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Identification and separation of components of calf thymus DNA using a CsCl-netropsin density gradient

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ABSTRACT

Calf thymus DNA containing satellite components of various densities was used as a model to study the effect of netropsin on the density of DNA in a CsCl gradient. The binding of netropsin resulted in a decrease in density which depended upon the quantity of netropsin added and on the average composition of the DNA. Differences in density of DNA components were higher in CsCl - netropsin gradients than in simple CsCl gradients. By use of netropsin a main band and four satellite bands could be differentiated in calf thymus DNA. Satellite DNA's were isolated using preparative CsCl - netropsin gradient centrifugation and were characterised by density and homogeneity in native and in reassociated state. Two of the satellite components, with densities of 1.722 and 1.714 g/cm^3, are probably of homogenous sequence, the other two components of densities 1.709 and 1.705 g/cm^3 appear to be heterogeneous.

INTRODUCTION

Separation of various components of DNA in CsCl or Cs_2SO_4 density gradients can be improved by the use of some substances which bind to DNA in a medium of high ionic strength, and in this manner cause density changes. For this purpose one can use ions of heavy metals (Ag^+, Hg^{2+})[1,2] and a number of antibiotics[3,4] including netropsin[4]. The binding of netropsin to DNA has been intensively studied[5 - 10]. Zimmer et al.[8] have reported that the binding is very strong and exists partially even in media of high ionic strength (7.2 M CsCl, 5 M LiCl). Netropsin is preferentially bound to regions of the DNA molecule rich in (A+T)[5-7,9-10]. This has been used for the isolation of (A+T)-rich satellite components of DNA from Drosophila melanogaster[4].

Studies of DNA from calf thymus using CsCl density gradient

centrifugation have shown that this DNA contains two satellite components of densities 1.714 and 1.721 g/cm^3 [11,12]. Using fractionation on MAK columns, in combination with partial denaturation , a further component has been isolated of density about 1.707 g/cm^3 (ref.13). The above methods were, however, unable to provide homogeneous preparations of satellite components which indicated the presence of still further components[13]. This assumption was corroborated by the work of Filipski et al.[14]. Using Ag^+ - Cs_2SO_4 gradient centrifugation six satellite components were isolated in calf thymus DNA of densities 1.704, 1.705, 1.709, 1.710, 1.714 and 1.723 g/cm^3 (ref.14). The two heaviest satellite components have been studied in detail and were shown to belong to the class of rapidly reassociating simple sequence DNA [15-18].

The present work took advantage of the complexity of calf thymus DNA to study factors influencing changes in DNA density produced by netropsin. Separation of calf thymus DNA components in a CsCl - netropsin gradient was compared with that achieved by other methods, and the CsCl-netropsin gradient centrifugation was used to isolate satellite components.

MATERIALS AND METHODS

Samples of DNA

Calf thymus DNA was isolated according to Kay et al.[19]. The concentration of DNA was determined by measuring absorbance at 260 nm using $A^{1\%}_{1cm} = 200$.

Netropsin

Netropsin sulphate and netropsin hydrochloride preparations were kindly provided by Dr. CH. Zimmer, Institute for Microbiology and Experimental Therapy, Jena.

Fractionation of DNA on MAK columns

DNA was fractionated by the method of Mandell and Hershey[20]. The columns were 70x130 mm with a capacity for 25-30 mg DNA. The details of this separation are described elsewhere[11,13]. DNA from the MAK column was pooled into three fractions labelled A, B and C in the order in which it was eluted from the column.

Refractionation with the same system of these fractions one or two times gave further subfractions enriched with various satellite components. These latter subfractions were labelled AA, AB, AAA, ABA, BB etc.

Analytical centrifugation in a CsCl density gradient

Centrifugation was carried out with an ultracentrifuge Spinco Model E using UV absorption optics, at 44 770 rpm in cells with an optical pathlength of 12 mm. The centrifugation time was 20-24 hrs. DNA from Pseudomonas aeruginosa - N^{15} of density 1.742 g/cm^3 (based upon the density of E.coli DNA equal to 1.710 g/cm^3) was added as density marker. The density of DNA samples was calculated according to Schildkraut et al.[21].

Analytical centrifugation in a CsCl - netropsin gradient

Preparation of DNA samples for centrifugation started with a solution of DNA (5 - 10 µg/ml) in 0.01 M tris pH 8.4, to which was added a solution of netropsin (200 µg/ml in 0.01 M tris pH 8.4 + 0.005 M EDTA) in various ratios in order to cover the required ratios of netropsin to DNA, and solid CsCl up to a final density of 1.640 - 1.700 g/cm^3. The density of the solution was calculated from the refractive index at 25°C, measured with a Zeiss refractometer. Centrifugation was carried out in an ultracentrifuge Spinco Model E in 12 mm cells at 44 770 rpm for 20 - 24 hrs. The density of DNA samples was determined from the initial density of the CsCl solution and the limiting isoconcentration distance[22]. All density values were related to the density of E.coli DNA set at 1.710 g/cm^3.

Preparative centrifugation in a CsCl - netropsin gradient

Samples of DNA for preparative centrifugation were prepared in the same manner as for analytical centrifugation. Maximum concentrations of DNA were about 40 µg/ml. Centrifugation was carried out on a preparative ultracentrifuge Spinco Model L with an angle rotor 50. Polyallomer tubes were filled with 4.65 ml of DNA solution which was covered with liquid paraffin, the amount of DNA per tube varying from 25 to 200 µg. Centrifugation time was 44 - 48 hrs, at 44 000 rpm. After the end of centrifugation, the tubes were punctured and the contents were removed from the

bottom of the tube with a peristaltic pump through a 1 mm flow-through cell attached to a Unicam SP 8000 spectrophotometer. Absorbance at 260 nm was recorded. The contents of the tubes were subdivided into 30-40 fractions, and fractions containing DNA were pooled into 3-5 larger fractions after evaluation of the absorbance records. Netropsin was removed by 5-6 extractions with 2-propanol saturated with a saturated solution of CsCl[4].

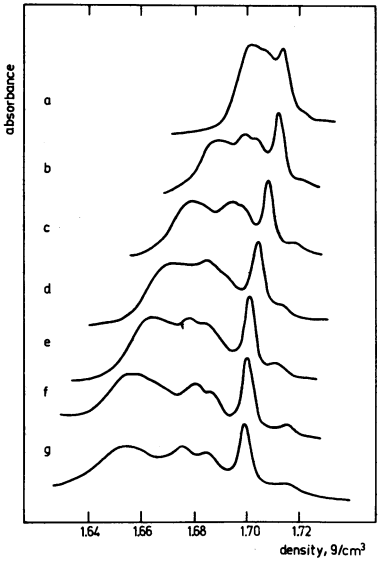

Fig. 1 Analytical centrifugation of MAK fraction A of calf thymus DNA in a CsCl gradient at various ratios of netropsin/DNA (mol netropsin/mol nucleotide phosphate): a - 0; b - 0.05; c - 0.1; d - 0.3; e - 0.6; f - 1.0; g - 1.5

RESULTS

The density of components of calf thymus DNA in a CsCl-netropsin gradient

The binding of netropsin to DNA resulted in a decrease in DNA density in the CsCl gradient. This effect was studied on a fraction of calf thymus DNA enriched with satellite components (fraction A). The pattern of this fraction in CsCl gradient without netropsin (Fig.1a) shows clearly the main band with the two satellite components of densities 1.722 and 1.714 g/cm^3 and a shoulder suggesting a further satellite components of density about 1.707 g/cm^3. Addition of netropsin (Fig.1b-g)

resulted in a decrease in the density of all components of DNA, in relation to the nature of the components and the netropsin/DNA

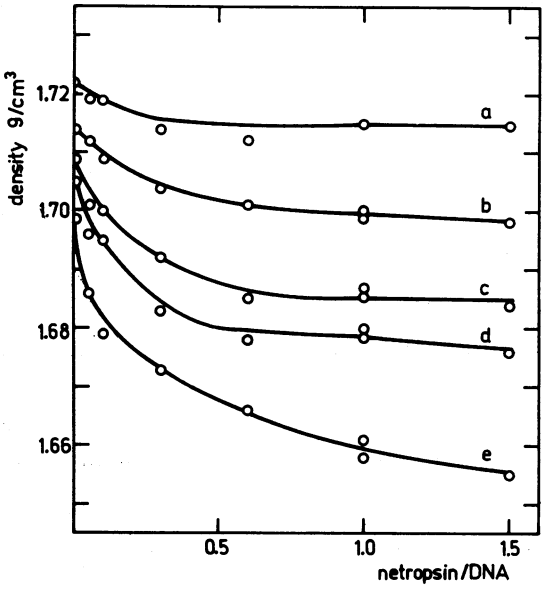

Fig. 2 The density of calf thymus DNA components as a function of the netropsin/DNA ratio. Component with CsCl density: a - 1.722 g/cm^3, b - 1.714 g/cm^3, c - 1.709 g/cm^3, d - 1.705 g/cm^3, e - main DNA band (about 1.700 g/cm^3)

ratio. It would appear that addition of the antibiotic does not change the order of the DNA bands. In other words, the amount of bound netropsin is primarily dependent upon the density of DNA determined in CsCl gradients without netropsin, which means that it is dependent primarily upon the DNA composition. Addition of netropsin results in a marked increase in density differences between the individual DNA components, and thus improves the resolving power of the CsCl gradient (Fig.1). The band of density about 1.707 g/cm^3, which in a simple CsCl gradient can be just barely distinguished from the main band, in fact subdivides into two bands (Fig.1b-g). In the CsCl - netropsin gradient this double band can easily be distinguished from the main band of DNA and from other satellite components at a netropsin/DNA ratio as low as 0.05.

The dependence of density of the individual DNA bands on

the netropsin/DNA ratio is shown in Fig.2. Density values corresponding to zero content of netropsin were measured after isolation of individual DNA components which has been described in detail in one of the following chapters. It is clear from Fig.2 that the density of components decrease with an increasing amount of netropsin reaching a limiting value at a netropsin/DNA ratio of about 0.6 for the satellite components. In the case of the main band DNA the limiting density value is achieved at a ratio of about 1.0 or higher.

Characterisation of calf thymus DNA and its fractions by analytical centrifugation in a CsCl - netropsin gradient

From the above results it follows that the increase in resolving power of CsCl gradients by addition of netropsin improves the use of this method in determining the presence and content of various components in a DNA sample. Fig.3a shows

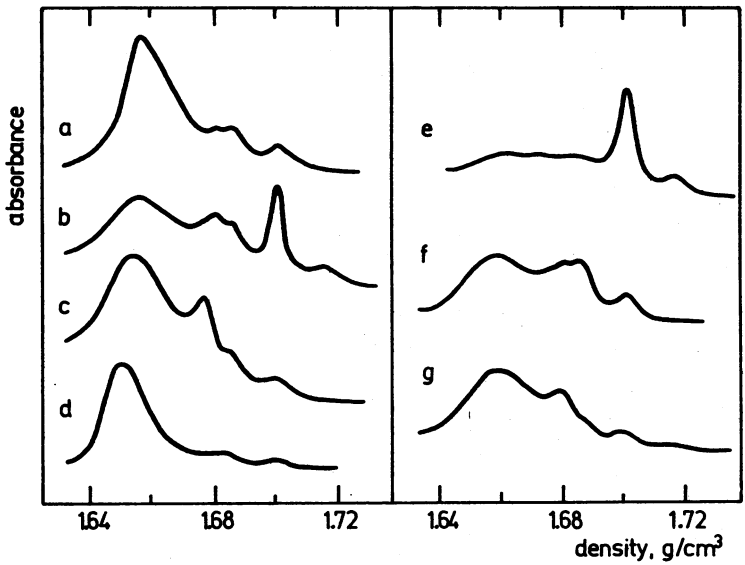

Fig. 3 Analytical centrifugation of calf thymus DNA and its fractions in a CsCl - netropsin gradient with a netropsin/DNA ratio of 1.0. DNA was fractionated as described in Materials and Methods. a - unfractionated sample, b - fraction A, c - fraction B, d - fraction C, e - fraction AA, used for isolation of components with densities 1.722 and 1.714 g/cm^3, f - fraction ABB used for isolation of the component with density 1.709 g/cm^3, g - fraction BB, used for isolation of the component with density 1.705 g/cm^3

that centrifugation of unfractionated calf thymus DNA in a CsCl-netropsin gradient allows differentiation of three satellite bands. In addition to the latter, calf thymus DNA contains a further band which can be seen only in the centrifugation pattern of fraction A, enriched with satellite components (Fig.3b). In all curves of Fig.3, and particularly in Fig.3a, a marked asymmetry of the main DNA band can be seen, which along with its width bears evidence of density heterogeneity. Apparently this is also the reason for a slught variation in the position of the main DNA band in various MAK fractions (Fig.3b-d). The positions of satellite DNA bands are relatively reproducible under constant conditions and do not depend on the ratio of the DNA components present. Reproducibility of the measurement is assured only if freshly prepared netropsin solutions are used. The use of stale solutions does not have an effect on resolution of bands, but results in somewhat higher values of densities, making identification of separate bands more difficult.

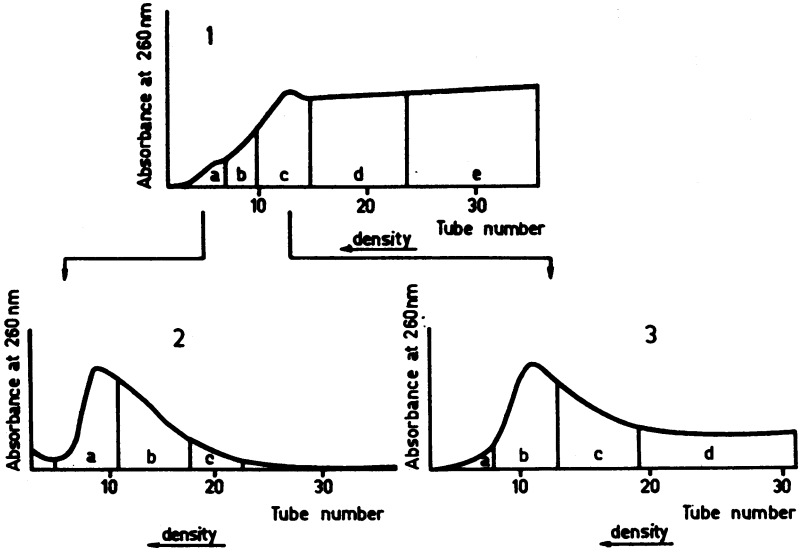

Fig. 4 Scheme of isolation of DNA components with densities 1.722 and 1.714 g/cm^3 by a two-step preparative centrifugation in a CsCl - netropsin gradient, netropsin/DNA = 0.3. 1 - first fractionation step (MAK fraction AA); 2 - second fractionation step (fraction 1a); 3 - second fractionation step (fraction 1c).

Isolation of calf thymus satellite DNA's using preparative CsCl-netropsin density gradient centrifugation

CsCl - netropsin centrifugation was further used as a preparative method for isolating components of DNA corresponding to the identified satellite bands. Since the content of satellite DNA's in unfractionated DNA sample is relatively low (Fig.3a), samples enriched with satellite components on MAK columns were used as initial material. Fig.3 shows the patterns of these fractions in analytical CsCl - netropsin gradients.

The scheme of isolation of the two heaviest satellite components is shown in Fig.4. An isolation procedure consisting in two steps of preparative centrifugation in a CsCl - netropsin gradient was used. In comparison with analytical centrifugation (Fig.3e) resolution of DNA bands in preparative centrifugation was far less complete (Fig.4, curve 1). Despite this, there was effective separation, as shown by the patterns of analytical

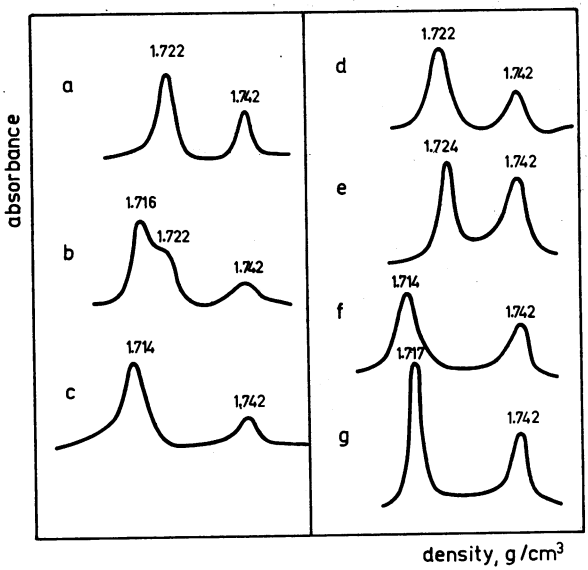

Fig. 5 Analytical centrifugation in a CsCl gradient of fractions obtained by preparative centrifugation in a CsCl - netropsin gradient. For fraction symbols see Fig.4. a - fraction 1a; b - fraction 1b; c - fraction 1c; d - fraction 2a; native, e - fraction 2a; melted and reannealed (C_0t = 0.2 mol s l^{-1}, 0.25 M Na^+); f - fraction 3b, native; g - fraction 3b; melted and reannealed (C_0t = 0.29 mol s l^{-1}, 0.25 M Na^+).

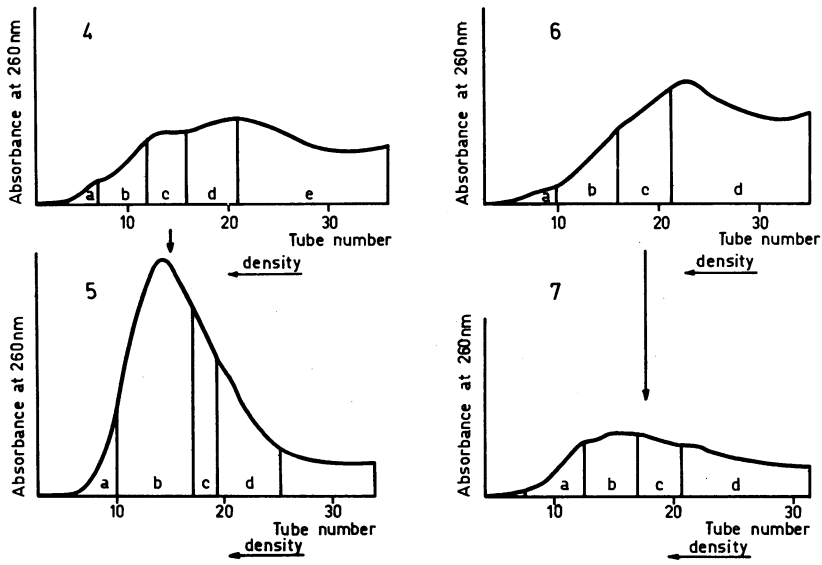

Fig. 6 Scheme of isolation of components with densities 1.709 and 1.705 g/cm^3 by two-step preparative centrifugation in a CsCl-netropsin gradient. 4 - first fractionation step (MAK fraction ABB), netropsin/DNA = 0.3; 5 - second fractionation step (fraction 4c), netropsin/DNA = 0.3; 6 - first fractionation step (MAK fraction BB), netropsin/DNA = 0.3; 7 - second fractionation step (fraction 6c), netropsin/DNA = 0.6.

centrifugation of some selected fractions (Fig.5a-c). The first fractionation step gave sufficiently homogeneous material (Fig.5a-c) so that identification of the two heaviest satellites, of densities 1.714 and 1.722 g/cm^3, was possible. After rebanding (Fig.4, curves 2 and 3) the isolated satellite DNA's vere fully homogeneous in terms of density in CsCl (Fig.5d,f). After denaturation and reassociation a single sharp band was formed in both cases, the density of which was only slightly higher than that in the native state (Fig.5e,g). Both satellite DNA preparations are therefore homogeneous also in terms of reassociation behaviour.

Two-step isolation procedures for the two lighter satellite components are shown in Fig.6. The patterns of analytical centrifugation in CsCl - netropsin gradient of the initial fractions enriched with the corresponding components are in Fig.3f,g. Fractions corresponding to DNA satellite bands (Fig. 7a,e) have densities in CsCl of 1.709 and 1.705 g/cm^3, and

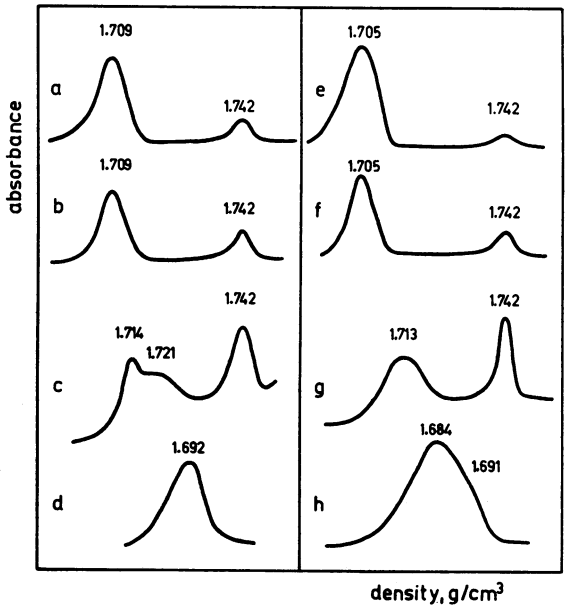

Fig.7 Analytical density gradient centrifugation of fractions obtained by preparative centrifugation in a CsCl - netropsin gradient. Symbols as in Fig.6a-c, e-g - CsCl gradients: a - fraction 4c; b - fraction 5b, native; c - fraction 5b, melted and reannealed (C_0t = 0.28 mol s l^{-1}, 0.25 M Na^+); e - fraction 6c; f - fraction 7b, native; g - fraction 7b, melted and reannealed (C_0t = 0.31 mol s l^{-1}, 0.25 M Na^+). D,h - CsCl - netropsin gradient, netropsin/DNA = 1.0: d - fraction 5b, h - fraction 7b.

after rebanding in a CsCl - netropsin gradient (Fig.7b,f) they would appear to be homogenous in terms of density. Fraction 5b of density 1.709 g/cm^3 was almost homogeneous even in the analytical CsCl - netropsin gradient (Fig.7d) which confirms its identification with the heavier component of the double band. After denaturation and subsequent reassociation, however, this DNA was split into two bands (Fig.7c). The DNA band of density 1.714 g/cm^3 corresponds to a rapidly reassociating component, but that of density 1.721 g/cm^3 obviously did not reassociate under the given conditions. DNA of fraction 5b, forming a single satellite band in a CsCl-netropsin gradient, is therefore composed of at least two DNA components of very similar densities which show marked differences in their reassociation behaviour. The fraction 7b of density 1.705 g/cm^3 can be identified with the lighter component of the double band

but the result from a CsCl - netropsin gradient suggests that an admixture of a heavier component has not been completely removed (Fig.7h). After denaturation and reassociation this DNA forms a single broad band of density 1.713 g/cm^3 (Fig.7g). It would appear that all the DNA of fraction 7b is partially

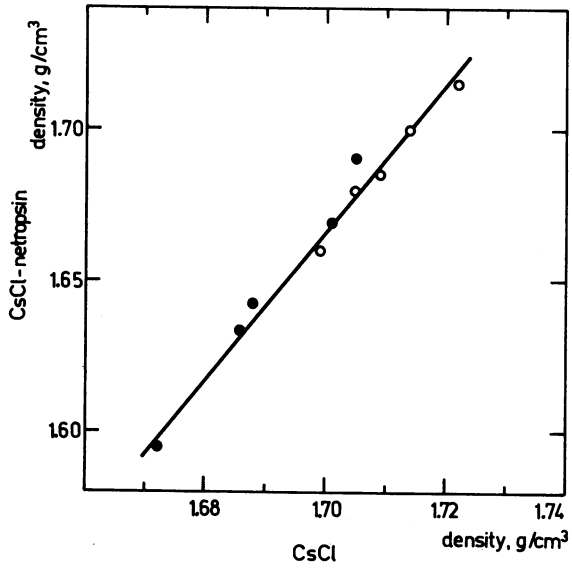

Fig. 8 Relation between density of DNA components in a CsCl - netropsin gradient (netropsin/DNA = 1.0) and density in a simple CsCl gradient. O - calf thymus DNA, ● - Drosophila melanogaster DNA (Peacock et al.[4])

reassociated because this component, unlike that of density 1.709 g/cm^3, shows no high density material after denaturation and reassociation.

DISCUSSION

Binding of netropsin to DNA in a CsCl gradient

Complexes of netropsin with DNA are stable even in a high ionic strength, thus making possible the use of the DNA - netropsin interaction in CsCl[4] and Cs_2SO_4 [10] gradients. A decrease in DNA density in CsCl and Cs_2SO_4 due to netropsin occurs under conditions in which other methods show only very small changes [4,8,9,10]. The method of density gradient centrifugation is therefore a very sensitive one, capable of

demonstrating even very small amounts of bound netropsin.

A decrease in density occured in all studied components of DNA, but to various degrees. It would appear that, unlike the binding of Ag^{+}, Hg^{2+} (ref.14,23,24) or actinomycin[4], the binding of netropsin is mainly influenced by the composition of the DNA sample and does not show a clear sequence specificity. This situation is reviewed in Fig.8, where the densities of homogeneous DNA components in CsCl - netropsin are plotted against their densities in simple CsCl. This figure also includes the results of Peacock et al.[4], who determined the density of (A+T)-rich satellite components of DNA from Drosophila melanogaster in both types of gradient. Densities of (G+C)-rich satellite components represent limiting values. Because of the dependence of density in CsCl on (G+C) content[21] the relationship on Fig.8 is in agreement with the results of Zimmer et all.[5,6,8] and Wartell et al.[10] who found that the greater the (A+T) content, the greater the amount of netropsin bound to DNA. Results obtained with homopolynucleotides[10] show that small effects of sequence specificity cannot be completely excluded.

Luck et al.[9] reported that even under conditions favourable for complex formation, there is binding of at most one molecule of netropsin to six nucleotides. In a CsCl-netropsin gradients fairly high netropsin/DNA ratios were used, usually those leading to limiting density values (0.3-1.0). Despite the fact that no quantitative data on the binding of netropsin under these conditions are available, it would appear very probable that complex formation is highly supressed. Hence the major portion of the netropsin is free in solution and in equilibrium with bound netropsin. This can explain the finding that density values of satellite DNA's are relatively reproducible and do not depend upon the ratio of individual components of DNA in the mixture. This property of CsCl-netropsin gradients simplifies identification of the bands and is an advantage in analytical use of this method.

The use of DNA-netropsin interaction in studies of calf thymus DNA

Calf thymus DNA contains a number of satellite components differing in composition and sequence complexity[11-14,23,25]. The most effective method for their isolation has thus far been centrifugation in Ag^+ - Cs_2SO_4 gradients[14,23,25], which has been used to isolate six satellite components[14] characterised in detail. In a CsCl-netropsin gradient one can differentiate four satellite bands corresponding in density to the principal satellite components. Since the interaction of netropsin with DNA does not show sequence specificity, there is no separation of satellite DNA's with the same or very similar composition but with different sequences. Probably there is also some fractionation of the main band DNA.

Fractions corresponding to DNA satellite bands distingiushable in analytical CsCl-netropsin gradients were isolated using preparative CsCl-netropsin gradient centrifugation. Density of fractions homogeneous in CsCl gradient without netropsin were 1.705, 1.709, 1.714 and 1.722 g/cm^3. The two heavier fractions can be identified with satellite components isolated from calf thymus DNA using CsCl density gradient centrifugation[12,15], MAK fractionation combined with partial denaturation[11,13] and Ag^+ - Cs_2SO_4 gradient centrifugation[14,23,25]. The present isolation method would seem to be of advantages as compared with the other methods in providing very pure products because of easy separation of the two heavy satellites from each other and from other components. According to reassociation behaviour these DNA components consist of homogeneous repeating sequences with kinetic complexities of the order of hundreds of nucleotides[16,18].

Satellite DNA of density 1.709 g/cm^3 consisted of two components, one of which could be classified as a rapidly reassociating DNA, while the other showed little reassociation under the given conditions. The reassociating part is in all probability identical with that described previously[13,16] as a repetitive portion of 1.707 g/cm^3 satellite DNA isolated by MAK

fractionation in combination with partial denaturation. This is supported by the agreement in densities of both components after denaturation and reannealing and by the results of CsCl-netropsin centrifugation of fractions obtained by the previous isolation method[13] (H.Votavová, unpublished). The non-reassociating component of density 1.709 g/cm^3 has not thus far been isolated in pure state. Both components of this density could not be separated even in Ag^+ - Cs_2SO_4 gradients as shown by the presence of high density DNA after denaturation and reannealing[14].

The satellite component of density 1.705 g/cm^3 formed a single broad band after denaturation and reassociation suggesting the presence of a number of components differing in density in the reassociated state. The native density suggested that this satellite DNA might contain a rapidly reassociating DNA characterised by Yasmineh and Yunis[23] and Filipski et al.[14] as the lightest component in the Ag^+ - Cs_2SO_4 gradient. Preliminary results obtained with this method show, however, that the component of low density in Ag^+ - Cs_2SO_4 is only a minor portion of our 1.705 g/cm^3 satellite DNA. A larger portion may be formed by DNA which was characterised by Filipski et al.[14] as a component with density 1.704 g/cm^3.

As shown above, centrifugation in a CsCl-netropsin gradient can serve as an analytical tool for studying the heterogeneity of DNA and as a preparative method for isolation of some DNA components. This method has some advantages over gradient centrifugation using interaction with heavy metals or with actinomycin. First, there is no change in the relative positions of bands on interaction with netropsin, which allows a simple identification of individual components without previous isolation. Second, in a CsCl-netropsin gradient the densities are insensitive to small changes in experimental conditions (concentration, ratio of components in the mixture). Centrifugation in a CsCl-netropsin gradient, however, does not allow separation of different components of about the same average composition, which is sometimes possible on the basis

of sequence specific interaction with actinomycin or with heavy metals. It would appear that in a general case of an eukaryotic DNA best separation of various components will be achieved by a proper combination of interacting substances in density gradients.

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