Divergence, differential methylation and interspersion of melon satellite DNA sequences

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Melon (*Cucumis melo*) satellite DNA consists of two components, Q and S, each with a buoyant density in CsCl of 1.707 g/ml, but differing by 9°C in 'melting' temperature. These physical properties appear to be in contradiction, since both depend on G + C content. In order to resolve this anomaly, base compositions were directly determined for isolated fractions. The low-'melting' component S contains 41.8% G + C, with 6% of C present as 5-methylcytosine, whereas Q DNA contains 54% G + C, with 41% of C methylated. Analyses of restriction-site loss agreed well with the direct determinations of methylation and divergence, and indicated some clustering of methylated sites in Q DNA. Analysis of restricted main-band DNA by hybridization with RNA complementary to Q satellite DNA ('Southern transfer') showed satellite Q tandem arrays interspersed in DNA of main-band density. Sequence divergence and extent of methylation did not appear to depend on whether a repeat array was present as satellite or interspersed in main-band DNA. Hybridization *in situ* indicated considerable heterogeneity in the genomic proportion of the Q-DNA sequences in melon fruit nuclei, implying over- and under-representation consistent with extensive unequal recombination in satellite Q tandem arrays. The cucumber, *Cucumis sativus*, contains less than 8% as much Q-homologous DNA per genome as the melon, suggesting rapid evolutionary gain or loss of these tandem repeat sequences.

Melon (*Cucumis melo*) DNA contains a symmetrical DNA satellite peak at 1.707 g/ml in CsCl isopycnic gradients, well-resolved from main-band DNA at 1.693 g/ml (Sinclair *et al.*, 1975). The quantity of this satellite fraction varies between varieties of melon, melon tissues and during development (Pearson *et al.*, 1974).

This satellite DNA consists of two principal components, a thermostable component which re-associates very rapidly, indicating several million

copies of about 450 base-pairs complexity, and a thermolabile heterogeneous component which re-associates almost 10000 times more slowly, indicating a few hundred copies with a sequence complexity of 3×10^6 base-pairs (Bendich & Anderson, 1974; Sinclair *et al.*, 1975). The heterogeneity of the second fraction is due in part to ribosomal and mitochondrial DNA sequences, which are present in this region of the CsCl gradient. The total complexity, however, indicates that a very great variety of intermediate repetitive DNA is presented in the satellite peak.

It is surprising that these two components of identical buoyant density (ρ) in CsCl differ by 9°C in 'melting' temperature (T_m), since both ρ and T_m normally depend on DNA base composition. Satellite DNA species with anomalous ρ and T_m values have been noted (Corneo *et al.*, 1968), but the reasons for this are not clear. Evidence has been presented which indicates that the two satellite components are not interspersed with each other, but that some of the satellite short-repeat arrays are integrated within main-band DNA (Bendich &

Abbreviations used: cRNA, complementary RNA transcribed *in vitro* from DNA template by using RNA polymerase holoenzyme and nucleotides; cRNA_Q, complementary RNA to Q DNA; SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0; SDS, sodium dodecyl sulphate; RNAase, ribonuclease.

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Taylor, 1977). It remained unresolved, however, whether interspersions could account for the anomalous physical properties of these fractions.

In the present paper we report restriction-enzyme analyses which confirm and extend earlier observations on the structure, complexity and evolutionary relationships of satellite DNA and its interspersions within main-band DNA, base-composition analyses for the two principal components and the cytological localization of these sequences.

Experimental

Preparation of melon satellite and main-band DNA

Total DNA was prepared from seeds or fruit of the melon (*Cucumis melo*, var. Spanish winter melon) (Sinclair *et al.*, 1975). Satellite (1.707 g/ml) and main-band DNA (1.693 g/ml) were separated and purified by three and two preparative cycles, respectively, of CsCl equilibrium centrifugation (Fig. 1 in Sinclair *et al.*, 1975). The molecular weight of the double-standard DNA was approx. 10^7 .

Separation of the Q and S fractions of melon satellite DNA

The two components of melon satellite were separated on the basis of their different 'melting' temperatures. Satellite DNA in 0.12 M-sodium phosphate buffer (pH 7.0) was sonicated to 800–1000 base-pairs (Sinclair *et al.*, 1975) and 0.62 mg of DNA was heated to 91.5°C and loaded on a 0.25 ml column of hydroxyapatite (Bio-Rad, DNA grade) at 91.5°C. Denatured DNA (component S) was washed through the column with 0.12 M-phosphate buffer, pH 7.0, and bound native DNA (Q) was eluted with 0.5 M-phosphate buffer. After the first cycle the two components were separated with 10–15% overlap. Each fraction was then recycled through a second hydroxyapatite column to yield Q and S fractions at least 98% pure. S DNA was bound to hydroxyapatite 60°C in 50 mM-phosphate buffer, washed with this buffer, and then eluted with 0.5 M buffer at 95°C. The fractions were dialysed against 10 mM-Tris/HCl (pH 8.3)/1 mM-EDTA, and precipitated with ethanol.

Base-composition analysis

DNA samples (90–120 µg) were dissolved in 90% (v/v) formic acid, and hydrolysed for 30 min at 175°C in sealed glass tubes. Formic acid was removed by repeated evaporation, and the hydrolysate, dissolved in 2 µl of 1 M-HCl, was chromatographed on cellulose thin-layer sheets (Eastman Kodak MN 300) with propan-2-ol/conc. HCl/water (85:22:18, by vol.) in the first dimension and butan-1-ol/methanol/water/conc. NH_3 (sp.gr. 0.88) (180:60:60:1, by vol.) in the second (Randerath & Randerath, 1967). The u.v.-absorb-

ing spots were cut out, along with concentric peripheral rings of equal area as background controls, and both were eluted with 0.1 M HCl. Eluates were scanned in a Unicam SP.8000 spectrophotometer for quantification.

Preparation and fractionation of cucumber DNA

Total DNA was prepared from cucumber (*Cucumis sativus*) and fractionated by CsCl equilibrium centrifugation. Total satellite DNA (1.706 and 1.701 g/ml) was separated from the main-band DNA (1.693 g/ml) (see Fig. 1b of Timmis & Ingle, 1977).

Restriction analyses

Incubation with endonuclease *Hind*III or *Hind*II + *Hind*III included 1–2 µg of DNA in 8 µl of 50 mM-NaCl/30 mM-Tris/HCl (pH 7.4)/10 mM-MgCl₂/10 mM-β-mercaptoethanol. *Hae*III-endonuclease digestions were in 6.6 mM each of NaCl, Tris/HCl (pH 7.4), MgCl₂ and β-mercaptoethanol. *Xho*I- and *Eco*RI-endonuclease digestions were in 100 mM-NaCl/10 mM-MgCl₂/40 mM-Tris/HCl (pH 7.4)/10 mM-β-mercaptoethanol. One enzyme unit digests 1 µg of λ-phage DNA in 1 h at 37°C.

Electrophoresis was in 2 or 3% agarose gel, in E buffer (36 mM-Tris base, 30 mM-NaH₂PO₄, 1 mM-Na₂EDTA, pH 7.7). Samples were mixed with 0.5 vol. of 40% (w/v) sucrose/20 mM-EDTA/1% SDS/1% agarose/0.2% Bromophenol Blue, at 70°C, before loading. Gels were stained for 1 h in ethidium bromide (0.5 mg/l) in E buffer and photographed under u.v. illumination through a red filter. Photographic negatives and radioautographs were scanned on a Joyce-Loebl microdensitometer and peak areas were determined by weighing, after subtraction of background fluorescence in an adjacent lane.

Preparation of cRNA

cRNA was transcribed *in vitro* from satellite DNA template by using RNA polymerase holoenzyme from *Escherichia coli* (Boehringer, Mannheim, Germany) in the presence of [³²P]GTP or [³H]GTP (The Radiochemical Centre, Amersham, Bucks., U.K.) and unlabelled ATP, UTP and CTP (Shmookler Reis & Biro, 1978). The product was asymmetrically transcribed, less than 10% of the cRNA being double-stranded (RNAase-resistant) after self-reassociation.

'Southern transfer' of DNA and hybridization with cRNA

DNA restriction fragments separated in 2–3% agarose gels or 6% acrylamide gel were transferred to nitrocellulose sheets (Schleicher and Schuell) as described by Southern (1975b), by using 10 × SSC as the transfer buffer. Hybridizations to [³²P]cRNA were for 4 h, in sealed, pre-washed dialysis tubing

containing 1.2×10^6 c.p.m. of [^{32}P]cRNA (approx. $0.4 \mu\text{g}$) per 4 ml of $5 \times \text{SSC}$, suspended in 2 litres of $5 \times \text{SSC}/0.2\%$ SDS at 72°C . The filters were washed thoroughly in $5 \times \text{SSC}$ at 72°C and in $2 \times \text{SSC}$ at 20°C . Radioautographs were scanned on a Joyce–Loebl microdensitometer.

Hybridization in situ

Nuclei were isolated from fruit and placental tissue of melon by finely slicing fresh material in 45% (v/v) acetic acid. After removal of large cell debris, nuclei were recovered by centrifugation at 200g for 2 min, dried on to glass slides and further fixed in ethanol/acetic acid (3:1, v/v) for 1 h. Nuclei were denatured in $0.1 \times \text{SSC}$ at 100°C for 30 s, dehydrated and immediately hybridized to [^3H]cRNA at $3 \mu\text{g}/\text{ml}$ in $4 \times \text{SSC}$ at 68°C for either 1 or 6 h. The slides were treated with RNAase in $2 \times \text{SSC}$ and extensively washed in $2 \times \text{SSC}$. After coating with Ilford K2 photographic emulsion (diluted 1:1 with water) and exposure under desiccation at 4°C for 21 days, the slides were developed and stained in Giemsa or Aceto-Carmine and finally air dried and mounted in Clearmount.

Silver-grain numbers were estimated by using a Vickers M85 integrating microdensitometer. Calibration of the microdensitometer showed that the area readings obtained were directly proportional to the number of silver grains up the highest values that could be counted directly by using photographs. The optical density threshold of the instrument was raised to a value which enabled only the area of totally opaque silver grains to be measured, with no contribution from the low extent of histological stain.

Results

Characterization of melon satellite DNA components

Satellite DNA comprised 31% of the DNA prepared from seeds, but only 17% of DNA from fruit. In seed satellite DNA 27% was the high-‘melting’ component, Q DNA, compared with 49% in fruit satellite DNA (Fig. 1). Although the amounts of total satellite DNA were quite different for fruit and seed, the quantity of the high-‘melting’ component Q was essentially constant, at 8.3 and 8.5% of total DNA respectively. The heterogeneous low-‘melting’ satellite fraction (S DNA) represented 8.7% of melon fruit DNA, but 22.6% of melon seed DNA. Cleavage of satellite DNA (S plus Q) with restriction endonucleases *HindII* + *HindIII*, *HaeIII* or *XhoI* produced fragments at 360 base-pairs and integral multiples thereof (Fig. 2). This agrees well with the kinetic complexity of component Q, as 3×10^5 daltons or 450 base-pairs (Sinclair *et al.*, 1975). Moreover, the proportion of the satellite DNA present as the 360-base-pair repeat was 22–25% for seed satellite DNA (Figs. 3d and 3e) and approx. 50% for fruit satellite DNA (Figs. 3g and 3h), very nearly the proportions of component Q observed by thermal denaturation of satellite DNA from these tissues (see Fig. 1).

Purified high-‘melting’ component Q was cleaved by *HindII* + *HindIII* endonucleases virtually quantitatively into fragments at integral multiples of 360 base-pairs (Figs. 3a and 3b). Low-‘melting’ component S, after reassociation and *Hind*-endonuclease digestion, showed a broad distribution, with less than 1% of material banding at 360 base-pairs

Fig. 1. Thermal denaturation of melon seed (b) and fruit (a) satellite DNA

Denaturation in $0.25 \times \text{SSC}$ is expressed as the percentage of maximal hyperchromicity at 260 nm (corrected for the expansion of water), as a function of temperature in a Unicam SP.8000 dual-beam spectrophotometer. Cuvette temperature was monitored with a thermistor.

Fig. 2. Restriction-enzyme digestion of melon seed satellite and main-band DNA

Satellite DNA was digested with endonucleases: (a) *HindII* + *HindIII*; (b) *XhoI*; or (c) *HaeIII*. Two preparations of main-band DNA were digested with endonucleases: (d) and (g) *HindII* + *HindIII*; (e) and (h) *XhoI*; or (f) and (i) *HaeIII*. A partial *EcoRI* restriction digest of mouse satellite DNA provided size markers (Southern, 1975a) as indicated at the left (b.p., base-pairs). Electrophoresis was in 3% agarose gel for 5 h at 10 V/cm, from top to bottom of the Figure.

and multiples thereof (Fig. 3c), although Q-DNA sequences could be efficiently cleaved after denaturation and reassociation (Fig. 3f). Clearly the satellite fraction Q, defined by its high 'melting' point, is the same fraction that is cut at 360-base-pair intervals by the *Hind* enzymes. Digestion of satellite DNA with pure *HindIII* endonuclease produced a fractionation identical with that by endonucleases *HindII* + *HindIII* (Figs. 3d and 3e), indicating that Q DNA contains only the *HindIII* site. The largest fragments resolved after partial

Table 1. Base compositions of melon seed main-band and satellite DNA components Q and S

5-Methylcytosine (5MeC), cytosine, guanine, adenine and thymine were determined as described in the Experimental section.

	Composition (%)		
	Q	S	Main-band
5MeC	11.8	1.3	4.7
C	15.6	20.5	13.7
G	26.5	20.0	18.9
A	23.2	28.6	30.7
T	22.8	29.6	31.9
G + C + 5MeC	53.9	41.8	37.5
5MeC/(C + 5MeC)	0.43	0.06	0.25

Hind-endonuclease digestion extend beyond the 22-unit repeat (7920 base-pairs), implying tandem arrays of the basic Q-DNA repeat unit with at least this length (Fig. 3b).

Base-composition analyses of melon satellite DNA components

Purified S and Q components were hydrolysed and their base compositions determined. The two components had different guanine plus cytosine (G + C) contents, 41.8% for component S, and 54.0% for Q, and Q DNA had a prominent spot which chromatographed as 5-methylcytosine and had the absorption spectrum of 5-methylcytosine (Table 1). In two independent preparations of Q DNA, 38% and 43% of total cytosine residues were methylated, in contrast with only 6% in satellite S DNA. No spot was seen in any chromatogram corresponding to 6-methylaminopurine.

The amounts of 5-methylcytosine present in total satellite DNA of fruit and seed are close to the values predicted from their relative proportions of Q and S components (49 and 25% Q respectively) and the 5-methylcytosine contents of seed Q and S satellite DNA (Table 2), suggesting that the extents of methylation in components Q and S are similar in both fruit and seed, although the proportions of the two components differ markedly.

Restriction analysis of melon satellite Q DNA

Q DNA contained cleavage sites for restriction endonucleases *HindIII* (5'-AAGCTT-3'), *HaeIII* (5'-GGCC-3') and *XhoI* (5'-CTCGAG-3'), all with very nearly the same 360-base-pair periodicity (Fig. 2, tracks a-c). Fragment sizes after double-enzyme digestions were determined (Fig. 4) and used to locate the restriction sites on the DNA with respect to one another (Fig. 5).

The *HindIII* site is close to a *HaeIII* site. Comparison of the overlapping fragments A

Fig. 3. Scans of restricted melon satellite DNA

Fraction-Q satellite DNA was digested to completion (a) or partially (b) with endonucleases *Hind*II + III. Fraction S satellite DNA was renatured to C_0t 100 mol·l⁻¹·s and digested to completion with *Hind*II + III endonuclease (c). Total seed satellite DNA was digested to completion with pure *Hind*III (d) or *Hind*II + III endonuclease (e). Total seed satellite DNA, denatured at 100°C for 3 min in 10 mM-Tris/HCl (pH 8.3)/1 mM-EDTA, was renatured to C_0t 100 and digested with *Hind*II + III endonuclease. Total fruit satellite DNA was digested to completion (g) or partially (h) with *Hind*II + III endonuclease. Complete digestions used 4 units of enzyme to 1 µg of DNA, in a final volume of 8 µl of buffer (50 mM-NaCl/10 mM-MgCl₂/10 mM-β-mercaptoethanol/20 mM-Tris/HCl, pH 7.5), incubated for 24 h at 37°C. Partial digestions contained 0.4 unit of enzyme per µg of DNA, incubated for 2 h at 37°C. Broken lines indicate background fluorescence adjacent to the loaded channels. Electrophoresis was as described in Fig. 2, and the photographic negatives were scanned with a Joyce-Loebl dual-beam microdensitometer, the top of the gel being on the left.

Table 2. 5-Methylcytosine (5-MeC) and cytosine contents of satellite DNA from melon seed and fruit
For details, see the Experimental section.

		Content (%)					
		Seed total satellite DNA (25% Q)				Fruit total satellite DNA (49% Q)	
		Q	S	Predicted	Observed	Predicted	Observed
5MeC		11.8	1.3	3.9	4.6	6.5	7.9
C		15.6	20.5	19.3	19.4	18.1	20.0

(*Hind*III/*Xho*I digest, 83 base-pairs) and A' (*Hae*III/*Xho*I digest, 89 base-pairs) indicates that they are very close, essentially adjacent, whereas a comparison of fragments B (*Xho*I/*Hind*III digest, 280 base-pairs) and B' (*Xho*I/*Hae*III digest, 266 base-pairs) shows a larger gap between these sites (Fig. 5). This discrepancy may be taken as evidence for a second *Hae*III site near the first (as depicted in Fig. 5). Bands produced by *Hae*III alone con-

sistently ran a little faster than those from *Hind*III or *Xho*I digestions (Fig. 2), and this was independent of *Hae*III enzyme concentration or incubation time, thus ruling out effects of exonuclease activity. We interpret this as supporting evidence for two adjacent *Hae*III sites, but realize that an effect of staggered versus flush ends on mobility could also account for these observations.

Complete digestion by *Hind*III and *Hae*III

Fig. 4. Double digests of melon seed satellite DNA with restriction enzymes

DNA (1 μ g) was digested with endonucleases (a) for 48 h at 37°C with 2 units of *Hind*II + III and 2 units of *Xho*I, in *Hind* buffer, (b) for 24 h at 37°C with 2 units of *Hae*III in *Hae* buffer, then for 24 h with 4 units of *Hind*II + III in *Hind* buffer, and (c) for 24 h at 37°C with 2 units of *Hae*III in *Hae* buffer and then for 24 h with 4 units of *Xho*I in *Hind* buffer. Products were separated by electrophoresis on 6%-acrylamide slab gels from top to bottom of the Figure. Replicate sets of tracks are presented, and the numbers at the sides indicate the size of the fragments in base-pairs, calculated from mouse satellite-DNA markers as described in Figs. 2 and 5.

Fig. 5. Map of the Q DNA repeat sequence
Fragment sizes [in base-pairs (b.p.)] were determined from double enzyme digestions electrophoresed in 6%-acrylamide gels (Fig. 4) with reference to mouse satellite-DNA partial-*Eco*RII-digestion products, calibrated in agarose-gel electrophoresis as integral multiples of 240 base-pairs (Southern, 1975a).

endonucleases was confirmed by doubling the enzyme input and incubation time, but this could not be assured for *Xho*I, because of the very high enzyme/DNA ratio required. The fraction of altered (uncleavable) sites, *P*, calculated from the ratio of dimer to monomer peak areas in complete digestions was $8.1 \pm 0.5\%$ for *Hind*II + *Hind*III digestion of seed satellite DNA, and similar values were obtained with pure *Hind*III endonuclease and with *Hind*-endonuclease cleavage of fruit satellite DNA (Table 3).

In contrast, digestion with *Hae*III endonuclease reached a limit with a much higher fraction of the Q component remaining as oligomers rather than monomer, with 41–44% of repeat units not being cleaved (Table 4). Since the mapping evidence indicates two adjacent *Hae*III sites in the typical Q-DNA repeat unit (Fig. 5), 64–66% of these sites would need to be eliminated in order to have $P^2 = 41\text{--}44\%$ of the basic units resistant to endonuclease *Hae*III, a value consistent with the observed extent of cytosine methylation.

The relationship of the proportions of oligomers to the probability of scission can be expressed in a linear form (Slack, 1974), provided that neighbouring cleavages are independent of each other. Results from four complete endonuclease-*Hae*III digestions (Figs. 6c–6f) indicated a consistent departure from linearity, with the monomers being present in excess of that predicted for random cleavage. Results from *Hind*- and *Xho*-endonuclease digestions did not suggest any excess of monomers (Figs. 6a and 6b).

Satellite Q sequences in DNA of main-band density

Main-band melon seed DNA was prepared free of any satellite-density contamination by two suc-

EXPLANATION OF PLATE 1

Hybridization in situ of cRNA from satellite DNA to isolated melon nuclei

Hybridization was with $3\mu\text{g}$ of cRNA/ml at 68°C in $4\times\text{SSC}$ for 1 h (a, c and g) or for 6 h (b, d, e, f and h). Nuclei were isolated from fruit tissue (a–g) or placental tissue (h) of melon. All the photographs are the same magnification, the bar representing $10\mu\text{m}$.

Table 3. *HindIII*-restriction-site divergence in melon satellite Q DNA

DNA-cleavage products were electrophoresed and photographed in agarose gel as shown in Fig. 2, and densitometric tracings were made of DNA channels with background absorptions scanned between the lanes (as in Fig. 3). Peak areas were determined by weight, from which P and d were calculated. The fraction of altered restriction sites is P , and the fraction of cleavable restriction sites is $1-P$. The probability of obtaining a monomer is thus $(1-P)^2$, and for a dimer it is $P(1-P)^2$. The expected ratio of (dimer/monomer) DNA contents is then $2P(1-P)^2/(1-P)^2 = 2P$, or $P = \frac{1}{2}(\text{dimer/monomer})$. The *HindIII* site contains six base-pairs, and cleavage will be prevented by mutation in one or more of these. If the rate of mutational divergence is d , then the probability of a site being intact and cleavable is $(1-d)^6$, which by definition equals $(1-P)$. Thus $d = 1 - \sqrt[6]{1-P}$. Where applicable, values are shown as means $\pm$ S.E.M.

Source of melon DNA/enzyme	Number of assays	Percentage of total Q DNA		Fraction (%) of altered sites (P)	Divergence (%) per base-pair (d)
		Monomer	Dimer		
Seed/ <i>HindIII</i>	1	84.6	15.4	9.1	1.57
Seed/ <i>HindII</i> + <i>III</i>	7	86.0 $\pm$ 0.7	13.9 $\pm$ 0.7	8.1 $\pm$ 0.4	1.40 $\pm$ 0.08
Fruit/ <i>HindII</i> + <i>III</i>	2	86.2 $\pm$ 2.8	13.1 $\pm$ 2.8	7.6 $\pm$ 1.8	1.32 $\pm$ 0.33

Table 4. Oligomer yields of melon satellite Q DNA after digestion with *HaeIII* endonuclease

Digestions contained 2–4 units of *HaeIII* enzyme/ μ g of DNA, as indicated. The proportions of Q DNA present in each of the oligomer peaks was determined from gel scans (as in Fig. 3) and P (slope) and P (intercept) were calculated as described in Fig. 6.

Digestion	Proportion (%) of Q DNA in each oligomer peak					Fraction (%) of uncleaved repeat units	
	1	2	3	4	5	P^2 (slope)	P^2 (intercept)
24 h, 3 units	45.1	22.8	14.9	8.3	5.8	40.7	43.8
48 h, 3 units	45.4	21.3	14.9	9.0	5.6	42.4	43.8
24 h, 2 units	39.2	25.1	17.3	10.9	—	43.7	44.4
24 h, 4 units	42.7	23.7	17.7	9.4	—	40.7	41.8

$$P^2 = 42.6 \pm 0.5\% \text{ (S.E.M.)}$$

cessive isopycnic bandings in CsCl gradients, in which only the lighter main-band fractions, well removed from the dense satellite DNA, were pooled. Two independent preparations of main-band DNA and pure satellite DNA were digested with restriction endonucleases *HindII* + *HindIII*, *HaeIII* or *XhoI*, and electrophoresed in agarose gel. Monomer- and dimer-length fragments are just discernible in the total main-band DNA digests as observed by ethidium bromide fluorescence in the agarose gel (Fig. 2, tracks, *d*–*i*). [^3H]cRNA_Q hybridized to the 360-base-pair unit and multiples thereof in the main-band-DNA digestion patterns, with profiles very similar to those obtained for pure satellite DNA (Fig. 7). Thus pure main-band DNA contains sequences homologous to satellite Q DNA, arranged in tandem repeat arrays with a 360-base-pair periodicity.

Hybridization *in situ* of melon satellite Q DNA sequences

Isolated melon nuclei were heat-denatured and

hybridized *in situ* under conditions allowing only Q sequences to reanneal with excess [^3H]RNA which had been transcribed from melon satellite DNA. The conditions achieved near-saturation of all rapidly annealing sites in the nucleus, since incubation for 6 h produced only 13% more silver grains than after 1 h. Grain counts after 6 h were therefore taken to be proportional to Q sequences per nucleus. The resulting radioautographs showed striking heterogeneity among melon fruit nuclei in their content of Q sequences (Plates 1a–1g). Even nuclei within a few μm of each other could differ over 10-fold in their response to the probe (Plates 1a, 1c, 1f and 1g). Generally, smaller fruit nuclei showed the most intense labelling, but large nuclei also spanned the range from sparse to very dense label.

Placental nuclei, in contrast, showed much lighter and more uniform labelling (Plate 1h). Grain counts over placental nuclei gave a bimodal distribution, with peaks at approx. 57 and 115 grains per nucleus (Fig. 8a), presumably corresponding to the 2c and 4c DNA contents expected of G1 and G2 diploid

Fig. 6. Oligomer yields of melon satellite *Q* DNA after restriction-endonuclease digestion

P is the probability that a DNA repeat unit has lost its restriction site(s) for a particular enzyme, and if restriction cleavages on the DNA are random independent events of probability $(1 - P)$ per repeat unit, the expected yield of DNA in each oligomer of length n units is $a_n = nP^{n-1}(1 - P)^2$. Taking logarithms (Slack, 1974):

$$\log(a_n/n) = (n - 1)\log P + 2\log(1 - P).$$

The equation describes a line relating $\log(a_n/n)$ to $(n - 1)$, with slope $\log P$ and intercept $2\log(1 - P)$. Seed satellite DNA was digested: with *Hind*II + III endonuclease partially (●; 0.4 unit/μg of DNA for 2 h) or completely (○; 2 units/μg for 24 h) (a); with *Xho*I (8 units/μg of DNA for 24 h) (b); and with *Hae*III, with 2–4 units/μg for 24 or 48 h, as indicated (c–f). Values of P derived from the slopes and intercepts for the *Hae*III digestions are given in Table 4.

Fig. 7. Hybridization of *cRNA*_Q to restriction-endonuclease digestions of melon satellite and main-band DNA. Radioautographs of *cRNA*_Q hybridizations to satellite (a–c) and main-band (d–f) melon seed DNA digested with *Hind*II + III (a, d), *Xho*I (b, e) or *Hae*III (c, f) endonucleases were scanned on a Joyce–Loebl microdensitometer, the top of the gel being on the left.

Fig. 8. Frequency histograms of the number of radioautographic grains per nucleus in melon tissues. Nuclei from (a) placental tissue and (b) fruit tissue were denatured and hybridized *in situ* with radioactive RNA complementary to melon satellite DNA for 6 h at 3 μ g/ml in 4 $\times$ SSC at 68°C. After radioautography, grain numbers were estimated by microdensitometry. The arrows in (b) indicate the expected doubling series based on the 2c and 4c grain counts in placental nuclei (a).

Fig. 9. Density of radioautographic grains in placental and fruit tissues of melon. The diameters of nuclei from Fig. 8 were measured and the grains per unit area plotted as a frequency histogram.

nuclei. From these values, the average hybridization to fruit nuclei, which are highly endopolyploid, represents satellite Q-DNA sequences present at about 28c. The range of hybridization was very

Fig. 10. Comparison of cucumber and melon satellite DNA by restriction-endonuclease digestion. Satellite DNA (1 μ g) of melon seed (a, c, e, g) or cucumber (b, d, f, h) was digested with endonucleases: (a, b) *Hind*II + III (4 units, 24 h at 37°C); (c, d), *Hae*III (2 units, 24 h at 37°C); (e, f) *Xho*I (4 units, 24 h at 37°C); (g, h) *Eco*RI (8 units, 4 h at 37°C). Electrophoresis was in 2% agarose gel, from top to bottom of the Figure. The numbers at the side indicate the size of the fragments in base-pairs.

broad, with approx. 11% of fruit nuclei containing Q sequences equivalent to less than 8c, and 8% exceeding 64c (Fig. 8b).

Nuclear DNA content is well correlated with nuclear volume in plants (Baetke *et al.*, 1967), and fixed, dehydrated nuclei are essentially thin discs, so that grain density per unit area provides an approximate measure of the relative proportion of Q sequences in the DNA of each cell. Placental nuclei thus gave a unimodal distribution of grains/area (Fig. 9a), whereas the corresponding distribution for fruit nuclei appeared extremely heterogeneous (Fig. 9b). Label over the majority of fruit nuclei (62%) was less concentrated than even the most sparsely labelled placental nucleus, whereas about 5% of fruit nuclei had a high density of grains than any placental nucleus.

Presence in cucumber DNA of sequences homologous to melon satellite Q DNA

Digestion of purified cucumber satellite DNA by *Xho*I endonuclease produced a distinct band at 360 base-pairs (Fig. 10f), but no similar band was observed after digestion with endonucleases *Hind*II + *Hind*III (Fig. 10b). *Hae*III-endonuclease digestion reduced most of the DNA to small

fragments, primarily between 40 and 500 base-pairs in length, but no clear bands were visible at 360, 720 or 1080 base-pairs (Fig. 10d). Endonuclease-*Eco*RI-digestion produced two prominent bands at 3500 and 3900 base-pairs, and a third at about 390 base-pairs (Fig. 10h). The two larger bands were eluted and further digested with endonuclease *Hae*III. This resulted in two very similar banding patterns, but still no indication of a periodicity at 360 base-pairs.

The 360-base-pair component of cucumber satellite DNA after *Xho*I cleavage contained approx. 1.5% of the satellite DNA (Fig. 11c). Hybridization of *Hae*III and *Hind*II + *Hind*III digestions of cucumber satellite DNA with cRNA to melon Q DNA showed clear hybridization peaks at the 360-base-pair banding positions, confirming that cucumber satellite DNA contains small amounts of DNA homologous to melon satellite Q DNA, cleaved by the same three restriction enzymes that have known sites in the melon satellite Q repeat unit.

Discussion

Implications of the base-composition analyses for physical properties of melon satellite DNA components

The predicted denaturation temperatures (Marmur & Doty, 1962) of melon Q and S satellite DNA, calculated from the experimentally determined base compositions and corrected to 0.21M- Na^+ , are 91.6°C and 86.6°C respectively, compared with the observed 'melting' transitions of 96.8 and 87.6°C. The discrepancies, 1.0°C for component S and 5.2°C for Q, could be accounted for if each mole percent of 5-methylcytosine raised the T_m by about 0.5°C. Other satellite DNA species from mouse, guinea pig and crustacea have T_m values significantly higher than expected from their actual G+C contents, which could be due to either methylated bases or abnormal DNA conformation (Walker, 1971).

The predicted buoyant density of each component, calculated from base compositions (Schildkraut *et al.*, 1962), is 1.694 g/ml for S and 1.706 g/ml for Q. The value for satellite Q DNA, 1.706 g/ml, agrees with the buoyant density observed for both satellite components in CsCl, whereas the predicted value for S component is nearer to the density observed for melon main-band DNA, 1.693 g/ml (Ingle *et al.*, 1973). Correction for 5-methylcytosine content as described by Kirk (1967) does not account for the deviant densities of melon satellite DNA components. Marked discrepancies have also been reported between the (G+C) contents determined from chemical analyses, from CsCl densities and from denaturation temperatures for mouse and guinea-pig satellite

Fig. 11. Scans of restriction-endonuclease digestions of cucumber satellite DNA

Radioautographs of cRNA transcribed from melon Q DNA hybridized to cucumber satellite DNA digested with *Hind*II + III (a) or *Hae*III (b) endonucleases were scanned on a Joyce-Loebl microdensitometer. Cucumber satellite DNA was digested with *Xho*I endonuclease, and the negative of the u.v.-induced fluorescence after staining with ethidium bromide (Fig. 10f) was scanned on the microdensitometer (c). Electrophoresis was in 2% agarose gel, with the top of the gel on the left. The numbers indicate the size of the fragments in base-pairs.

DNA (Corneo *et al.*, 1968). They argued that the 3% 5-methylcytosine content (Bond *et al.*, 1967) of mouse satellite DNA would not be sufficient to explain their data, invoking some special features of the sequence. Melon satellite S DNA contains even less methylation, but it would be difficult to invoke sequence idiosyncracies with its kinetic complexity of 2×10^6 base-pairs unless it contains a simple underlying oligonucleotide repeat structure.

Although digestion of purified S satellite DNA with endonucleases *Hind*II + *Hind*III produced a broad range of DNA fragments averaging 2000 bases (Fig. 3c), close to the expected weight-mean size of 1750 nucleotides for a digest of very complex (random-sequence) DNA, the non-Q sequences in melon satellite DNA were resolved into complex patterns of discrete bands by polyacrylamide-gel electrophoresis after double enzyme digestions (Fig. 4). The evidence thus suggests that melon satellite S component includes a wide variety of diverged internal repeats, ranging from 400 to 2000 base-pairs in length, which are organized in disparate patterns to create longer arrays that appear extremely heterogeneous. Cucumber satellite DNA offers a clear example of how very similar sets of highly reiterated elements can be put together to form two distinct complex arrangements (bands at 3900 and 3500 base-pairs in Fig. 10h).

The principal repetitive elements of satellite S DNA do not appear to be related to the Q sequence, since $cRNA_Q$ did not hybridize strongly to any of the bands shown in Fig. 4, other than to cleavage products of Q DNA, and purified S DNA contains less than 1% of material banding with a periodicity of 360 base-pairs (Fig. 3c).

Restriction analyses of Q DNA as indices of divergence and methylation

The divergence of *Hind*III sites in Q DNA was estimated from the dimer/monomer ratio to be 8.1% of sites, or 1.4% per base-pair (Table 3), a value very close to the 0.8–1.3% divergence per base-pair suggested from the 'melting' of reassociated Q DNA (Sinclair *et al.*, 1975). Since 40.5% of cytosine residues are methylated in this DNA fraction, methylation of cytosine in the *Hind*III restriction site (5'-AAGCTT-3') cannot be an impediment to *Hind*III restriction, unless these particular cytosines are specifically spared methylation. Methylation of the distal adenine residues in the recognition site prevents restriction cleavage (Roy & Smith, 1973), and it may be calculated that just less than 2% of adenines are methylated *in vivo*.

Digestion of melon satellite DNA with restriction enzyme *Hae*III indicated a much higher proportion of uncleavable sites, calculated as 64–66% of restriction sites from the data presented in Fig. 6 and Table 4. The value of divergence (d) of 8% estimated

from *Hind*-endonuclease digestions, and the methylation (m) value of 40.5%, may be used to estimate the fraction P of sites uncleavable owing to divergence plus methylation, by using the relationship $P = 1 - (1 - d)^4(1 - m)^2 = 1 - (1 - 0.008)^4 \times (1 - 0.405)^2 = 0.657$. The data are therefore entirely consistent with cytosine methylation as the principal mechanism whereby satellite Q-DNA repeat units become resistant to endonuclease-*Hae*III cleavage. The excess of monomers observed in the linear plots (Fig. 6) over the proportion expected for randomly distributed alterations indicates a tendency for cleavable and resistant sites to be separately clustered in the DNA, which may be interpreted as a clustering of unmethylated *Hae*III sites. A similar concentration of unmethylated CpG sites has been observed in the *Xenopus* ribosomal-DNA spacer region (Bird & Southern, 1978).

Divergence and methylation of Q sequences in main-band and satellite-band DNA

Bendich & Taylor (1977) presented evidence that some satellite Q-DNA sequences are interspersed in main-band DNA, on the basis of renaturation between pure Q-DNA fragments and reassociation 'networks' obtained from high-molecular-weight main-band DNA. Hybridization of $cRNA_Q$, a specific probe of Q satellite DNA, to sequences in ultra-pure main-band DNA which are cleaved by restriction endonucleases to 360 base-pair units confirms the presence of sequences in main-band DNA which are homologous to Q DNA, and also shows that these sequences are present in tandem arrays of the basic Q-DNA repeat unit.

The extents of sequence divergence in main-band and satellite-band fractions was compared from their relative frequencies of repeat units resistant to *Hind*-endonuclease cleavage, since sequence divergence appears to be responsible for most *Hind*-site inactivation. The hybridization profiles are very similar for Q satellite DNA and for main-band DNA in their relative proportions of oligomers (Fig. 7), indicating that divergence must be similar for Q sequences in main-band surroundings, and for large blocks of satellite-density Q DNA.

The similarity of hybridization of $cRNA_Q$ to main-band and satellite Q DNA digested with *Hae*III endonuclease (Fig. 7) suggests similar extents of methylation of Q-DNA sequence, whether present as satellite or interspersed in main-band, since the loss of the *Hae*III restriction site is predominantly attributed to cytosine methylation.

Hybridization in situ to melon satellite Q sequences

Like many other satellite DNA sequences, melon Q DNA is localized over heterochromatic areas of interphase nuclei (Plate 1). A more specific localization at the chromosome level is not possible, owing

to the small size of the chromosomes. The parenchymal melon fruit cells showed striking heterogeneity in satellite Q-sequence content, and the grain counts per nuclear area were more widely distributed in fruit nuclei than in placental nuclei (Fig. 9). The range of hybridization densities, representing satellite Q-DNA repeat frequencies per genome, was considerably broader than we have previously encountered in hybridization of rRNA or satellite DNA probes to plant nuclei (Timmis *et al.*, 1975; J. N. Timmis, unpublished work) or human satellite probes to cultured cell nuclei (R. Shmookler Reis, unpublished work). These radioautographic observations of heterogeneity support and extend earlier cytological evidence that heterochromatin is generally over- and under-represented in individual *Cucumis* fruit nuclei (Pearson *et al.*, 1974). The increased and diminished representation of Q sequences in different nuclei of the same differentiated tissue suggests that heterogeneity may arise from a reciprocal process such as unequal recombination, considered to be an essential mechanism for the evolution of tandemly repeated DNA arrays (Tartof, 1973; Smith, 1973, 1976; Shmookler Reis & Biro, 1978; Shmookler Reis & Goldstein, 1980).

Satellite sequences in the cucumber homologous to melon Q DNA

Satellite DNA species are thought to be very sensitive indicators of evolutionary divergence, since distinctive differences have been seen between closely related animal species (Walker, 1971; Gall & Atherton, 1974; Southern, 1975a). The DNA of cucumber, *Cucumis sativus*, a related species in the same genus as melon, contains at most 0.65% of sequences homologous to melon satellite Q-DNA repeat, which accounts for 8.4% of the melon genome. In closely related species of sea urchin, satellite families differed by up to 20-fold in their genomic representation (Moore *et al.*, 1978). If rapid evolutionary changes between closely related species commonly occur through waxing and waning of the corresponding sequence families, this could mimic rapid divergence, since cross-reassociating DNA might arise predominantly from a related sequence family present in both genomes.

Extremely rapid fluctuations in satellite sequence content can also occur in differentiated human cells (Shmookler Reis & Goldstein, 1980), a phenomenon supported by the results *in situ* of hybridization reported herein. Both the evolutionary and somatic drifts in satellite-like DNA repeat numbers are to be expected if unequal recom-

binations are sufficiently frequent and extensive in tandem-repeat arrays (Shmookler Reis & Goldstein, 1980).

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