FRACTIONATION OF NONSATELLITE REPETITIVE DNA FROM CALF THYMUS

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About 25% of rapidly renaturing DNA was isolated using hydroxyapatite chromatography of calf-thymus DNA fraction containing no satellite components. This DNA was divided into six fractions differing in thermal stability, rate of renaturation and partly also in the CsCl density gradient centrifugation profile. Following the results of kinetic measurements a great part (about 80%) of this DNA can be identified as so-called intermediate DNA. In the renatured state all fractions are characterized by an incomplete matching of base pairs. After correction of the renaturation rate constants for the mismatching of base pairs, the maximum complexity of the intermediate DNA is shown to be within the range of 450 to 3300 base pairs. When compared with values found for satellite DNAs these results indicate that the two DNA types are not significantly different in their basic sequence complexities. However, considerable differencies exist in the degree of divergence of this basic sequence.

In the past few years it has been shown[1-6] that a considerable part of eukaryotic DNA consists of repetitive DNA. The amount of the repetitive fraction and the size of the repeated sequence differ even with DNA's of closely related species[7]. It is not known at present what purpose the repeated sequences might serve or what is their origin. According to Southern[8], families of repetitive sequences were formed from originally very simple short sequences by a divergence process caused by spontaneous mutations. It does not appear likely that sequences formed by this mechanism would possess a certain specific genetic function[9]. On the other hand, Britten and Davidson[10] ascribe the repetitive sequences a major role in the control of transcription.

Britten and Smith[11] analyzed the fractions obtained from calf-thymus DNA on the basis of different renaturation rates. They divided them into four types designated as fast, intermediate, slow and unique DNA. The slow and unique fractions contain nonrepeated sequences while the fast and intermediate fractions can be classified as repetitive DNA's. We showed recently[4] that the satellite fractions of calf-thymus DNA rich in guanine and cytosine belong to the fast fraction and, in view of their total amount in DNA (some 10%) they probably represent its major part. We showed further[12] that the isolated repetitive nonsatellite calf thymus DNA can be classified as intermediate as a whole. It would be interesting to explore whether, from the point of view of kinetic complexity, there exists a gradual transition from the fast to the intermediate DNA or whether the two types are distinctly different.

The present communication deals with further fractionation of renatured nonsatellite calf-thymus DNA based on different thermal stabilities of the fractions and shows that the corrected complexities of the basic sequence of satellite and intermediate fractions do not significantly differ.

EXPERIMENTAL

Isolation of DNA. DNA was isolated from calf thymus according to Kay and coworkers[13]. The concentration of DNA was estimated from the absorbance value on the assumption of $A_{cm}^{1\%} = 200$.

Fractionation of DNA *on methylalbumin* (MAK) columns. DNA was fractionated on columns of kieselguhr with adsorbed methylated serumalbumin (MAK) according to Mandell and Hershey[14] using a 70 × 130 mm column. The procedure is described in detail elsewhere[4].

Isolation of rapidly renaturing DNA *on hydroxyapatite.* DNA (about 10 mg) sonicated to a molecular weight of about 500 000 daltons was heated to 100°C for 15 min in 0·03M phosphate ($NaH_2PO_4 + Na_2HPO_4$, 1 : 1). The concentration of phosphate was then adjusted to 0·09M and DNA was annealed at 70°C under conditions which give the value of $c_0\tau$ (c_0 is molar concentration, τ time in seconds) equal to 0·27 mol s liter^{-1}, or 2·7 mol s liter^{-1}. After adjusting the concentration of phosphate to 0·12M, DNA was applied to a hydroxyapatite column (15 × 100 mm). Elution was done at 70°C, at first with 0·12M phosphate (denatured DNA is eluted), and further with a gradient of phosphate from 0·12M to 0·4M (renatured DNA is eluted).

Thermal chromatography of DNA was done according to Miyazawa and Thomas[14]. DNA (about 1·5 mg) dissolved in 0·12M phosphate was applied to a hydroxyapatite column (10 × × 20 mm). Temperature was gradually increased in small intervals and at each temperature the DNA was eluted with 14·5 ml 0·12M phosphate. Temperature was measured with a thermocouple placed in the column mantle.

The kinetics of renaturation was measured spectrophotometrically using a Hilger Uvispek spectrophotometer in 1 cm cells at 260 nm. The method has been described in detail elsewhere[4]. The values of the rate constants were calculated[1] from the value of $c_0\tau_{1/2}$ which was read from the plot of the degree of renaturation *vs* log $c_0\tau$.

The melting curves were measured with the Hilger Uvispek spectrophotometer in 1 cm cells at 260 nm. In most cases the curves were recorded only in the range from the annealing temperature to 100°C to establish the heat stability of the double-helical part of the product formed by annealing. These curves were recorded immediately after the kinetic measurements.

Equilibrium centrifugation in a CsCl *density gradient* was done in a Spinco E ultracentrifuge in cells with 12 mm optical path at 44 770 r.p.m. for 20—24 h. *Staphylococcus aureus* DNA of a density of 1·693 g/cm^3 was used as a density marker. The density was calculated according to Schildkraut and coworkers[15].

The sedimentation coefficients were determined in the Spinco E ultracentrifuge using cells with 30 mm optical path. The DNA was dissolved in an alkaline medium consisting of 0·9M-NaCl + + 0·1M-NaOH. The molecular weight of single-stranded DNA was calculated according to Studier[16].

RESULTS

Isolation and Fractionation of Nonsatellite Repetitive DNA

For isolating the nonsatellite repetitive DNA renaturing up to $c_0\tau = 0{\cdot}27$ mol s liter^{-1} we used a fraction from the descending part of the MAK fractionation elution curve containing no satellite DNA's[12]. This fraction yielded some 20% of the repetitive

component by hydroxyapatite chromatography after previous denaturation and annealing. The repetitive DNA was divided into two parts: fraction A eluted from hydroxyapatite with 0·12 – 0·14M phosphate (some 1/3) and fraction B eluted with 0·14M and higher concentrations of phosphate (some 2/3). Fraction A was used for further measurements as such, fraction B was further fractionated by thermal chromatography on hydroxyapatite. The elution profile of thermal chromatography (Fig. 1) is relatively broad in comparison with that of native calf-thymus DNA and, in contrast with the latter, it displays two peaks. The T_m of fraction B determined by thermal chromatography in 0·12M phosphate is 79·8°C which is by 7·1°C lower than the T_m of the native calf-thymus DNA determined in the same way. For further measurements, fraction B was divided into four approximately identical parts: fraction B I, eluted at temperatures below 74·1°C, B II eluted between 74·1 and 79·7°C, B III eluted from 79·7 to 84·1°C, and B IV eluted between 84·1 and 94·2°C.

To isolate the nonsatellite repetitive DNA renaturing to $c_0\tau = 2{\cdot}7$ mol s liter^{-1} we used unfractionated sonicated calf DNA from which the fraction renaturing to $c_0\tau = 0{\cdot}27$ mol s liter^{-1} had already been removed. A total of 5·1% of this rapidly renaturing fraction was obtained and it was designated as C.

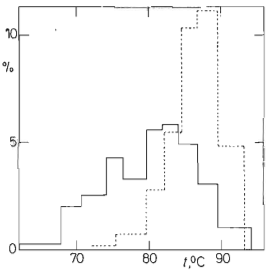

FIG. 1

Thermal Chromatography of Fraction B in 0·12M Phosphate

Full line, fraction B; broken line, native sonicated calf-thymus DNA. % Fraction of DNA eluted from hydroxyapatite, *t* temperature of elution from the column.

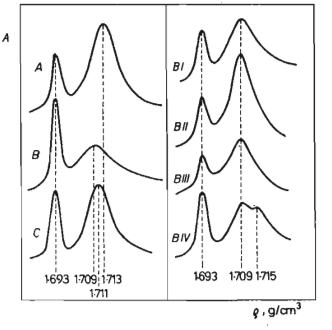

FIG. 2

Equilibrium Centrifugation in a CsCl Gradient of Calf-Thymus Repetitive DNA Fractions

Properties of the Fractions of Repetitive Nonsatellite DNA

Fig. 2 shows the CsCl density gradient centrifugation profiles of all fractions in the renatured state. The fractions display different densities: A 1·713 g/cm^3, B 1·709 g/cm^3 and C 1·711 g/cm^3. Fractionation of fraction B by thermal chromatography was not much reflected in the CsCl density gradient centrifugation profiles. Only fraction B IV is different in containing, besides the 1·709 g/cm^3 band, another band at 1·715 g/cm^3.

Table I shows the values of the sedimentation coefficients in the alkaline medium, of molecular weights and values of T_m. The range of the molecular weights of fractions is rather narrow ($1·2-3·3 . 10^5$ daltons). The values of T_m were determined as the temperature of 50% rise of absorbance in the range from the temperature of renaturation (60°C or 70°C) to 100°C. They are thought to characterize the heat stability of the double-helical part of the annealing product under the given annealing conditions. Changes in absorbance occurring at lower temperatures were not measured. However, these changes do not appear to be important for assessing the degree of matching of base pairs of renaturated DNA since at these lower temperatures mainly the structures formed by nonspecific matching of bases will be melted. The thermal stability of fractions rises in the sequence: A, B I, B II, B III and B IV, *i.e.* in the sequence in which the fractions were eluted from hydroxyapatite either by increasing the concentration of phosphate or by rising temperature. The hyperchromic effect is always lower than with native DNA and increases also in the same order. The thermal stability and the hyperchromic effect of fraction C are within the limits of error the same as with fraction A. With the exception of fraction B IV, where both

TABLE I

Some Physico-Chemical Parameters of Calf-Thymus Repetitive DNA Fractions

The values of T_m were determined from the increase of absorbance measured (*a*) in 2× SSC between 60 and 100°C, (*b*) in 2 × SSC between 70 and 100°C, (*c*) in SSC between 70 and 100°C.

Fraction	$s^{pH13}_{20,w}$	$M . 10^5$ (single strand)	T_m, °C		
			a	*b*	*c*
A	8·5	3·3	73·8	76·4	75·7
B	7·0	2·0		87·8	83·5
B I	6·2	1·6	73·6	77·1	76·2
B II	5·9	1·3	78·4	81·9	79·2
B III	5·7	1·2		87·9	83·0
B IV	7·4	2·3	91·6	91·2	88·1
C	7·5	2·4	73·7		

T_m values are the same within limits of experimental error, the T_m measured in the interval from 60 to 100°C is lower than that measured in the range from 70 to 100°C. This points to the low stability of the part of product obtained by annealing at 60°C.

The kinetics of renaturation of the fractions obtained was measured under different conditions of temperature and ionic strength. Fig. 3 shows the renaturation kinetics of fractions B I, B II, B III and B IV in 2 × SSC (SSC, standard saline citrate = 0·15M-NaCl + 0·015M trisodium citrate) at 70°C. Fractions A and C were not measured under these conditions but results obtained in 2 × SSC at 60°C indicate that their renaturation proceeds in analogy to the renaturation of fraction B I with a little different rate constant (Table II). According to the course of renaturation shown in Fig. 3, the fractions can be divided into two groups. The first group includes fractions B I, B II and A and C. The decrease of absorbance during annealing could be recorded here under suitable experimental conditions almost from the very beginning. This excludes the presence of very rapidly renaturing molecules. The second group includes fractions B III and B IV. The initial drop of absorbance could not be recorded even under low ionic strength when usually a considerable slowing down of renaturation is observed. This suggests the presence of a fully reversible DNA or of DNA renaturing much faster than satellite DNAs the renaturation of which readily measurable in 0·25 SSC (ref.[4]). The B IV sample was measured even in 0·1 SSC. Although during the annealing at renaturation temperature no drop of absorbance was observed, even here a pronounced hyperchromic effect was found, indicating the presence of double-helical DNA. On the basis of kinetic measurements, the amount of this DNA with unmeasurably fast renaturation rate, was assessed

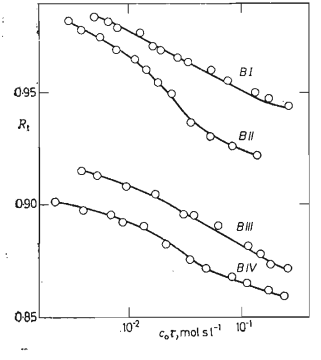

FIG. 3

Kinetics of Renaturation of Calf-Thymus Repetitive DNA Measured in 2× SSC at 70°C

R_t, relative absorbance.

to be 50% in both fractions. We proceeded from this estimation when calculating the renaturation rate constants for the kinetically measurable fraction contained in DNA B III and DNA B IV. To calculate the renaturation rate constant it is also necessary to know the value of the absorbance of renaturing DNA at zero time. This value was usually[4,12] taken to be identical with the absorbance value of the given sample in a completely denaturated state. When working with samples containing besides the measured DNA also DNA with unmeasurably fast renaturation rate a different procedure must be used, since the absorbance value at zero time is to be obtained only for the component participating in the kinetically examined reaction. For the kinetically measurable fraction of B III and B IV this value was obtained by extrapolating the renaturation curves measured in low ionic strength media to zero time. It is apparent that such an extrapolation affects unfavourably the accuracy of calculation and that, therefore, the renaturation rate constants of fractions B III and B IV are determined with a lower accuracy.

Table II shows the values of renaturation rate constants. Under comparable conditions the renaturation rates increase in the order A, BI, B II, B III and B IV. The renaturation rate of fraction C does not differ much from that of fractions A and B I. The temperature dependence is more pronounced in fractions with a lower rate constant. In these cases the annealing temperature of 70°C is too high and hence rather far from the temperature optimum. With fractions with a higher renaturation rate the difference in the rate constants at 60°C and 70°C is relatively small. Dependence on ionic strength was examined only in fraction B IV. It is remarkable that

TABLE II

Renaturation Rate Constants of Calf-Thymus Repetitive DNA Fractions

The rate constants were determined from kinetic data obtained (*a*) in 2 × SSC at 70°C (*b*) in 2 × SSC at 60°C, (*c*) in SSC at the temperature shown in the footnote. All the rate constants were expressed[17] per molecular weight of a single strand of $1{\cdot}7 \cdot 10^5$ daltons (500 nucleotides).

Fraction	k_2, l mol^{-1} s^{-1}		
	a	*b*	*c*
A		49	17[a]
B I	37	57	
B II	75	82	62[b]
B III	82		
B IV	155	181	161[b]
C		55	

[a] At 70°C, [b] at 60°C.

there is relatively little dependence of the renaturation rate constant on the ionic strength, much less than one would expect[4,11,17].

DISCUSSION

The results of our previous work[12] indicate that calf-thymus nonsatellite repetitive DNA is a heterogeneous mixture from the viewpoint of renaturation kinetics. This finding is supported by the fact that chromatography on hydroxyapatite yields fractions differing in their physico-chemical properties.

Repetitive DNA characterized in this work includes a greater part of calf-thymus DNA than the DNA III studied in previous work[12]. An attempt to assess the composition of nonsatellite repetitive DNA from its physico-chemical properties led during the isolation of DNA III to the application of conditions making it possible to obtain from the renatured DNA only the fraction exhibiting proper matching of base pairs. In this way, only about 50% of the total amount of calf-thymus repetitive DNA was obtained. Hence for further work we used the procedure[11] employed by most authors, taking as renatured DNA all the DNA eluted from hydroxyapatite at phosphate molarities greater than 0·12M. Besides fractions A and B isolated after annealing to $c_0\tau = 0{\cdot}27$ mol s liter^{-1}, fraction C was isolated after annealing of the remainder to $c_0\tau = 2{\cdot}7$ mol s liter^{-1}. DNA III described previously[12] corresponds to fraction B purified by further hydroxyapatite fractionation. The yield of calf-thymus nonsatellite DNA obtained under the present conditions represents 25% of the total amount of calf-thymus DNA. If the 10% of satellite DNA is added one obtains 35% of repetitive DNA which approaches the value of 40% reported by Britten and Smith[11].

The obtained fractions of repetitive DNA differ mainly in their thermal stability, in the magnitude of the hyperchromic effect and in the rate of renaturation, smaller differences being in the CsCl density gradient centrifugations profiles. The thermal stability of DNA may be affected by its molecular weight, composition and secondary structure. In this case it is possible to eliminate the effect of molecular weight since the differences in the molecular weights of fractions are very small. Since all the fractions were characterized in the renatured state, the differences in thermal stability must be due mainly to different degree of matching of base pairs. This is in accordance with the low values of hyperchromic effects and the low values of T_m of some of the fractions. The fraction B IV is an exception, its high thermal stability being partly due to the presence of molecules with a higher content of G + C. The sedimentation in the CsCl density gradient indicates that fraction B IV contains some 40% DNA with density of 1·715 g/cm^3 after renaturation. The remaining 60% have a density identical with that of B I, B II and B III. Since fraction B IV shows the highest T_m, the higher density of DNA in the band at 1·715 g/cm^3 cannot be due to incomplete renaturation but apparently indicates the presence of DNA with a higher content of G + C. With fractions B I, B II and B III, no great differences in composition are

assumed since their profiles in the CsCl density gradient are identical. Small differences cannot be eliminated, however, since the density of renatured DNA depends on both the composition and the matching of base pairs so that their effects can cancel each other. For this reason it is difficult to decide whether the higher density of fractions A and C is caused merely by the less perfect matching of base pairs or whether it involves also the higher content of G + C.

Kinetic measurements showed that the whole nonsatellite rapidly renaturing DNA contains three different types of DNA. Firstly, fractions B III and B IV contain a very rapidly, probably reversibly renaturing DNA which was found in a similar amount (some 3% of the total amount of DNA) in calf-thymus DNA by Britten and Smith[11]. Its properties and origin have not been further investigated but it appears that we are dealing here with a fraction analogous to the so-called cross--linked DNA found in samples of bacterial DNA and that we might be thus dealing with an artifact caused by the isolation procedure[18]. Secondly, fraction B IV was also found to contain a DNA with a higher density (1·715 g/cm^3 in renatured state), probably identical with one of the calf-thymus satellite DNA's[19].

The main fraction of nonsatellite, rapidly renaturing, DNA (about 80%, *i.e.* about 20% of the total calf-thymus DNA) includes fractions A, B I, B II, C, an irreversible part of fraction B III and possibly a small part of fraction B IV.

Table II indicates that the renaturation rate constants of all the fractions with the exception of B IV differ at most by a factor of 2 and that from the point of view of renaturation rate we are dealing with a single type of DNA which can be grouped under the category of intermediate DNA.

The calf-thymus intermediate DNA was investigated by Britten and Smith[11] who, by comparison of its renaturation rate with that of bacterial or bacteriophage DNA's, deduced its complexity to be about 17000 nucleotide pairs. Our kinetic results on DNA III which is a part of calf-thymus intermediate DNA with better base matching indicate a value about one-half of that. Both these values are relatively high when compared with values found for satellite[4] DNA. Fractions that can be identified as intermediate DNA are all characterized in the renatured state by an incomplete matching of base pairs which indicates that the reacting repetitive sequences are not fully identical. The fact that during annealing, molecules may react which are not complementary over their entire chain length may also affect the rate of renaturation. This problem was taken up in detail by Southern[20] who derived quantitative relationship for the correction of the renaturation rate constant for the degree of mismatching on the assumption that all the sequences were formed by random alteration of one short basic sequence. The size of this basic sequence can be assessed from the corrected renaturation rate constant. The validity of this relationship derived by Southern was confirmed for mouse satellite[21] DNA where the above assumption can be considered as justified. In the case of calf-thymus intermediate DNA the situation is more complex. The results of measurement of thermal stability suggest that the

degree of base matching in different fractions of DNA is different but it cannot be decided whether the different fractions were formed by divergence of a single or of different basic sequences. Nevertheless, we attempted to apply the correction for mismatching to the present results. For the calculation we used kinetic data of fractions A, B I and B II obtained in 2 × SSC at a renaturation temperature of 60°C and the calculation was done on the assumption that the fractions have a composition identical with that of unfractionated calf-thymus[12] DNA. The percentage of mismatching was calculated[22] from the difference between T_m of native DNA of the same composition and T_m of the given renatured fraction and the data were plotted according to Southern[20]. Extrapolation to zero value of mismatching can be done in two ways: either by drawing the best line through all the experimental points, or separately for each point using the slope according to Sutton[21]. If the present fraction represented a single sequence diverged to different extents, both procedures should give the same result. In the opposite case, if we were dealing with different families of sequences differing in their degree of divergence it would be probably more correct to use the correction separately for each fraction. In view of the relatively small number of points and the large experimental error it cannot be decided which of the alternatives is preferable. Application of the first correction procedure leads to a complexity of 3300 base pairs, that of the other to complexities of about 450–900 base pairs. These values can be taken in both cases for maximal since any heterogeneity of the sample or the presence of remainders of the nonrepetitive sequence would decrease the effective concentration of the renaturing component which is reflected in an apparent decrease of the rate constant and hence in an increase of the complexity obtained therefrom. The corrected complexity values can be compared with the complexity of calf-thymus satellite DNA. Our kinetic measurements lead to a complexity of about 2300 base pairs[4] but the value must be corrected for the heterogeneity of the sample (since a mixture of satellites was examined) and for the degree of mismatching. Preliminary results indicate that even a combination of the two corrections will not yield a value of complexity of less than about 400–500 base pairs. This comparison shows that the basic sequence complexities of the two types of DNA, satellite and intermediate, are not significantly different. However, differences exist in the degree of divergence of these basic sequences. This conclusion can be taken as support for Southern's theory[8] of the origin of repetitive DNA by gradual divergence of a simple basic sequence.

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