ISOLATION AND CHARACTERIZATION OF NATIVE AT-RICH SATELLITE DNA FROM NUCLEI OF THE ORCHID *CYMBIDIUM*

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1. Introduction

The DNA of many plant species can be resolved by centrifugation to equilibrium in neutral CsCl into two fractions, the minor component being called satellite DNA [1–3]. A better separation of native satellite DNA can often be achieved by complexing the DNA with Ag^+ or Hg^{2+} followed by density gradient centrifugation in Cs_2SO_4 [4–6]. We have recently described an unusually large proportion of satellite DNA in the protocorms of the orchid *Cymbidium* [3]. Such protocorms can be induced to differentiate in vitro by appropriate hormone treatments [7,8]. This paper describes the isolation and characterization of the satellite fraction. The satellite isolated by density gradient centrifugation in Ag^+/Cs_2SO_4 has the melting behavior of the low temperature component of total nuclear DNA. Its reassociation kinetics indicates that it consists of two fractions of different base sequence complexity.

2. Materials and methods

2.1. *Cultures*

Virus free *Cymbidium* protocorms were cultured aseptically in liquid medium at 25°C in continuous light without hormone addition [9]. Under these culture conditions, new protocorms are formed and the wet weight of the tissue is doubled in one week. Their nuclei contain large blocks of heterochromatin [10].

2.2. *Isolation of nuclei*

Protocorms were ground in a medium containing 0.5 M sucrose, 0.05 M Tris, 5 mM EDTA, pH 8 (2 mg/l g) according to the method described by Beridze [11]. To separate the nuclei from the cytoplasmic organelles 1 ml of this suspension was layered over 4 ml of 2 M sucrose, 0.05 M Tris, pH 7.8, and centrifuged for 1 h at 33 000 rev/min in the SW 65 Spinco rotor. Nuclei were precipitated at the bottom of the tube.

2.3. *Isolation of DNA*

Nuclear precipitates were suspended in a mixture of 0.05 M Tris, 1 M NaCl, 0.1 M EDTA, pH 7.8, 1% sodium dodecyl sulfate. DNA was purified with chloroform–isoamylalcohol (24:1) extraction and low-speed centrifugation. This step was repeated till the proteinic interphase disappeared. Ethanol precipitation, ribonuclease and pronase digestions, chloroform–isoamylalcohol treatment and further ethanol precipitations completed the procedure [12,13]. Removal of pigment and ribonucleotides from the DNA was assumed by passing the DNA over Sepharose 4-B columns [3].

2.4. *CsCl and Ag^+/Cs_2SO_4 gradient centrifugation*

The buoyant density of DNA samples was determined by CsCl equilibrium centrifugation in accordance with the procedure described by Schildkraut et al. [14].

For preparative and analytical Ag^+/Cs_2SO_4 gradient centrifugation, the pooled DNA fractions obtained after chromatography over Sepharose 4-B (Pharmacia), were dialysed against 0.1 M Na_2SO_4 buffer. The DNA fractions in 0.1 M Na_2SO_4, 0.005 M borate buffer, pH 9, were mixed with Cs_2SO_4 and 1 mM $AgNO_3$ water solution up to an inititial density of 1500 g cm^{-3} and a 0.4 M ratio of Ag^+ to DNA phosphate.

2.5. *Melting and reassociation measurements*

Melting and reassociation kinetics [15] of DNA fractions in 0.12 M phosphate buffer were followed optically in a Gilford 250 recording spectrophotometer. The DNA was sheared by repeated forceful extrusion through a narrow gauge needle, and the samples were overlaid with liquid paraffin to prevent evaporation from the cuvettes. Reassociation was recorded continuously by plotting the decrease of the optical density at 260 nm.

3. Results

Upon analytical ultracentrifugation in neutral CsCl the nuclear DNA forms a light satellite at 1680 g cm^{-3} and a main peak at 1692 g cm^{-3} (fig.1a). Repeated preparative centrifugation of the satellite shoulder results in an enrichment from 15% to about 70% satellite in the DNA, but does not result in a complete separation. When DNA was complexed with Ag^+ ions, a heavy satellite comprising 12 to 15% of the total DNA is completely resolved from the bulk DNA by preparative Cs_2SO_4 centrifugation (fig.2). The Ag^+/Cs_2SO_4 preparative fractionation was reproducible and similar to the UV profile obtained by analytical centrifugation (20 h, 44 000 rpm, 20°C). The two peak fractions from the preparative run were pooled, dialyzed for 3 days to eliminate Ag^+ ions and traces of Cs_2SO_4. The dialyzed fractions form clean single peaks upon centrifugation in neutral CsCl with densities of 1.680 g cm^{-3} for the pure satellite fraction (fig.1c) and 1692 cm^{-3} for the pure main band fraction (fig.1b). The G + C content for the two fractions as determined from their buoyant densities are 32% for the main band and 20% for the satellite DNA. Figure 3a represents the thermal denaturation profile of the total nuclear DNA of *Cymbidium* protocorms. This satellite fraction is found in the DNA of isolated nuclei of Cymbidium species but not in the DNA of isolated chloroplasts.

The two isolated fractions were transferred by dialysis to 0.12 M phosphate buffer for thermal denaturation. Their melting profile is given in fig.3b. The AT-rich satellite fraction melts in a very narrow temperature range between 72 and 78°C with a T_m of 74.5–75°C and a hyperchromicity of 41%. The main band fraction melts between 75 and 95°C with a T_m value of 83.3–83.7°C and a hyperchromicity of 38–40%.

Main band and satellite fractions were renatured separately at temperatures 23°C below their respective Tm values. The renaturation kinetics of the satellite DNA are reproducibly biphasic. They can be approximated by the second order kinetics of two differently

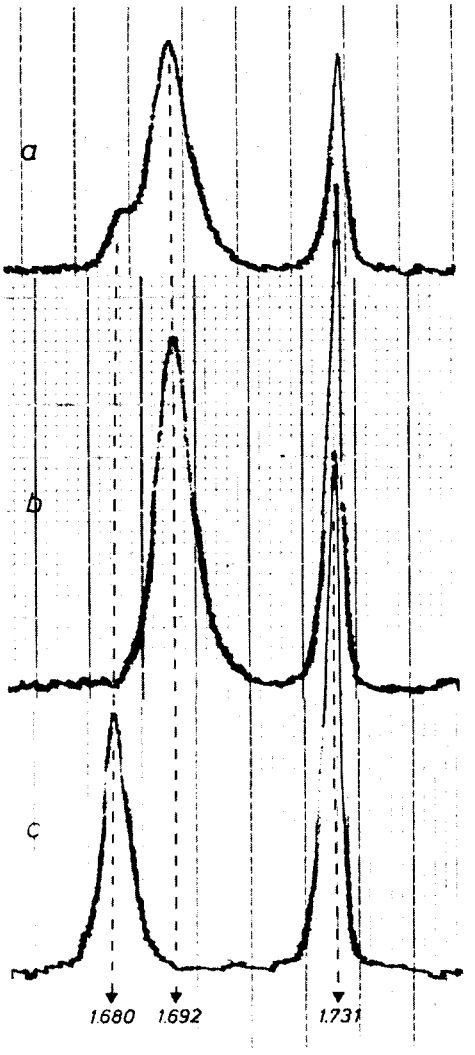

Fig.1. Photoelectric scans of DNA from *Cymbidium* nuclei centrifuged to equilibrium in neutral CsCl. Buoyant densities [14] are calculated using Micrococcus lysodeikticus DNA as a marker (1731 g cm^{-3}). Model E analytical ultracentrifugation at 44 000 rpm, 20°C for 20 h. (a) Nuclear DNA; (b) purified main band DNA and (c) purified AT-satellite DNA.

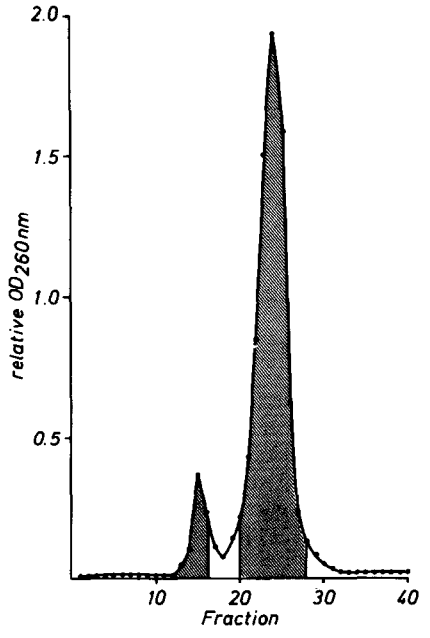

Fig.2

Fig.2. Preparative ultracentrifugation of nuclear DNA (70 μg) in Ag^+/Cs_2SO_4. The initial density of Cs_2SO_4 was 1500 g cm^{-3} with a 0.4 M ratio of Ag^+ to DNA phosphate, in 0.01 M Na_2SO_4 and 0.005 M sodium borate buffer, pH 9. Centrifugation at 35 000 rpm in a SW 65 rotor for 68 h at 20°C. Fractions were collected from the bottom. Samples (0.11 ml) were diluted with 0.1 ml buffer and the absorbance determined in a Zeiss spectrophotometer.

Fig.3. Melting curve of native nuclear DNA from *Cymbidium* protocorms in 0.12 M phosphate buffer. Gilford 250 spectrophotometer with continuous recording. Temperature increase was 0.5°C per min. (a) Isolated nuclear DNA; (b) •—• AT-rich satellite DNA, ○- - -○ main band DNA.

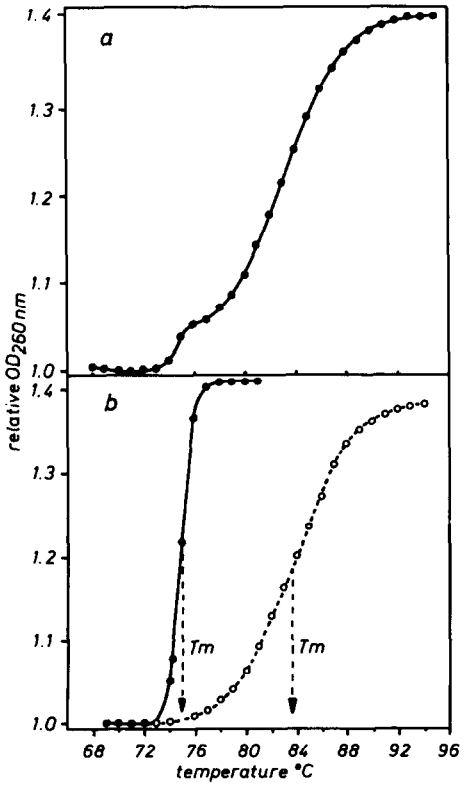

Fig.3

repetitive fractions with the following characteristics: Fraction 1, corresponding to 37.5% of the satellite has a corrected Cot $\frac{1}{2}$ value of 2×10^{-3} and therefore contains a basic sequence 940 nucleotide pairs in length repeated 2.2×10^5 times in each haploid genome. Fraction 2, corresponding to 62.5% of the satellite has a corrected Cot $\frac{1}{2}$ value of 0.3 and therefore contains a basic sequence 1.4×10^5 nucleotide

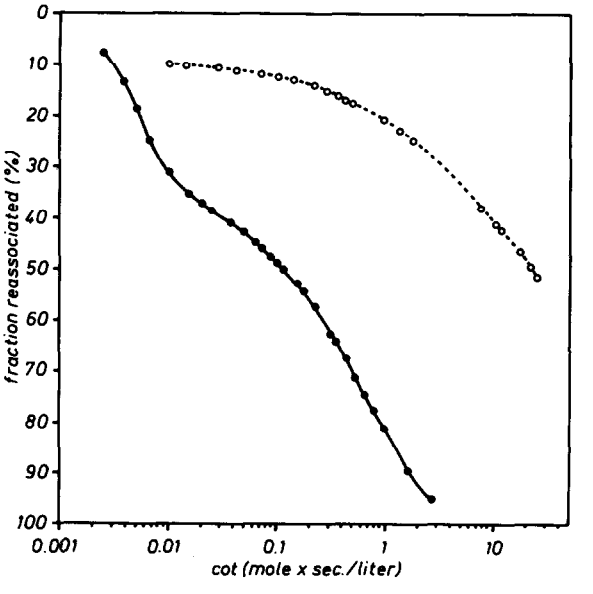

Fig.4. Renaturation of satellite DNA (•—•) and main band (○- - -○) from *Cymbidium* nuclei in 0.12 M phosphate buffer. Satellite DNA (14 μg/ml) at 52°C, main band DNA (38 μg/ml) at 61°C.

pairs in length which is repeated 2600 times in each haploid genome. The haploid genome of the *Cymbidium* strain used here contains 4.05 pg DNA corresponding to a total length of 3.6×10^9 nucleotide pairs.

4. Discussion

These experiments show that the AT-rich satellite observed by thermal denaturation of nuclear DNA is identical to that observed by analytical ultracentrifugation. While the base composition of this fraction is rather uniform when studied by thermal denaturation and ultracentrifugation, DNA renaturation curves reveal that the satellite consists of two fractions with different base sequence complexity.

The muskmelon satellite DNA studied by Bendich and Anderson [12] shows a similar division in two sets of repetitive sequences. These two sets have complexities very similar to the ones formed for *Cymbidium*. However, there is no homology between these satellites. The muskmelon satellite is a GC-rich fraction with different proportions of G + C in the two subsets, while *Cymbidium* contains an AT-rich satellite with a homogeneous base composition. In addition, the *Cymbidium* satellite is restricted to this genus alone and no similar fraction has been found in other orchid species. The evolution of such fractions is therefore a fast event limited to a narrow range of related species. This limitation has to be considered in the discussion of the physiological significance of satellite fractions. In Cymbidium the appearance of the satellite seems to be correlated with a high but variable amount of heterochromatin which is hormone dependent [7,10]. This observation will be the basis for further work on the significance of satellite DNA in plants.

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