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Characterization and localization of cryptic satellite DNAs in barley (*Hordeum vulgare*)

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Summary. Three satellites on the heavy side of the main band and two satellites on the light side were isolated in a pure from by preparative ultracentrifugation of *H. vulgare* DNA in Ag^+/Cs_2SO_4 density gradients. The satellites were characterised in terms of their buoyant densities in CsCl and their thermal dissocation temperature in both native and reassociated forms to Cot 4. In CsCl gradients, heavy satellites formed a single peak whereas light satellites resolved into more than one component. Thermal transitions of some satellites indicated the presence of more than one molecular species. The multicomponent nature of thermal denaturation profiles was evident on differential analysis. Radioactive RNAs complementary to the three heavy satellites of *H. vulgare* were localised by in situ hybridization onto its nuclei and chromosomes. One heavy satellite (H_3) was found to be distributed on all chromosomes, although one pair showed less hybridization compared to the others. The other satellite (H_1) appeared to be present in a much lower amount on the chromosomes.

Key words: Ag^+–Cs_2SO_4 gradient – Buoyant density – Cryptic satellites in situ hybridization – Thermal denaturation

Introduction

In genomes of many organisms the pattern of repetition of a given sequence (at least one hundred thousand tandem repeats, Salser et al. 1976) results in DNA banding in a CsCl gradient at a different density than that of the bulk of the DNA (main band). The additional band(s) is referred to as a satellite band. In some cases satellite band(s) can be observed only after the binding of DNA with antibiotics (such as actinomycin D, Dunsmuir 1976; Dennis et al. 1979) or heavy metals like Hg^{2+} and Ag^+ (Corneo et al. 1968, 1970; Nandi et al. 1965; Ranjekar et al. 1976; Timmis et al. 1975; Wall and Bryant 1981; Fillipski et al. 1973) followed by centrifugation in CsCl or Cs_2SO_4 gradients respectively. Satellites of this type are called 'cryptic' satellites.

A great deal is already known about satellite DNA from animal genomes (Walker 1971; Bostock 1971; Rae 1972; Appels and Peacock 1978; John and Miklos 1979). However, the information about the satellite DNA component of plant genomes is limited to inferences of whether the DNA of plants and animals share common characteristics.

Amongst the plants studied, DNA from many dicotyledonous species form satellites in CsCl gradients while monocotyledons in general do not show such satellites (Ingle et al. 1973, 1975; Nagl 1976; Coudray et al. 1970) except in *Cymbidium* (Capesius et al. 1975; Capesius 1976). Cryptic satellites in monocots have, however, been resolved in barley (Ranjekar et al. 1976; Deumling et al. 1976; Peacock et al. 1977a; Chakrabarti et al. 1978), wheat (Huguet and Jouanin 1972; Ranjekar et al. 1976; Deumling et al. 1976; Peacock et al. 1977a, b), rye (Appels et al. 1978) and in *Scilla* (Timmis et al. 1975).

Very few of these plant satellites have been characterized and localized (*Scilla*, Timmis et al. 1975; rye, Appels et al. 1978; and wheat, Gerlach and Peacock 1980; Peacock et al. 1977a, b). Peacock et al. (1977a) made a passing reference to the isolation and localization of one satellite from *Hordeum vulgare*. In the same species Ranjekar et al. (1976) reported two satellites, one of which was characterized (Ranjekar et al. 1978a) in terms of thermal denaturation and reassociation kinetics.

Comparative data concerning the physicochemical properties of satellite DNA from related species within the same genus are not available.

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Our studies with six species of *Hordeum* (Chakrabarti 1979) revealed similarities in the satellite patterns of closely related diploid species which are distinct from those of the polyploid series. This paper deals with the characterization of purified satellite DNAs from *H. vulgare* and the in situ localization of heavy satellites on the chromosomes.

Material and methods

Hordeum vulgare var. 'clipper' (2x) was obtained from Dr. R. Oram, CSIRO, Division of Plant Industry, Canberra, Australia.

DNA extraction

Shoots of seven to ten-day old seedlings (grown aseptically, Chakrabarti et al. 1978) were used for extraction of DNA. The procedure is similar to one already described (Subrahmanyam and Azad 1978) except that the ribonuclease step was omitted as advised by Marmur (1963) since the DNA was to be used as a primer for complementary RNA (cRNA) synthesis.

Preparative Ag^+/Cs_2SO_4 gradient centrifugation

The optimum R_F for *H. vulgare* is 0.3 (Chakrabarti and Subrahmanyam, unpublished). Silver nitrate (10 mM) was added to DNA to achieve the desired R_F value; the mixture was gently shaken for 2 h and then Cs_2SO_4 was added to an initial density of 1.50 g cm^{-3}. A sample was first run in an analytical ultracentrifuge to check the resolution and the remainder of the DNA was used for preparative centrifugation in a Beckman L2-65B ultracentrifuge, using a 50 Ti fixed angle rotor for 60 h at 44,000 rpm at 20 °C. 350 µg of DNA was placed in each tube. Each gradient was fractionated using a Gilford 2580 density gradient scanner recording the absorbance profile (Fig. 1) by a Gilford 2400 spectrophotometer. Fractions corresponding to each satellite were chosen in such a way as to reduce contamination from adjoining peak(s). Fractions corresponding to each satellite from several gradients were pooled and samples were run in an analytical ultracentrifuge to check for purity. The preparative step was then repeated except that the initial density of the Cs_2SO_4 was the same as that of the pooled fractions and no adjustment was made. UV profiles of the second preparative gradients are also shown in Fig. 1. A third preparative step was necessary for the purification of some satellites (H_3, L_1 and L_2, Fig. 1). Fractions containing pure satellites were pooled, dialysed extensively against SSC (0.15 M NaCl, 0.015 M Na-citrate, pH 7.0) and then stored at −20 °C.

Buoyant density

DNA in SSC was mixed with CsCl to an initial density of 1.705 g cm^{-3} and analysed in a Beckman Model E analytical ultracentrifuge (44,000 rpm, 20 °C, 18 h). Buoyant density was calculated with reference to *Micrococcus luteus* DNA and/or poly (dA-dT) as standards. Thermal dissociation of satellite DNA was carried out with a Gilford 250 spectrophotometer equipped with a Gilford 2527 thermoprogrammer, 6046 analog multiplexer and 6051 recorder. DNA solutions in 0.12 M phosphate buffer (0.18 M Na^+, pH 6.8, 0.3 ml) were added to cuvettes fitted with a tapered teflon stopper to prevent evaporation. The temperature of the cuvette chambers was increased by 1 °C/min and both temperature and hyperchromicity were recorded. The Tm of *E. coli* DNA was simultaneously determined for each experiment and was used for correction taking 90.5 °C as the standard.

The amount of *H. vulgare* H_2 satellite recovered after purification was too low for use in a Gilford 250 and its thermal dissociation was determined using a Zeiss spectrophotometer. Derivatives of hyperchromicity ($\Delta H/\Delta T$) were calculated by using a Digital Docwriter II computer and were plotted against temperature by a Hewlett-Packard 7200 A graphic plotter following the procedure of Vitek et al. (1974).

Reassociation

The denatured DNAs (100 °C for 5 min) were allowed to reassociate at the appropriate temperature (Tm −25 °C) for 16 h (Cot 4).

Synthesis of ^3H labelled complementary RNA

Satellite DNA (100 µl, 3–4 µg) in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.4) was denatured in a boiling waterbath for 5 min. ^3H-Nucleoside triphosphates (UTP, CTP, GTP and ATP; 100 µl each; 1 mci/ml; specific activity 10 Ci/mmol, Amersham) were mixed in a tube and evaporated under vacuum. Denatured DNA (100 µl in TE) was added to the tube. The solution was then adjusted to 40 mM Tris (pH 7.9), 10 mM $MgCl_2$, 160 mM KCl, 0.2 mM dithiothreitol, and 1 mM ATP (by adding cold ATP). *Escherichia coli* RNA poly-

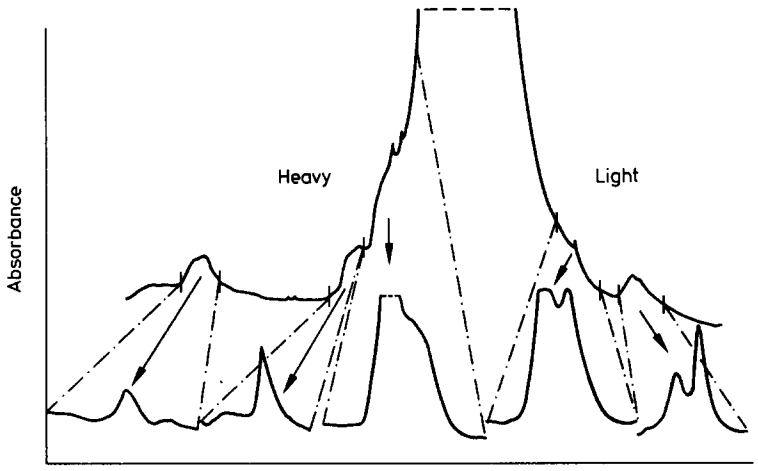

Fig. 1. Optical density (A_{260}) profiles of *Hordeum vulgare* DNA after preparative ultracentrifugation in Ag^+/Cs_2SO_4 gradients. The *upper figure* represents the scan after the first preparative run. Pooled fractions for each satellite marked by vertical line from several gradients were centrifuged again and theirs absorbance profiles are shown in the *lower figures*

merase (10 units, Sigma, USA) was added and the reaction was allowed to proceed at 37 °C with polymerisation (incorporation of label in TCA precipitable material) being monitored at appropriate times. Yeast carrier RNA (150 µg, BDH) was added after 1 h and the reaction was stopped by adding DNase (10 µg; Worthington, DPFF RNase-free). The mixture was left at room temperature (21 ± 1 °C) for 30 min before adjusting it to a final concentration of 0.1% SDS and 10 mM EDTA. An equal volume of TE saturated phenol was added and the mixture was gently stirred for 10 min and then centrifuged at 500 × g for 10 min. The aqueous phase was collected and the phenol layer re-extracted with distilled water (0.2 ml). The pooled aqueous phases were then passed through a column (0.9 cm × 15 cm) of Sephadex G-75 (equilibrated with 0.1 × SSC) to separate unincorporated ^{3}H-nucleotides from the ^{3}H-cRNA. Fractions (about 0.5 ml each) were collected and all label in the exclusion volume was found to be in the cRNA (TCA precipitable material). Fractions containing cRNA were pooled and concentrated by evaporation under vacuum. The solution was made 6 × SSC and stored at −20 °C. Radioactivity was measured in a Beckman LS250 scintillation counter using a toluene based scintillant (0.5% W/v PPO in toluene).

In situ hybridization

a) Preparation of plant materials. Root-tips were collected in distilled water (0 °C), left overnight at 0 °C and then fixed in acetic acid : ethanol (1 : 3) for a minimum of 1 h at 21 ± 1 °C. The fixative was removed and the root-tips were washed in distilled water and treated with 1 M HCl at 60 °C for 5 min. After removal of HCl, 2% cellulysin (in water) was added and the mixture was incubated at 37 °C for 10 min. After washing with water, the root-tips were squashed in a drop of 45% acetic acid using siliconized slides and coverslips. The preparation was scanned under a phase contrast microscope to check for the spread of chromosomes. The slide was placed on dry ice for at least 15 min and the coverslip was removed using a blade. The slides were passed through two changes of absolute ethanol for 5 min each, subsequently air dried, and stored (0°–4 °C) in clean boxes.

b) Hybridization procedure. Preparations were dehydrated in absolute ethanol and air dried. The slides were incubated in 0.2 M HCl at 37 °C for 30 min, followed by dehydration through an alcohol series (50%, 70%, and 100% twice, 2 min each). Labelled cRNA (6–7 µl) was placed on the preparation and covered with a clean coverslip. After sealing the edges of the coverslip with a rubber solution, the slides were incubated in a waterbath at 65 °C for 6–7 h. The slides were finally treated in the following manner:

(i) washed twice in 3 × SSC at 45 °C, 10 min each;
(ii) washed twice in 2 × SSC at 45 °C, 10 min each;
(iii) transferred to RNase, 10 µg/ml in 2 × SSC at 37 °C, for 30 min;
(iv) washed six times in 2 × SSC at room temperature (21 ± 1 °C), 10 min each;
(v) dehydrated through 70% ethanol and 100% ethanol twice, 2 min each and then air dried.

c) Autoradiography. All steps were carried out under an Ilford red light. Ilford K2 emulsion in gel form was heated at 43 °C for 10 min. A 1 : 1 dilution was made with 2% glycerol kept at 43 °C and the mixture was kept at 43 °C for another 5 min. The slides were dipped one at a time and were left on the bench in the dark for at least 2 h to dry. After drying, the slides were placed in a black slide box, sealed with black tape, and kept at 4 °C for various exposure times (10 days to 2 months).

The autoradiographs were developed at 20 °C for 3 min in a developer very similar to that of Kodak D19b, rinsed in water, to which a dash of developer had been added, and fixed in Ilford Hypam rapid fixer, diluted 1 : 4 with distilled water. The slides were washed in running water for 10 min followed by distilled water and were finally air dried.

d) Staining. The preparation was stained with 5% Gurr R66 Giemsa for 20 min followed by differentiation with 0.066 M phosphate buffer (pH 6.8) for 4–5 min. The slides were rinsed in distilled water and air dried.

Results

Buoyant density analysis

The five satellites were isolated in pure form by repeated preparative centrifugations (Fig. 1). Approximate percentages of satellite to total DNA and their buoyant densities in CsCl are presented in Table 1. Each heavy satellite of *H. vulgare* formed a single peak in CsCl gradients, whereas one light satellite (L_1) resolved into two and the other (L_2) into 4 components (Fig. 2). No attempt was made to purify the individual components of the light satellites observed in CsCl gradients.

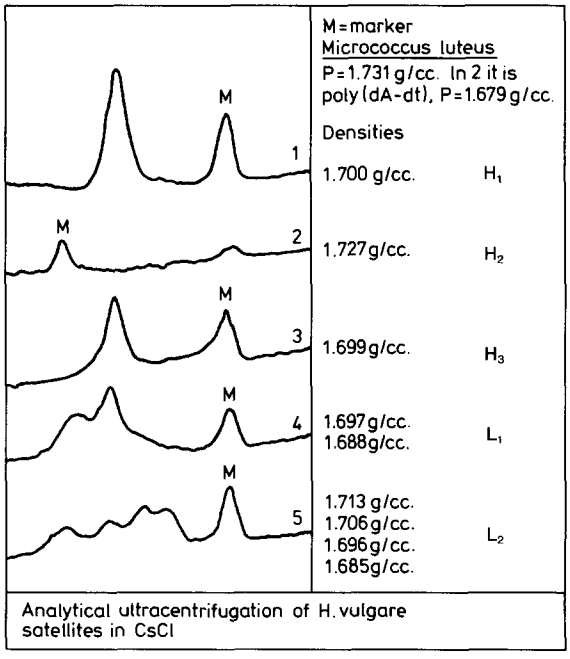

Fig. 2. Optical density (A_{265}) profiles of native satellite DNAs of *H. vulgare* in analytical CsCl gradients. M refers to the maker DNA *Micrococcus luteus* (p = 1.731 g cm^{-3}) or poly (dA–dT) (p = 1.680 g cm^{-3}). Densities of individual components are shown to the right

The amount of H_2 recovered after purification was low, therefore, detailed analysis was not feasible with this satellite. The other four satellite DNAs (H_1, H_3, L_1 and L_2) were denatured (100 °C for 5 min) and then allowed to reassociate to Cot 4. The reassociated H_1, H_3 and L_2 satellites gave single sharp peaks (Fig. 3) in CsCl gradients. Satellite L_1 showed shoulders on the lighter side of a sharp peak (Fig. 3). Buoyant densities of reassociated satellites are given in Table 1.

With the exception of H_1, buoyant densities of the reassociated satellites were higher than their respective native forms. The native L_2 DNA gave four components but the reassociated DNA gave only one, whereas the native L_1 DNA clearly resolved into two components and its reassociated DNA showed heterogeneity in the form of shoulders to a major peak.

In alkaline CsCl gradients, each of the satellites showed a broad peak but no strand separation was evident.

Thermal dissociation profiles and Tms of native and reassociated satellite DNAs are presented in Figs. 4–7 and Table 2, and the data were examined for heteroge-

Table 1. The relative amounts and buoyant densities of *Hordeum vulgare* satellites in CsCl gradients

Satellite[a]	%[b]	No. of components	Buoyant density g/cm³		G+C[c] content
			Native	Reassociated	
H_1	0.86	1	1.700	1.700	46.1
H_2	1.88	1	1.727	–	69.2
H_3	–	1	1.699	1.703	40.1
L_1	2.30	2	(a) 1.697	1.708	38.2
			(b) 1.688		28.6
L_2	1.30	4	(a) 1.713		54.4
			(b) 1.706	1.712	47.6
			(c) 1.696		37.5
			(d) 1.685		25.7

[a] H refers to satellites on the heavy, and L refers to satellites on the light side of main band DNA
[b] Calculated by comparing the area of satellites to an area occupied by a known amount of *M. luteus* DNA in an analytical CsCl gradient (average of five measurements)
[c] Calculated from buoyant density of Native DNA according to Mandel et al. (1968)
– Not estimated

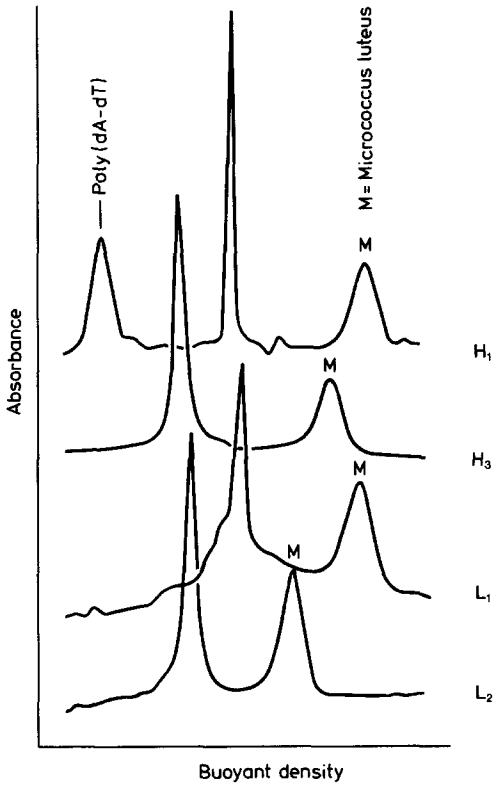

Fig. 3. Optical density (A_{265}) profiles of reassociated (Cot-4) satellite DNAs of *H. vulgare* in analytical CsCl gradients. M represents marker DNA *Micrococcus luteus* ($p = 1.731$ g cm^{-3}) and/or poly (dA–dT) $p = 1.680$ g cm^{-3}

Table 2. Melting temperature of native and reassociated satellite DNAs of *H. vulgare*

Satellite	Native satellite		Reassociated satellite	
	Tm[a]	Thermal components (minimum)[a]	Tm[a]	Thermal components (minimum)
H_1	87[c], 93	2	71, 75.5[a,c]	4
H_2	91[b]	1	not done	not known
H_3	83[c], 91.5	2	67, 71, 77[c]	3
L_1	86[c], 90	3	80, 87, 92.5, 95.5[c]	4
L_2	86.5, 91, 95[c]	3	87, 91, 95[c]	3

[a] Determined from differential plot
[b] Determined from thermal denaturation profile
[c] Major component (occupying maximum area under the curve)

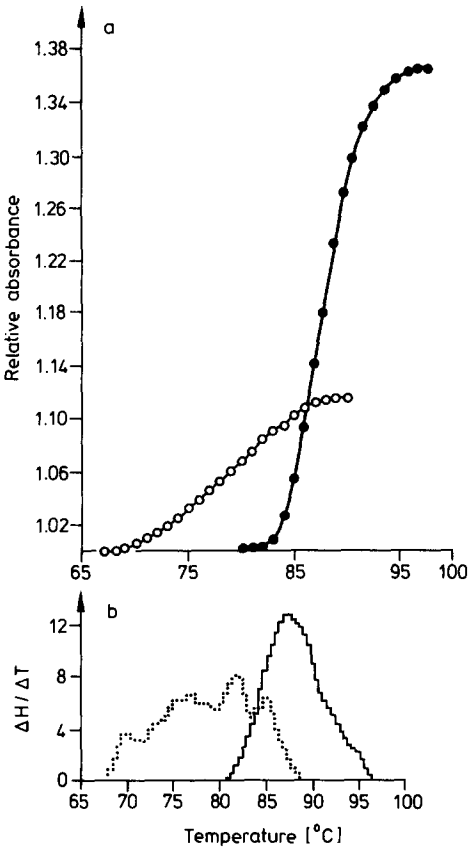

Fig. 4. Thermal denaturation profiles of native (●—●) and reassociated (○—○) H_1 satellite DNA of *H. vulgare* and their differential analysis below

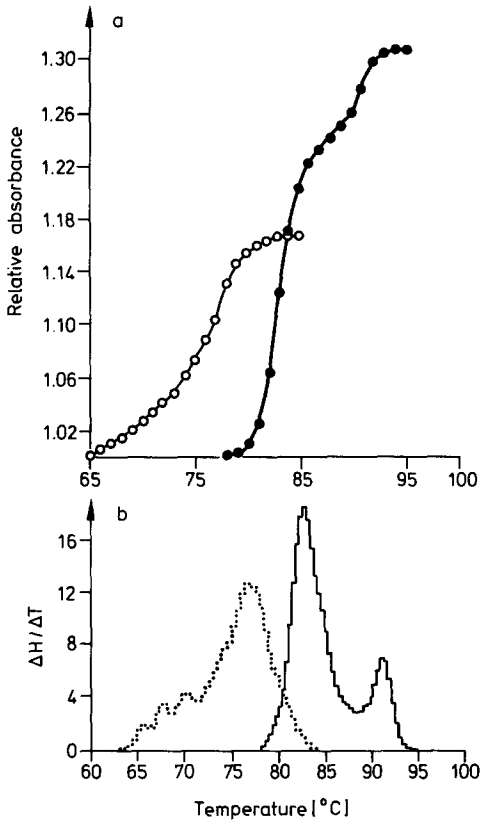

Fig. 5. Thermal denaturation profiles of native (●—●) and reassociated (○—○) H_3 satellite DNA of *H. vulgare* and their differential analysis below

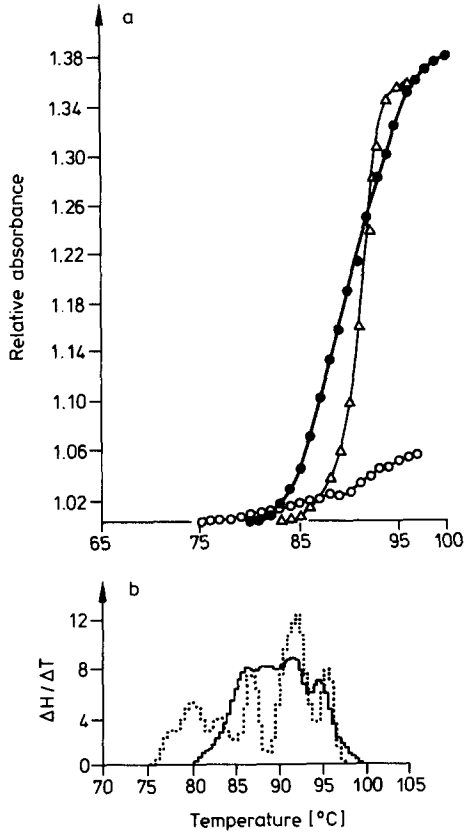

Fig. 6. Thermal denaturation profiles of native (●—●) and reassociated (○—○) L_1 satellite DNA of *H. vulgare* and their differential analysis below (△—△) native *E. coli* DNA

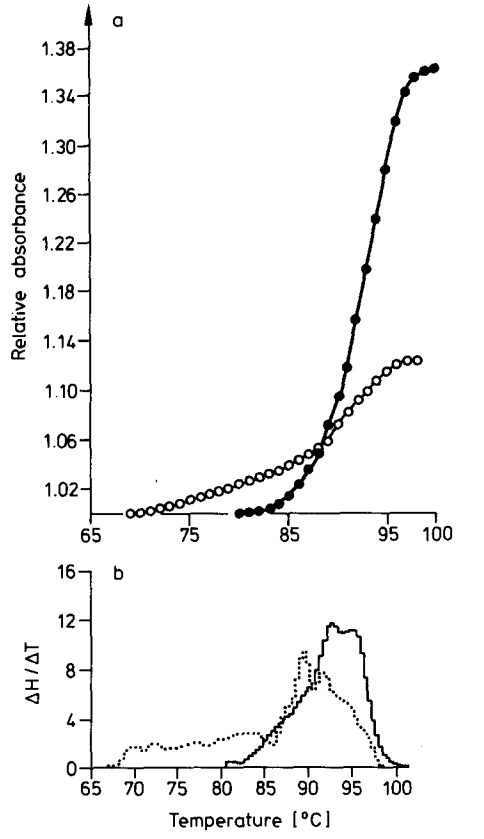

Fig. 7. Thermal denaturation profiles of native (●—●) and reassociated (○—○) L_2 satellite DNA of *H. vulgare* and their differential analysis below

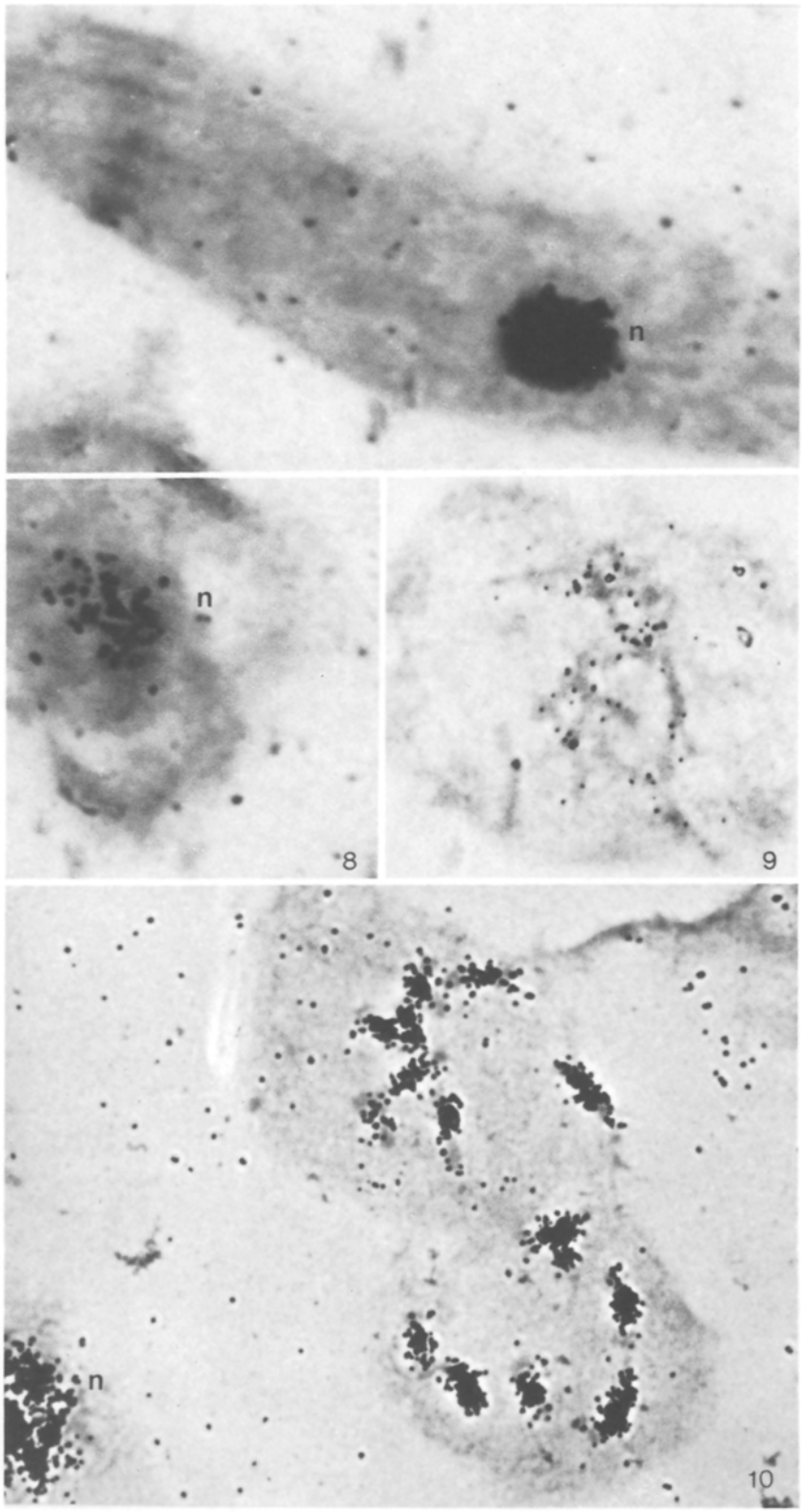

Fig. 8. Distribution of ^3H RNA complementary to H_1 satellite of *H. vulgare* in the nuclei of the same species

Fig. 9. Distribution of H_2 satellite sequences of *H. vulgare* on mitotic chromosmes of the same species. ^3H c-RNA (1×10^4 cpm/μl) was used for in situ hybridization

Fig. 10. Distribution of H_3 satellite sequences of *H. vulgare* on mitotic chromosomes of the same species ($2n + 1^4 = 15$). ^3H c-RNA (7×10^4 cpm/μl) was used for in situ hybridization

neity. Two components were evident from the thermal transition profile and differential analysis of the H_3 satellite DNA (Fig. 5). It was difficult to estimate the number of components from the denaturation profile of L_1 DNA (Fig. 6), but differential analysis (Fig. 6) revealed at least three thermal components. In general, all satellite DNAs contained more than one thermal component. Thermal transitions of all reassociated DNAs were broad and more complex than the original melts in showing more components and with lower Tms.

In situ hybridization

The nuclear localization of the H_1, H_2 and H_3 satellites is clear (Figs. 8–10) since ^{3}H cRNA made from these satellites appears only in nuclei. Although attempts were made to hybridize ^{3}H-RNA complementary to H_1, H_2 and H_3 satellites of *H. vulgare* to chromosome preparations of the same species, only H_3 gave satisfactory results (Fig. 10). All chromosomes of *H. vulgare* contained the H_3 sequences in regions adjacent to centromeres while some chromosomes showed additional sites. The localization of H_2 satellite at some of the centromeres was also apparent (Fig. 9). However, the amount of label of H_1 and H_2 probes on chromosomes was insufficient to permit further interpretation of the distribution of sites. Light satellites did not show hybridization.

Discussion

Density gradient analysis revealed that the light satellites are more complex in their base composition than the heavey ones. The formation of a single band by the native and reassociated heavy satellite in a CsCl gradient and the similarity of the buoyant densities indicate very little sequence mismatch. Each satellite was purified as a single component in a preparative Ag^+/Cs_2So_4 gradient, yet the light satellites revealed more than one component in CsCl gradients (Table 1, Fig. 2). A similar observation has been reported for the light satellite of rye (Appels et al. 1978). The complicated nature of these two light satellites is also apparent from the buoyant densities of reassociated satellite DNAs.

At alkaline pH (9.2 used in this study), Ag^+ binds preferentially to the (A+T)-rich fraction of calf DNA (Filipski et al. 1973; Macaya 1976). If this is true for all eukaryotic DNA then heavy satellites, as a whole, must be different from the light satellites in their base composition because these two types of satellites show quite different densities in a Ag^+/Cs_2SO_4 gradient and are separated by the main band. The buoyant densities of two of the three heavy satellites are very close to that of the main band DNA. The H_2 satellite of *H. vulgare* is (G+C)-rich (69%) compared to that (41%) of other heavy satellites. On the other hand, light satellites have density components (in CsCl) which appear to be (A+T)-rich. Moreover, the G+C content obtained for each satellite using two established procedures (buoyant density and Tm) was markedly different. The anomalous nature of satellite DNAs could be explained by assuming that "the normal relationships between base composition, buoyant density and temperature of denaturation do not necessarily apply to satellite DNA" (Corneo et al. 1968). Ranjekar et al. (1978) suggested that satellite DNA may be conformationally different from main band DNA in barley and wheat.

The purified 1.701 g cm^{-3} component of the Cot 0.2 fraction of rye DNA is known to produce a hypersharp peak in CsCl and electron microscopic evidence suggests that this is due to aggregate formation, a characteristic of renatured DNA where the repeating sequence is shorter than the fragment length (Appels et al. 1978). If this is a general phenomenon for highly repeated plant DNA, this would explain the observed hypersharp nature of reassociated satellite DNAs of barley. The L_2 satellite of *H. vulgare* shows the presence of four components in CsCl, but after renaturation it bands as a single sharp peak. This could only be explained by assuming that the base compositions of the individual components are similar enough to allow cross hybridization. Similarly, it can be argued that two components of the L_1 satellite of *H. vulgare* are similar in base sequences.

Earlier studies of thermal denaturation of satellites utilized plant species where only one satellite is present. In *H. vulgare* only one of the two satellites (Ranjekar et al. 1976) was studied by Ranjekar et al. (1978). When thermal denaturation profiles of satellite DNAs from different species are compared, some of them are monophasic (Capesius 1976; Timmis and Ingle 1977; Ranjekar et al. 1978) while others are biphasic (Bendich and Anderson 1974; Sinclair et al. 1975; Chilton 1975; Deumling and Nagl 1978). The satellites of *Hordeum vulgare* also fall into these two classes. The use of differential analysis in the present work revealed a more complex situation than the thermal transition profile suggests.

The H_3 satellite in the present study probably corresponds to the satellite I of *H. vulgare* described by Ranjekar et al. (1978). The density of this satellite is very close to that of the main band DNA. The biphasic thermal transition, Tm's of the two components and Tm's of reassociated satellites are similar in the two studies.

The low Tm of reassociated DNA is caused by base mismatching (Bonner et al. 1973). Since native and reassociated satellites of *H. vulgare* are multicomponent, it is difficult to estimate the base mismatch (usually calculated from the difference in the Tm's of native and reassociated DNA). Since binding of Ag^+ to DNA is specific at alkaline pH (Filipski et al. 1973), the forma-

tion of satellites on the lighter side of the main band indicates that these sequences are compositionally or conformationally different from the rest of the DNA and probably GC rich. Composition and conformation may not be mutually exclusive, since the former may influence the latter.

The buoyant densities of component (a) of L_1 and (c) of L_2 are similar to those reported for plant chloroplast DNA (Wells and Ingle 1970; Kung 1977). Component (b) of L_2 DNA has a buoyant density which is similar to the density of mitochondrial DNA of plants (Wells and Ingle 1970). The similarities of the densities may be coincidental, further work is necessary to elucidate such a possibility.

In situ hybridization studies using ^3H cRNA of satellites show the location(s) of the satellite sequences on the chromosomes and give a characteristic pattern to individual chromosomes. In rye, the heterochromatic regions (C-band positive) of the chromosomes are the sites where the highly repeated sequences are mostly located (Appels 1978). In *Scilla* too, satellite DNA has been reported to be localized at the heterochromatic parts of the chromosomes which are again distal (Timmis et al. 1975).

The C-banding pattern has already been published for *H. vulgare* (Noda and Kasha 1976; Linde-Laursen 1976). According to Noda and Kasha (1976), C-bands are mainly located adjacent to centromeres and the distal regions are devoid of C-bands. In 'Emir' barley telomeres of some chromosomes and secondary contrictions of chromosome 6 and 7 are C-band positive (Linde-Laursen 1976). However, in both studies the bulk of heterochromatin (C-bands) was found to be located near centromeric regions.

Radioactive RNA complementary to the H_3 satellite of *H. vulgare* hybridize mainly to regions adjacent to centromeres. In addition to the centromeric locations of the H_3 sequences, some chromosomes of the *H. vulgare* complement showed additional sites. No hybridization of ^3H cRNA of light satellites on the chromosomes was observed. This failure of hybridization may imply that the light satellites do not contain highly repeated DNA as has been suggested for the light satellite of rye (Appels et al. 1978). These sequences could also be highly interspersed among non-repeated DNA. Further experiments are required to elucidate these aspects.

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