NONSATELLITE RAPIDLY RENATURING FRACTION OF DNA FROM CALF TISSUES

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By hydroxylapatite chromatography of sonicated renatured calf DNA, a fraction was obtained which during annealing at the renaturation temperature partially restored its double helical secondary structure. In a CsCl density gradient this fraction gives three bands. Two of these bands correspond to the described satellite components found in DNA from calf tissues (DNA I and DNA II). The third band (DNA III) contains a DNA which in native calf DNA is not separated as a satellite band. DNA III has been found in all fractions obtained by fractionation of calf thymus DNA on the methylated albumin-kieselguhr (MAK) column. By repeated hydroxylapatite chromatography of fractions eluted from the methylated albumin column by higher molarities of NaCl, renatured DNA III was isolated in a relatively pure state without the admixture of satellite DNA's. In a CsCl density gradient DNA III forms a band at a density of 1·706 g/cm^3. Its average composition which follows from physico-chemical measurements is 40% (G + C). The value of the renaturation rate constant indicates that DNA III is composed of repeating sequences shorter than $11 \cdot 10^6$ daltons (double stranded).

The DNA's of higher organisms contain fractions capable of rapid renaturation[1-7]. These fractions are probably formed by DNA which consists of reiterated short, identical or very similar, sequences. In a CsCl density gradient some of the repetitive DNA's are separated as satellite bands[3-6], whereas others are parts of the main band[4]. So far very little is known about their biological function. The DNA of calf tissues contains up to 40% of the repetitive fraction[2,7] which represents a multicomponent mixture[4]. Two of its components have been characterized relatively well, namely the satellite DNA's with a density in the native state of 1·714 g/cm^3 and 1·721 g/cm^3. The rest of the repetitive DNA has been isolated as yet in renatured state only and very little is known about its properties[4].

We have studied earlier[4] the fractionation of sonicated renatured calf thymus DNA on hydroxylapatite. The fraction undergoing reassociation on annealing can be resolved on hydroxylapatite into the renatured and the merely reassociated part. Sedimentation experiments in a CsCl density gradient have shown that the isolated renatured fraction contains, in addition to a mixture of satellite DNA's, still another component which, in the native state does not form a separated satellite band. The aim of the present paper is to report on the isolation and characterization of this component.

EXPERIMENTAL

DNA *samples.* Calf thymus DNA was isolated by the method of Kay and coworkers[8], calf liver nuclear DNA was prepared by the method of Marmur[9]. The concentration of DNA was determined by absorbance measurement at 260 nm using the value of $A_{1cm}^{1\%} = 200$. DNA *was fractionated on methylated albumin-kieselguhr (MAK) column* according to Mandell and Hershey[10]. The procedure has been described in detail in our previous paper[4].

Isolation of rapidly renaturing fractions on hydroxylapatite. The rapidly renaturing fractions were isolated either from unfractionated DNA or from fractions obtained by separation on MAK column. DNA (800–1 000 μg) was first degraded by sonical treatment to a molecular weight of 500 000–600 000 daltons, equilibrated with 0·03M phosphate ($NaH_2PO_4 + Na_2HPO_4$, 1 : 1) by passage over a Sephadex G-50 column, and denatured 10 min at 100°C. After cooling the concentration of phosphate was adjusted to 0·09M and the sample was annealed at 70°C until the value of $c_0\tau$ (*i.e.* the product of DNA concentration in mol of nucleotide P per liter and time in s [ref.[1]]) became 0·27. The DNA solution was then diluted to a 0·03M concentration of phosphate and applied to a 15 × 30 mm column of hydroxylapatite. The elution was effected by a linear gradient of phosphate ($NaH_2PO_4 + Na_2HPO_4$, 1 : 1) at 70°C. In certain cases the isolated fraction required one additional fractionation without the preceding annealing.

Measurement of kinetics of renaturation. The kinetics of renaturation was followed spectrophotometrically at 260 nm in Hilger Uvispek Spectrophotometer using cells of 1 cm optical path. DNA was subjected to alkaline denaturation as follows. To the sample dissolved in 0·01 × SSC (standard saline citrate, *i.e.* 0·15M-NaCl + 0·015M trisodium citrate) was added 1/10 of the volume of 1M-NaOH, the sample was set aside for 10 min at room temperature, then cooled, neutralized by the addition of 1/10 of the original volume of 2M-NaH_2PO_4, and equilibrated with 0·01 × SSC by dialysis. The renaturation kinetics was measured in SSC as described elsewhere[4].

Optical rotatory dispersion measurement and other methods. Optical rotatory dispersion (ORD) measurement was carried out in Jasco ORD/UV Spectropolarimeter using 1 cm cells. DNA was dissolved in 0·1M-NaCl + 0·01M phosphate ($NaH_2PO_4 + Na_2HPO_4$, 1 : 1). The remaining methods used, *i.e.* isopycnic centrifugation in a CsCl density gradient, measurement of sedimentation coefficients in neutral and alkaline media, and measurement of melting curves and hyperchromic spectra have been described elsewhere[4].

RESULTS

Isolation of Rapidly Renaturing Fractions by Chromatography

As shown before[4], the sonicated calf thymus DNA previously denatured and annealed yields three peaks when subjected to chromatography on hydroxylapatite. Peak A contains denatured DNA, peak B partly reassociated DNA, and peak C renatured DNA.

As obvious from the CsCl density gradient pattern of renatured fraction C (Fig. 1), this fraction is a mixture of three components with densities 1·709 g/cm^3, 1·716 g/cm^3, and 1·724 g/cm^3. We designated these bands as follows: DNA I – the band of density 1·716 g/cm^3, DNA II – the band of density 1·724 g/cm^3, and DNA III – the band of density 1·709 g/cm^3. The densities of DNA I and DNA II show that these components are renatured satellite DNA's[4]. By contrast, DNA III is not represented

by a discrete satellite band in the CsCl density gradient pattern of the unfractionated native sample. Fig. 1 shows that this component exists in rapidly renaturing fractions isolated from DNA both from whole cells and from the isolated nuclei.

The elution profile of the fractionation of calf thymus DNA on the MAK column (not shown in the Figure) can be divided into three parts: the ascending one, the one around the peak, and the descending one. These fractions were used for isolation of rapidly renaturing DNA's. The elution profiles of the hydroxylapatite fractionations are essentially identical with those obtained with unfractionated[4] DNA. The rapidly renaturing DNA contained in the fractions which fall into the ascending part of the elution curve characterizing the fractionation on the MAK column (these fractions contain molecules with a higher (G + C) content and of lower molecular weight), however, is eluted from the hydroxylapatite column only partly by a gradient of phosphate. Its residue could be displaced only by 0·12M phosphate

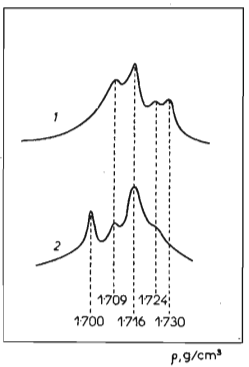

FIG. 1

Isopycnic CsCl Density Gradient Centrifugation of Rapidly Renaturing Fractions Isolated from Calf DNA

1 Rapidly renaturing DNA fraction from whole calf thymus cells; 2 rapidly renaturing fraction of nuclear DNA from calf liver. The band at density 1·700 g/cm^3 corresponds to T 4 DNA marker, the band at density 1·730 g/cm^3 to *Streptomyces chrysomallus* DNA marker.

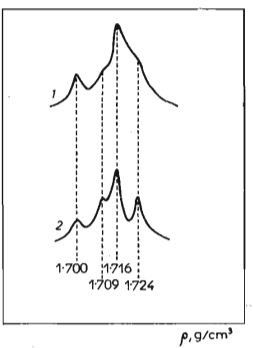

FIG. 2.

Isopycnic CsCl Density Gradient Centrifugation of Rapidly Renaturing Fraction of Calf Thymus DNA Isolated by Hydroxylapatite Chromatography from Fractions Corresponding to Ascending Part of the MAK Chromatography Elution Curve

Fraction eluted from hydroxylapatite by: *1* phosphate gradient at 70°C; 2 0·12M phosphate at 96°C and then annealed 12 h at 70°C in SSC. The band at density 1·700 g/cm^3 corresponds to T 4 DNA marker.

at 96°C. By this procedure, however, DNA is obtained in denatured form. This sample was therefore renatured 12 h at 70°C in SSC. Fig. 2 shows the CsCl density gradient pattern of both parts of rapidly renaturing DNA isolated from fractions of the ascending part of the MAK fractionation elution curve. As obvious from the Figure, both DNA's are essentially identical and contain all three rapidly renaturing DNA's found in the unfractionated sample. A better resolution of both satellite DNA's in the fraction characterized by curve 2 is most likely caused by its higher molecular weight. In Fig. 3 (curve *1* and *2*) are shown the CsCl density gradient patterns of rapidly renaturing DNA's obtained from fractions around the peak and from fractions corresponding to the descending part of the MAK chromatography elution curve. These samples are predominantly formed by DNA III of density 1·709 g/cm^3 and

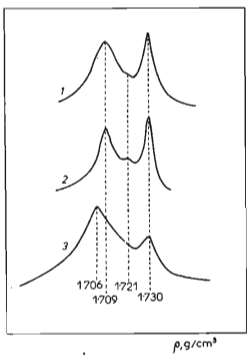

FIG. 3

Isopycnic CsCl Density Gradient Centrifugation of Rapidly Renaturing Fractions Isolated by Hydroxylapatite Chromatography from Fractions of Calf Thymus DNA Obtained by MAK Fractionation

Rapidly renaturing DNA isolated from: *1* fractions around the peak of the MAK fractionation elution curve; *2* fractions corresponding to the descending part of the MAK fractionation elution curve; *3* pooled fractions from the peak and the descending part of the MAK fractionation elution curve and purified by additional fractionation on hydroxylapatite. The band at density 1·730 g/cm^3 corresponds to *Streptomyces chrysomallus* DNA marker.

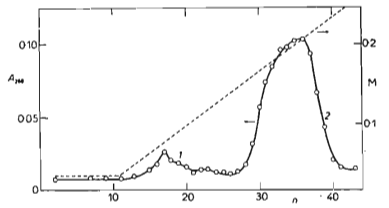

FIG. 4

Elution Diagram of Refractionation of Rapidly Renaturing DNA on Hydroxylapatite

The rapidly renaturing DNA was obtained by hydroxylapatite chromatography of fractions from the peak and the descending part of the MAK chromatography elution curve. Peak *1* denatured DNA; peak *2* renatured DNA. M molarity of phosphate buffer.

do not contain satellite DNA. They contain a small amount of DNA with density 1·721 g/cm^3, *i.e.* with density lower than that of renatured satellite DNA II (1·724 g/cm^3).

Isolation and Properties of Renatured DNA III

The results given show that the rapidly renaturing DNA III can be obtained in relatively pure form if it is isolated from fractions from the MAK column rather than from the unfractionated sample. Fractions corresponding to the peak and the descending part of the MAK chromatography elution curve (total quantity approximately 20 mg of DNA) were used for isolating the rapidly renaturing fraction on hydroxylapatite. Isolated fraction was purified by one additional hydroxylapatite fractionation which led to the separation of the rest of denatured DNA (Fig. 4). A comparison of the CsCl density gradient patterns (curve *3* with curves *1* and *2* in Fig. 3) shows that the removal of the residual denatured material by the second fractionation resulted in a decrease of the density of DNA III from 1·709 g/cm^3 to 1·706 g/cm^3 and in the disappearance of the band at density 1·721 g/cm^3, obviously containing denatured DNA. The band of DNA III is considerably broad, probably due to its low molecular weight, and somewhat asymmetric toward the side of higher densities. The asymmetry of the band is obviously caused by the heterogeneity of the sample with respect to its composition or renaturation degree. Since DNA III can be obtained by hydroxylapatite chromatography in renatured form only, its composition cannot be calculated from its buoyant density or the T_m-value either. The calculation based on the hyperchromic spectrum leads to a value of 38% (G + C).

Information on the secondary structure of isolated DNA III can be obtained from the course of its melting curve or ORD curve. The melting curve (Fig. 5) shows that DNA III is renatured to a considerable degree. The T_m-value is relatively high

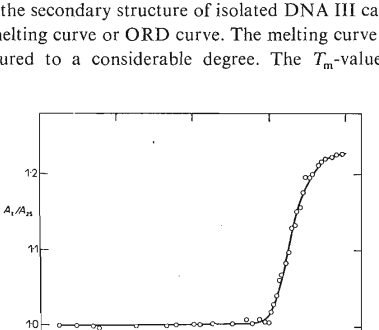

FIG. 5
Melting Curve of DNA III Measured in SSC at 260 nm

(85·5°C), comparable with the T_m-value of unfractionated native calf thymus DNA. The hyperchromic effect is lower than with native DNA's and equals 22·5%. Unlike the rapidly renaturing fractions isolated by one single fractionation on hydroxylapatite[4], the twice fractionated DNA III does not contain material denaturing at temperatures below 79°C. The dependence of the ORD curve on the composition of DNA (Fig. 6) is qualitatively identical with the data of Samejima and Yang[11], yet slightly less pronounced. The ORD curve of denatured calf thymus DNA measured after the cooling (not shown in the Figure) differs from the ORD curve of native DNA mainly in the shift of the trough[12] from 256 nm to 260 nm. The ORD curve of DNA III shows a peak at 290 nm and a trough at 257 nm and its overall

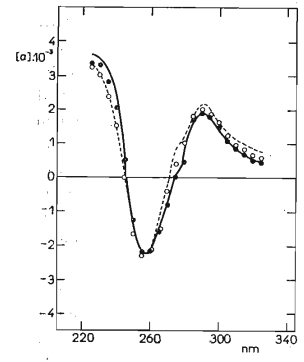

Fig. 6

Optical Rotatory Dispersion

●DNA III; ○ fraction of calf thymus DNA rich in satellite components (DNA I + + DNA II, ref.[4]); full line, unfractionated calf thymus DNA; broken line, *Streptomyces chrysomallus* DNA. Measured in 0·1M-NaCl + 0·01M phosphate.

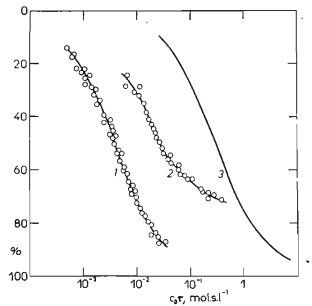

FIG. 7

Renaturation Kinetics

1 Mixture of satellite calf thymus DNA's (DNA I + DNA II); *2* DNA III; *3* DNA T 4; % stands for reassociation. The data for curve *1* are taken from paper[4], the data for curve *3* from paper[1]. All the data were recalculated[7] for 0·18M-Na^+. The size of DNA molecules was in the case of DNA III and DNA T 4 approximately the same (the molecular weight of DNA III is $1·4 . 10^5$ daltons in a single stranded state, Britten and Kohne[1] report for T 4 DNA a value around $1·9 . 10^5$ daltons), for the mixture of satellite DNA's the size of the molecules was approximately twice larger ($3·4 . 10^5$ daltons).

shape is essentially the same as the shape of the curve of unfractionated native calf thymus DNA.

The renaturation kinetics (Fig. 7) shows that DNA III renatures more slowly than the mixture of satellite DNA's I and II, yet, however, faster than phage DNA. The course of the renaturation seems to indicate that the system is not entirely homogeneous. Approximately 20% of DNA III does not renature, obviously due to degradation of the sample during the thermal treatment or due to incomplete separation of the residual nonrepetitive DNA. The renaturation rate constant of DNA III in 0·18M-Na^+ equals 58·8 mol s/l, as follows from the value of $c_0\tau_{1/2}$ (ref.[1]).

DISCUSSION

The rapidly renaturing fraction contained in DNA from calf tissues is a relatively complex mixture whose individual components differ in composition, rate of renaturation, and extent of restoration of the double helical structure on annealing[4]. This fraction in renatured state can easily be isolated by fractionation on hydroxylapatite at elevated temperatures. In a CsCl density gradient it yields three bands, of which two correspond to the satellite DNA. The third one (DNA III) contains the rapidly renaturing fraction which in native state is not separated as a discrete band. DNA III is contained in the DNA isolated from whole cells and also in the DNA isolated from nuclei and cannot thus be of mitochondrial origin. This conclusion is also supported by the fact that the yields of DNA III (around 5%) are higher than the average content of mitochondrial DNA in animal cells (around 1%) (ref.[13]). Unlike the satellite DNA I and DNA II which have been found only in fractions of calf thymus DNA rich in (G + C), DNA III is contained in all fractions obtained by separation of the unfractionated sample on the MAK column. It can be thus concluded that the sequences corresponding to DNA III are in native calf DNA distributed in molecules of different average (G + C) content and as an individual fraction can be obtained from the degraded sample only. This conclusion is in accordance with the observation of Britten and Smith[7] that repetitive and nonrepetitive sequences are interspersed in calf thymus DNA.

DNA III has been isolated so far only in renatured form and its composition cannot therefore be determined by conventional physico-chemical methods, *i.e.* from T_m or buoyant density. The composition calculated from hyperchromic spectra (38% (G + C)) characterizes merely the double helical region and does not involve parts of the molecules with nonrestored secondary structure which do not display an increase of absorbance on heating. From the ORD curve and the melting curve follows that DNA III does not contain a larger amount of unordered, single stranded regions and that the composition of DNA III is close to the composition of unfractionated calf thymus DNA (42% (G + C)). Hence, eventhough we do not know the exact chemical composition of DNA III, we can postulate from the results given

above that it varies around 40% (G + C). DNA III differs in this respect substantially from satellite DNA I and II which belong to DNA's rich in (G + C) as indicated by their T_m-values (ref.[14,15]), buoyant densities[4,14,15], hyperchromic spectra[4,16], and ORD.

The renaturation of calf thymus DNA has been studied recently by Britten and Smith[7]. These authors found that the total content of the rapidly reassociating fraction in calf DNA is approximately 40%. Under our experimental conditions ($c_0\tau$ = = 0·27 mol s/l, 70°C) approximately half of this value is obtained. Britten and Smith[7] classify calf thymus DNA with respect to reassociation rate as three fractions: fast, intermediate, and slow ones. They suppose that the slow fraction contains nonrepetitive DNA. Some 25% of the fast fraction (~2% of total DNA) reassociates at very low $c_0\tau$ values and the authors regard this fraction as DNA in which complementary sequences occuring at different sites of the same strand react during reassociation. The remaining 75% of the fast fraction and the whole intermediate fraction contain repetitive DNA. The renaturation rate constant of DNA III (58·8 mol s/l) is in very good agreement with the value of renaturation rate constant of the intermediate fraction (60·6 mol s/l) (ref.[7]). It follows from this finding that the two fractions are most likely identical. The values of the renaturation rate constant serve for the calculation of the size of the repeating sequence[1] which for this DNA is $11 \cdot 10^6$ daltons (ref.[7]. double stranded). The satellite DNA's I and II, which renature faster than DNA III, are components of the fast fraction reported by Britten and Smith[7] and the length of their repeating sequence is $1{\cdot}5 \cdot 10^6$ daltons (double stranded) at the most.

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