between native and renatured M-DNA of these species should be considered real. The density values suggest that these renatured DNA's contain between 10 and 20% single strands. This conclusion is supported by electron microscope observations. Sinclair et al. (1967) observed puddles (i.e., single-stranded regions) in yeast M-DNA that had been reannealed or 6 hr, while frog M-DNA reannealed for similar periods of time was entirely free of puddles. Since the size and homogeneity of yeast M-DNA are not known, it is possible that this DNA is less homogeneous than vertebrate M-DNA, making less complete renaturation explainable. However, chick and guinea pig M-DNA have the same size and physical structure as frog M-DNA (Kroon et al., 1966; Borst et al., 1967; Dawid and Wolstenholme, 1967), so that the apparent difference in the extent of renaturation of these M-DNA's cannot be explained at present.

The concatenation reactions which take place during reassociation of strands of M-DNA provide the basis for a sequence homology test by which limited but reliable information on M-DNA relations can be obtained. The M-DNA's of *X. laevis* and the chick appear to be related, but those of *X. laevis* and the yeast are not. A correlation with evolutionary distance is suggested. This conclusion is supported by preliminary hybridization experiments using complementary RNA and the filter technique of Nygaard and Hall (1963). A homology of about 5% between frog and chick or calf M-DNA was found, similar to the homology between the nuclear DNA's of these species (Denis, 1966). The absence of homologies between the M-DNA's of frogs and yeast is also in accordance with the experience regarding nuclear

DNA of very distant species (Hoyer, McCarthy, and Bolton, 1964; Britten and Waring, 1965). In view of the present results it seems likely that the homology between M-DNA's, where it occurs, is a result of evolutionary relatedness rather than due to functional requirements of mitochondria so stringent that they require maintenance of identical nucleotide sequences in M-DNA. Evolutionary continuity of M-DNA has previously been suggested by Reich and Luck (1966) on the basis of experiments showing the preservation of M-DNA during subsequent generations and in crosses of *Neurospora*. The results reported in the present paper support this view and extend it across a larger evolutionary distance.

Before discussing our results on the relation of nuclear and mitochondrial DNA, the conflicting evidence of DuBuy and his collaborators must be mentioned. In reporting the properties of DNA from mitochondrial preparations of the mouse and *Leishmania enriettii*, DuBuy, Mattern, and Riley (1966) showed that the material studied renatured only to an extent of about 20%. Virtually complete renaturation of M-DNA has been observed in the present work in agreement with the reports of others (Borst and Ruttenberg, 1966; Corneo et al., 1966). Whereas the frog M-DNA used here has been characterized as circular DNA of unique physical properties (Dawid and Wolstenholme, 1967), no structural information regarding the DNA they used has been reported by DuBuy et al. (1966). It appears likely that these authors' preparations consisted of mixtures of nuclear and mitochondrial DNA. The high degree of homology reported by DuBuy and Riley (1967) between this material and nuclear DNA seems to reflect the degree of contamination of their preparations with nuclear DNA rather than any homology of that DNA with M-DNA. It may also be pointed out that it is highly unlikely on grounds of a priori considerations for 40% of all nuclear sequences to be homologous to M-DNA as reported by DuBuy and Riley (1967), because of the very large number of nuclear sequences as compared to the small size and high homogeneity of M-DNA.

Turning to the results reported in the present paper, two aspects should be considered. First, the nucleus may contain one or more "master copies" of M-DNA. If these M-DNA sequences were present free in the nucleus, i.e. not covalently linked to other DNA molecules, they would renature together with the added M-DNA without providing links to different molecules of nuclear DNA; these sequences would remain undetected in the experiments reported here. The existence of such master copies could only be shown in hybridization studies by more conventional methods comparing radioactive M-DNA or its complementary RNA with the DNA from highly purified nuclei. A single copy of M-DNA per nucleus would represent about 10^{-5} of the total DNA only, making such experiments difficult. The experiments reported here are relevant only with respect to *general* homologies between nuclear and M-DNA. In view of their absence the relation between the many molecules that are obtained from the nuclear DNA of an animal cell should next be considered. During isolation, the DNA of a nucleus breaks into some 10^5 mole-

cules whose average molecular weight is about 10^7. It seems that most of these molecules have regions that share sequences with some other molecules in the population (Britten and Waring, 1965; Britten and Kohne, 1966). These similarities are the basis of interactions during reannealing of nuclear DNA that lead to extensive network formation. Such similarities are believed to have resulted from evolutionary interchange, like duplication, translocation, etc. Lack of a detectable relation to the other sequences in the cell can be found in certain fractions of nuclear DNA, e.g. the mouse satellite DNA, and in M-DNA. Their different localization and presumed different history distinguishes these two types of "non-cross-concatenating" DNA's. Material like the mouse satellite is believed to have arisen recently in evolution by the extensive multiplication of a specific sequence. Evolutionary change is expected to eventually transform such material into a family of sequences which would be related to other DNA sequences in the genome (Britten and Kohne, 1966). Although M-DNA appears to have a long evolutionary history, it has not become related to the rest of the genome in a way that allows cross-concatenation. This fact may be due to its cytoplasmic localization and evolution, or, if nuclear master copies exist, to some other mechanism which restricts interchange of these sequences with the rest of the genome.

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