The Complexity of Satellite Deoxyribonucleic Acid in a Higher Plant

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Purified satellite DNA from melon (*Cucumis melo*) was shown to contain at least two components from thermal-denaturation and renaturation studies. Two components were separated after partial renaturation, a fast-renaturing fraction similar in complexity to mouse satellite DNA, and one with 6000 times greater complexity. Both components renatured very accurately, indicating a minimum of sequence divergence. Centrifugation of the purified satellite DNA in $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradients resolved two major and several minor fractions. The two major fractions were only slightly enriched for fast- or slow-renaturing sequences.

The presence of nuclear satellite DNA has been observed in many animal (Arrighi *et al.*, 1970) and plant species (Ingle *et al.*, 1973). Although several animal satellite DNA types have been well characterized, much less is known about the physical-chemical properties of those present in plants. Previous studies suggested that plant satellite DNA may be more complex, since no strand separation occurred in alkaline CsCl , and the rate of renaturation, which was slower than that of mouse satellite DNA, indicated the presence of at least two components within the satellite fraction (Ingle *et al.*, 1973). The present paper describes the properties of purified melon satellite DNA, showing that it contains at least two sequences, differing more than 1000-fold in their rates of renaturation.

Materials and Methods

Preparation of satellite DNA

Total DNA was prepared from melon (*Cucumis melo*) fruit or melon seed tissue by homogenization in a detergent medium, deproteinization with chloroform, digestion with ribonuclease and Pronase and recovery by high-speed centrifugation (Wells & Ingle, 1970). The satellite component was separated from the main band after preparative CsCl equilibrium centrifugation (Scott & Ingle, 1973) and recycled through a second preparative gradient to remove any traces of main-band DNA. All DNA preparations were monitored by equilibrium centrifugation in a CsCl gradient in the model E analytical ultracentrifuge (Wells & Ingle, 1970). Samples were also analysed by centrifugation in $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradients in the model E analytical centrifuge (Jensen & Davidson, 1966). The preparative procedure yielded DNA with mol.wt. approx. 10×10^6 (double-stranded). For denaturation and renaturation studies the DNA was sheared by several rapid

passages through a syringe needle to approx. 2×10^6 mol.wt., and both double- and single-stranded sizes of the DNA preparations were estimated as a routine by zone sedimentation in the model E centrifuge (Studier, 1965).

Thermal denaturation

Samples of satellite DNA and bacteriophage T_4 DNA in either 0.5 or $0.25 \times \text{SSC}$ (SSC is 0.15 M-NaCl–0.015 M-sodium citrate, pH 7.2) were dialysed extensively against the appropriate SSC solution and heated in a Unicam SP.800 spectrophotometer containing a four-cell, temperature-controlled block. The temperature of the block was increased $1^\circ\text{C}/4\text{min}$ and the E_{260} of three of the cells was measured every minute. The fourth cell contained a thermistor to monitor the temperature. A computer program was used to correct for the expansion of water to 60°C , and to differentiate the 'melting' curve.

Renaturation

The samples of DNA in the spectrophotometer cuvettes were denatured by boiling for 10 min, and the rate of renaturation was followed optically at 260 nm in the SP.800 spectrophotometer at 65°C . A computer program was used to calculate the reciprocal of the remaining hyperchromicity at each time-interval, and this was plotted against time to determine the second-order rate constant of renaturation (Wetmur & Davidson, 1968). Rate constants were not corrected for differing G+C content, since the base compositions of the satellite components, which do not appear to be directly related to T_m (temperature of 50% denaturation) or buoyant density, have not yet been determined. In any case the relationship between composition and renaturation rate appears uncertain (Gillis *et al.*, 1970). Rates were not correlated to a particular size because of the anomalous relationship between renaturation rate and fragment

Fig. 1. Analytical ultracentrifugation of melon fruit satellite DNA

Total DNA, with main band and satellite at 1.693 and 1.707 g/ml respectively, was prepared from melon fruit tissue (a), and the satellite fraction was isolated by two cycles through preparative CsCl gradients. Native satellite (1.707 g/ml, b) with mol.wt. 7.8×10^6 , was heat-denatured and the density increased to 1.721 g/ml (c). After 6h renaturation at $10 \mu\text{g/ml}$ at 65°C in $1 \times \text{SSC}$ the density was 1.709 g/ml (d). A sample of satellite DNA was sheared to mol.wt. 2.9×10^6 , denatured and re-natured, and two peaks at 1.708 and 1.711 g/ml were resolved (e). Buoyant densities in CsCl gradients were determined relative to *Micrococcus lysodeikticus* DNA at 1.731 g/ml after 20h centrifugation at 44000 rev./min at 25°C . Total sheared melon fruit DNA ($20 \mu\text{g/ml}$) was complexed with Ag^+ , at a molar ratio of Ag^+ to DNA P of 0.3, and centrifuged to equilibrium in Cs_2SO_4 in the absence of marker *M. lysodeikticus* DNA (f).

size with simple sequences (Chilton, 1973). The complexity, or mass of the non-repeating sequence, of a satellite component was determined by direct comparison of the rate constant with that of bacteriophage T₄ DNA, which has a complexity of 110×10^6 daltons (Friedlander, 1970).

Results

Satellite DNA prepared from melon fruit tissue DNA (Fig. 1a) with mol.wt. 7.8×10^6 formed a symmetrical peak in CsCl at 1.707 g/ml (Fig. 1b). The density was increased to 1.721 g/ml on denaturation (Fig. 1c) and it returned to a single peak at 1.709 g/ml after 6 h renaturation at $10 \mu\text{g/ml}$ (Fig. 1d).

Slightly different results were obtained when this experiment was repeated with different satellite DNA preparations. A preparation with mol.wt. 2.9×10^6 (1.5×10^6 single-stranded) behaved in a similar way when analysed in the native and denatured states, but the renatured DNA was resolved into two sharp peaks with densities of 1.711 and 1.708 g/ml (Fig. 1e). Resolution of these two renatured peaks was, however, lost when this satellite DNA was further decreased in size.

Native satellite was resolved into two components when total melon fruit DNA (3×10^6 mol.wt.) was analysed by centrifugation in $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ (Fig. 1f). The two fractions comprised 20% of the total DNA, compared with 19% satellite in CsCl (Fig. 1a). Subsequent analysis of CsCl gradient-purified satellite DNA by centrifugation in $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradients (see Fig. 5) confirmed that the apparently homogeneous CsCl satellite could be resolved into several components.

The thermal denaturation of melon seed satellite

Fig. 2. Thermal denaturation of melon seed satellite DNA

Purified satellite DNA at $19 \mu\text{g/ml}$ (●) and bacteriophage T₄ DNA at $17.5 \mu\text{g/ml}$ (■) were denatured in $0.5 \times \text{SSC}$ with 1°C increase in temperature per 4 min. The E_{260} values were corrected to 60°C .

Fig. 3. Renaturation of total melon seed satellite DNA

Purified satellite DNA at $55.4 \mu\text{g/ml}$ (●) and bacteriophage T₄ DNA at $21 \mu\text{g/ml}$ (■) were denatured at 100°C for 5 min and then renatured in $0.5 \times \text{SSC}$ at 65°C . The reciprocal of the remaining hyperchromicity, $1/(A_t - A_n)$, where $A_n = E_{260}$ of native DNA and $A_t = E_{260}$ of renatured DNA at time t , was plotted against time. Both samples renatured by 37% over these periods.

DNA was clearly biphasic, 62% 'melting' with a T_m of 83.5°C and the remaining 38% 'melting' at 92°C , compared with bacteriophage T₄ DNA, which 'melted' at 82°C in $0.5 \times \text{SSC}$ (Fig. 2). Renaturation of the denatured satellite DNA similarly showed a biphasic second-order rate plot, whereas renaturation of bacteriophage T₄ DNA was linear with time for a comparable degree of renaturation (Fig. 3). The extent of the fast renaturation, about 30%, suggested that it might correspond to the high- T_m component of satellite DNA, and this was confirmed by thermal denaturation of partially renatured (30%) satellite DNA. A comparison of denaturation of native and partially renatured DNA, plotted as the differential of increasing E_{260} against temperature, showed two peaks at 83.5 and 92°C for native DNA, but only a single peak at 91°C for the partially renatured product (Fig. 4a). The increase in hyperchromicity was 29% of that of the native denaturation. After more complete renaturation (70%) the reassciated DNA 'melted' to produce two peaks at 83.5 and 91°C (Fig. 4b). The fast-renaturing component was therefore almost exclusively composed of the high- T_m sequences, the lower- T_m sequences renaturing much more slowly. The stabilities of the reassciated duplexes, as assessed by thermal denaturation, were very similar to those of native DNA, indicating a minimum of base mismatching during renaturation. The difference in T_m for the native and reassciated slow component (ΔT_m) was essentially zero, and that of the fast fraction was 1°C . This may be compared

Fig. 4. Differential denaturation plots of native and re-natured melon seed satellite DNA

Comparison of denaturation of purified melon satellite DNA, denatured and renatured to 30% (a, ○) or to 70% (b, ○), with that of the native DNA (●). (c) Comparison of denaturation of native (●) and renatured mouse satellite DNA (○). The ordinate,

$$R = \frac{A_{i2} - A_{i1}}{A_d - A_n} (t_2 - t_1)$$

where A_{i1} , A_{i2} are the E_{260} values at consecutive temperatures t_2 and t_1 , and A_d , A_n the E_{260} of denatured and native DNA respectively. All E_{260} values are corrected for the expansion of water to 60°C.

with the denaturation of native and renatured mouse satellite DNA, where the ΔT_m was 4°C (Fig. 4c).

The resolution of satellite DNA into two components by denaturation, with T_m values of 83.5 and

Fig. 5. Ag^+/Cs_2SO_4 analysis of purified melon fruit satellite DNA

(a), (b) Samples from one preparation of purified satellite DNA, complexed with Ag^+ at a molar ratio of Ag^+ to DNA P of 0.2. (c), (d), Samples from a different preparation, complexed with Ag^+ at a molar ratio of 0.4. Samples were centrifuged to equilibrium in Cs_2SO_4 at 44000 rev./min, 25°C for 20h.

92°C (Fig. 2), the latter corresponding to the fast component of the biphasic renaturation (Fig. 3), and the presence of two components when melon DNA

Fig. 6. *CsCl*-gradient analysis of fractions from an $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradient

Fractions were isolated from a preparative $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradient analogous to that shown in Fig. 5(b). DNA from peaks 1, 2 and 3 was extensively dialysed against $0.1 \times \text{SSC}$ and centrifuged in *CsCl* gradients containing *Micrococcus lysodeikticus* DNA (1.731 g/ml) at 44000 rev./min , 25°C , for 20 h (a, b, c respectively). Comparable samples were denatured at 100°C for 5 min, renatured in $1 \times \text{SSC}$ at 65°C overnight, and analysed by *CsCl* centrifugation (d, e, f respectively).

was complexed with Ag^+ and centrifuged to equilibrium in a Cs_2SO_4 gradient (Fig. 1f), suggested that these two components in the $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ fractionation may be equivalent to the low- T_m and high- T_m components which appeared to correspond to the

slow- and fast-renaturing sequences respectively. Satellite DNA purified from *CsCl* gradient fractionation was therefore complexed with various concentrations of Ag^+ and centrifuged in Cs_2SO_4 . Two major components, in a ratio of 1:2, were resolved together

with several minor bands (Fig. 5a). The fractionation obtained, however, was not very reproducible. A sample of the same satellite DNA, which had previously been removed from the bulk of the preparation and stored separately, was resolved into three components, in a ratio of 1:1:0.25 (Fig. 5b). The only known difference between these satellite DNA preparations was that the latter sample had a slightly smaller mol.wt., 2.2×10^6 compared with 2.9×10^6 . Purified satellite DNA prepared from similar melon fruits at another time was resolved into two major peaks, completely separated from each other, and a series of minor components (Fig. 5c). This same preparation of satellite DNA, after further centrifugation in a preparative $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradient, yielded a completely different profile (Fig. 5d).

The major components resolved by $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ centrifugation were further characterized. After extensive dialysis DNA from peaks 1 and 2 from Fig. 5(b) equilibrated at a density of 1.707 g/ml in CsCl (Figs. 6a and 6b). DNA from peaks 1 and 2 from Fig. 5(a) similarly had densities of 1.707 and 1.708 g/ml respectively. Peak-3 DNA from Fig. 5(b) contained components with densities of 1.719 and 1.710 g/ml in CsCl (Fig. 6c). The total satellite DNA, when denatured and renatured in SSC overnight, was resolved into two components with densities of 1.711

(57% of total) and 1.707 (43%) g/ml in CsCl, similar to the fractionation shown in Fig. 1(e). Denaturation and renaturation of peak-1 DNA (Fig. 5b) yielded a major component at 1.713 g/ml, with 27% of the DNA at 1.707 g/ml (Fig. 6d), whereas renatured peak-2 DNA showed a single peak at 1.707 g/ml (Fig. 6e). Renatured peak-3 DNA showed a major component at 1.711, with a shoulder at 1.708 g/ml (Fig. 6f). On these criteria peak-2 DNA appeared homogeneous, whereas peak-1 DNA still contained at least two components.

DNA from peaks 1 and 2 (Fig. 5a) was denatured in $0.25 \times \text{SSC}$ to determine whether they were equivalent to the two components resolved by thermal denaturation (Fig. 2). Denaturation of peak-1 DNA showed 80% of the DNA 'melting' with T_m 79.5°C, similar to that of the low-'melting' component of total satellite DNA; the remaining 20% 'melted' at 88°C, similar to the high-'melting' component (Fig. 7). These T_m values of 79.5 and 88°C in $0.25 \times \text{SSC}$ are equivalent to the T_m values of 83.5 and 92°C in $0.5 \times \text{SSC}$ (Fig. 2). Peak-1 DNA was therefore enriched with the low-'melting' component, which was increased from 60 to 80% of the total. This was consistent with the CsCl profile of renatured peak-1 DNA (Fig. 6d). Denaturation of peak-2 DNA showed that 40% of the DNA 'melted' gradually from 75 to 85°C, and 60% 'melted' with T_m 88°C, similar to the high-'melting' component (Fig. 7). Peak-2 DNA was therefore enriched for the high-'melting' component, from 40 to 60% of the total, the remaining sequences being more heterogeneous, and not analogous to the low-'melting' component. Denaturation of DNA from peaks 1, 2 and 3 from Fig. 5(b) showed similar enrichments, with the difference that the initial melting of peak-2 DNA was more comparable with that of the low-'melting' component (T_m 79.5°C) and the more heterogeneous sequences were present in peak-3 DNA. The major fractions obtained from $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ equilibrium centrifugation did not therefore correspond directly to the two components seen in the denaturation and renaturation profiles.

Values for the renaturation rate constants for the fast- and slow-renaturing components of the melon satellite DNA may be estimated from the two slopes of the second-order rate plot for total satellite DNA (Fig. 3), assuming that the fast component corresponds to the high- T_m fraction, comprising 38% of the total hyperchromicity, and that the differences in rate are great enough to make cross-interference negligible. From these assumptions the rate constants were 1460 and $9 \text{ mol}^{-1} \cdot \text{s}^{-1}$ for the fast- and slow-renaturing components respectively. These values were compared with those obtained for the fast- and slow-renaturing components separated by hydroxyapatite chromatography. Total melon seed satellite DNA was renatured to approx. 30% in $0.5 \times \text{SSC}$, the reaction was stopped by dilution into 0.06 M -

Fig. 7. Thermal denaturation of fractions from an $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradient

Fractions were isolated from a preparative $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradient analogous to that shown in Fig. 5(a). The DNA from peaks 1 (∇) and 2 ($\square$) and total satellite DNA ($\bullet$) were dialysed against and denatured in $0.25 \times \text{SSC}$ (see Fig. 2). The results are expressed as a percentage of the total hyperchromicity.

Fig. 8. Rate of renaturation of the separated components of melon seed satellite DNA

Melon fast-renaturing component at $9.6 \mu\text{g/ml}$ (a, ●), mouse satellite DNA at $6.2 \mu\text{g/ml}$ (a, ■), bacteriophage T_4 DNA at $21 \mu\text{g/ml}$ (b) and melon slow-renaturing component at $306 \mu\text{g/ml}$ (c) were denatured at 100°C for 5 min and renatured at 65°C in $0.5\times\text{SSC}$. The reciprocal of remaining hyperchromicity (see Fig. 3) was plotted against time.

sodium phosphate, pH 7.8, and the mixture was fractionated on hydroxyapatite at 65°C . The single-stranded material was washed from the column with 0.06M -phosphate, followed by 0.12M -phosphate, and the renatured double-stranded DNA was then eluted with 0.3M -phosphate. Both fractions were recovered by centrifugation at $100000g$ for 16 h, dissolved in and dialysed against $0.5\times\text{SSC}$, and renatured at 65°C . The rate constant for the separated fast-renaturing component, $434\text{mol}^{-1}\cdot\text{s}^{-1}$, was very similar to that of mouse satellite (650 ; Fig. 8a). The separated slow-renaturing component, which still contained 15% of much-faster-renaturing sequences, renatured at a rate slower than that of bacteriophage T_4 DNA, with rate constants of 0.072 and $1.18\text{mol}^{-1}\cdot\text{s}^{-1}$ respectively (Figs. 8c and 8b). The complexity, or mass of the non-repeating sequence, of the fast-renaturing component was 0.3×10^6 daltons relative to a bacteriophage T_4 DNA complexity of 110×10^6 daltons, only marginally greater than that of mouse satellite DNA at 0.2×10^6 daltons or 300 base pairs. The complexity of the slow-renaturing component, 1800×10^6 daltons, was 6000 times greater than that of the fast component, approaching that of the bacterial genome. Telophase nuclei of melon seeds contain 2pg of DNA (Pearson *et al.*, 1974) or 12×10^{11} daltons, of which 25%, or 3×10^{11} daltons, is satellite DNA. The fast- and slow-renaturing components, 0.3×10^6 and 1800×10^6 daltons, are therefore present as approx. 4×10^6 and 100 copies respectively.

Discussion

The satellite DNA prepared from total tissue con-

tained as a contaminant mitochondrial DNA, which has a similar buoyant density (Wells & Ingle, 1970). Since mitochondrial DNA comprises less than 1% of the total DNA, or less than 5% of the satellite, it would not be detected in the studies described here. With a complexity of approx. 100×10^6 daltons (Wells & Birnstiel, 1969; Kolodner & Tewari, 1972) the mitochondrial DNA would certainly not interfere with the kinetics of the fast-renaturing component, but may be part of the faster-renaturing contamination present in the slow fraction. Contamination of satellite with bacterial DNA was not significant, since both fruit tissue and seeds from melons have been shown to be sterile at the time of opening (Pearson & Ingle, 1972).

The melon satellite DNA differs from many of the animal satellites in containing at least two components. In terms of the biphasic 'melting' curve it resembles *Drosophila melanogaster* satellite (Gall *et al.*, 1971). The fast-renaturing component of the melon satellite appears analogous with the high- T_m fraction (38%) of the denaturation. However, the rate constant for the fast fraction estimated from renaturation of total satellite DNA (Fig. 3) was 3–4-fold greater than that of the isolated fast fraction, and in fact a value similar to the latter, $434\text{mol}^{-1}\cdot\text{s}^{-1}$, and a more linear second-order rate constant plot, was obtained if 45% rather than 38% of the total satellite was assumed to be fast renaturing. The slow-renaturing component contained mainly the lower- T_m sequences, but the fast and the slow components accounted for only 70% of the total satellite, and it is not certain whether additional intermediate and

slower sequences are present within the satellite. The estimation of a second-order rate constant of $9 \text{ mol}^{-1} \cdot \text{s}^{-1}$ for the slow component in the renaturation of total satellite DNA (Fig. 3), compared with 0.07 for the separated slow component (Fig. 5), illustrates the difficulties involved in analysing such biphasic second-order rate constant plots. The very large difference in renaturation rate of the two components means that even the final few per cent of fast-component renaturation very significantly affects the estimation of the renaturation of the slow fraction. Such biphasic plots can therefore at best give a minimal estimation for the complexity of the slow component.

Both the fast and slow components renatured accurately with very small T_m differences between native and renatured molecules. This again differs quite markedly from most animal satellite DNA where the difference in denaturation T_m of native and renatured duplexes suggests a large degree of mismatching, which is interpreted in terms of divergence from the common ancestor of the sequence (Sutton & McCallum, 1971). The rate of renaturation is decreased by such mismatching, so the complexity of the sequence is estimated from the observed renaturation rate and the degree of mismatching, the ΔT_m (Sutton & McCallum, 1971). Although the renaturation rates for the mouse satellite DNA and melon fast-renaturing component are very similar, the complexity of mouse satellite is decreased approximately threefold to correct for mismatching ($\Delta T_m = 4\text{--}5^\circ\text{C}$), whereas only a minimal correction is necessary for the melon fast component ($\Delta T_m = 1^\circ\text{C}$). The fast component of the melon therefore appears to be two or three times more complex than mouse satellite.

The presence of at least two components in melon satellite DNA, on the basis of denaturation, renaturation rate and $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ analysis, is rather difficult to reconcile with the single peak in the CsCl gradient. Previous work showed that the melon satellite was extremely homogeneous in terms of buoyant density (Ingle *et al.*, 1973). The buoyant densities of the two components should be 1.704 and 1.725 g/ml on the basis of T_m values (83.5 and 92°C), compared with the observed native buoyant density of 1.707 g/ml. However, it has previously been demonstrated that the normal relationships between base composition; buoyant density and temperature of denaturation do not necessarily apply to satellite DNA (Corneo *et al.*, 1968). Although analysis of the native satellite showed only a single component in CsCl , analysis of the renatured satellite did, under certain conditions, resolve two peaks at 1.711 and 1.706–1.708 g/ml. The satellite DNA is resolved into two major and several minor components after centrifugation in $\text{Ag}^+/\text{Cs}_2\text{SO}_4$. The two major components, which both have a native density of 1.707 g/ml in CsCl , are en-

riched for the high-‘melting’ fast-renaturing and low-‘melting’ slow-renaturing sequences. The renatured densities of the major fractions from $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ gradients indicate that the high-‘melting’ fast-renaturing sequences plus heterogeneous sequences renature completely back to 1.707 g/ml, whereas the slow-renaturing low-‘melting’ sequences renature back to 1.711–1.713 g/ml. The great variability of the $\text{Ag}^+/\text{Cs}_2\text{SO}_4$ fractionations (Fig. 5), and the fact that the major fractions do not contain either pure fast- or slow-renaturing sequences, suggest that the fast- and slow-renaturing sequences are integrated, at least with the more heterogeneous sequences, which possibly make up 30% of the total satellite.

The heterogeneity and complexity of the satellite DNA in melon suggests that it may have different and additional functions to the simple, homogeneous satellite DNA in animal cells. The presence of at least two components in the satellite, on the basis of either denaturation or renaturation, is not unique to melon, having been observed also in flax (*Linum usitatissimum*), runner-bean (*Phaseolus coccineus*) and cucumber (*Cucumis sativas*) satellite DNA (J. Sinclair, R. Wells, B. Deumling & J. Ingle, unpublished work). Preliminary results indicate, however, that the other plant satellite DNA types differ quite markedly from melon in their complexities and apparent degrees of divergence.

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