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Analysis and in situ hybridization of cryptic satellites in *Hordeum arizonicum*

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Summary. Three satellites, one (H_1) on the heavy side of the main band of *Hordeum arizonicum* DNA and two (L_1, L_2) on the lighter side were purified using preparative silver-cesium sulphate density gradients. The native and the reassociated satellite DNAs were analysed in terms of buoyant densities and thermal dissociation. In cesium chloride gradients the H_1 and L_1 satellites formed single peaks corresponding to buoyant densities of 1.700 and 1.701 g · cm^{-3} respectively while the L_2 satellite gave two peaks (1.680 and 1.661 g · cm^{-3}). The H_1 satellite showed three thermal components (Tm = 82.5 °C, 87 °C and 91.5 °C) while the L_1 and L_2 had three (86.5, 92, 97.5 °C) and two (86, 95 °C) respectively. The H_1 satellite was localized on the nuclei and chromosomes. The distribution of H_1 onto approximately on third of the complement may reflect the genome specific origin of this satellite.

Key words: DNA characterization – Cryptic satellites – Cytological localization – Buoyant density – Thermal denaturation – *Hordeum*

Introduction

It has been shown (Chakrabarti and Subrahmanyam 1985) that six *Hordeum* species contain both light and heavy cryptic satellites. Although DNA satellites have been reported for several plants, very few have been characterized in detail. Thermal denaturation profiles of satellites from wheat (Ranjekar et al. 1978 a) flax (Timmis and Ingle 1977) and *Cymbidium* (Capesius 1976) show monophasic transitions, whereas those of melon (Bendich and Anderson 1974; Bendich and Taylor 1977; Sinclair et al. 1975), tomato (Chilton 1975), cucumber (Timmis and Ingle 1977) and *Tropaeolum* (Deumling and Nagl 1978) are biphasic, indicating the presence of two components. Upon reassociation, however, all these satellites revealed the presence of two components.

Chromosomal localization of satellites have been reported for three species of plants, *Scilla* (Timmis et al. 1975), rye (Appels et al. 1978) and wheat (Peacock et al. 1977 a, b). In at least two of these (*Scilla* and rye) the satellites are localized in distal heterochromatic regions.

Peacock et al. (1977 a) make passing reference to the isolation and localization of one satellite from *H. vulgare* but no details are provided. In *H. vulgare* two satellites have been reported by Ranjekar et al. (1976), one of which was characterized in terms of thermal denaturation and reassociation (Ranjekar et al. 1978 a) and revealed the presence of two components. A comparison of the physical properties of one wheat satellite with one of the two satellites of barley revealed that they differ in thermal dissociation profiles and most probably in the kinetic complexities of the major fast reassociating DNA fractions (Ranjekar et al. 1978 a).

No comparative data concerning the physiochemical properties of satellite DNA from related species within the same genus is available. This paper describes the isolation and purification of the satellites from *H. arizonicum*, a hexaploid wild species representing the end product of a polyploid series. Purified satellite DNAs have been analyzed in terms of their physical properties and their localization on the chromosomes by in situ hybridization.

Material and methods

Hordeum arizonicum (6x) and its trihaploids were obtained in earlier studies (Subrahmanyam 1980). All plants were maintained in a greenhouse at 24° ± 3 °C.

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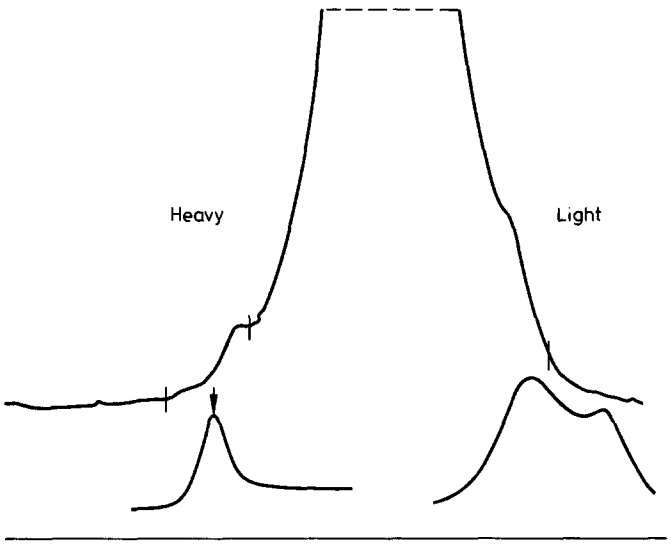

Fig. 1. UV absorbance profiles of *H. arizonicum* DNA after preparative ultracentrifugation in $Ag^+Cs_2SO_4$ gradients. *Upper figure* represents the first preparative step. Fractions selected for each satellite are shown by *vertical lines* on the figure. The preparative step was repeated using pooled fractions for each satellite from several gradients; their absorbance profiles are shown in the *lower figure*. Originally, the lower figure was of the same scale as the upper one but is now shown in reduced size. A third preparative step was necessary to separate L_1 and L_2

Table 1. Buoyant densities of *Hordeum arizonicum* satellite DNAs in CsCl gradients

Satellite[a]	No. of components(s)	Buoyant density (g/cm³)		% G+C[b] (native)
		Native	Reassociated	
H_1	1	1.700	1.703	41.06
L_1	1	1.701	1.698	42.35
L_2	2	i) 1.680	1.694	21.03
		ii) 1.661	1.691	1.60

[a] H refers to the satellite on the heavy side of the main band DNA and L refers to the satellite on the light side
[b] Calculated from buoyant density of native DNA according to Mandel et al. (1968)

DNA extraction

Shoots of two- to three-week old tillers of *H. arizonicum* were used for the extraction of DNA following the procedure described earlier (Subrahmanyam and Azad 1978) but omitting the ribonuclease step as advised by Marmur (1963) in cases where DNA is to be used as a primer for complementary RNA (cRNA) synthesis.

Preparative Ag^+/Cs_2SO_4 gradient centrifugation

R_{FS} for best resolution were obtained by analytical Ag^+/Cs_2SO_4 centrifugation (Chakrabarti and Subrahmanyam 1985). The optimum R_F for *H. arizonicum* was 0.2. The details of the procedure have been described in the preceding paper (Chakrabarti and Subrahmanyam 1986). The absorbance profile of the fractionation of the gradient is shown in Fig. 1. 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 Cs_2SO_4 was the same as the pooled fractions and no adjustment was made. Absorbance profile of the second preparative gradient is also shown in Fig. 1. A third preparative step was necessary for the purification of L_1 and L_2 satellites (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 −10 °C.

Buoyant density determinations, thermal dissociation, reassociation kinetics, labelling of complementary RNA, in situ hybridization and staining procedures have been described in detail in the preceding paper (Chakrabarti and Subrahmanyam 1986).

Results

Buoyant density analysis

One heavy and two light satellites were purified by repeated preparative centrifugation in Ag^+/Cs_2SO_4 (Fig. 1). Buoyant densities of the satellites in CsCl are listed in Table 1, and the UV absorbance profiles are presented in Fig. 2. One heavy and one light satellite (L_1) each produced rather a broad peak in CsCl; the other light satellite (L_2) resolved into two peaks.

After denaturation, each satellite DNA was allowed to reassociate to a Cot of about 3 and then centrifuged in an isopycnic CsCl gradient (Fig. 3). Both H_1 and L_1 gave sharp single peaks, probably with a small shoulder

Table 2. Melting temperatures (Tm's) of native and rassociated satellites of *Hordeum arizonicum*

Satellite	Native		Reassociated	
	Tm (°C)[a]	Thermal components	Tm (°C)[a]	Thermal components
H_1	82.5[b], 87, 91	3	67, 71.5, 75[b], 82	4
L_1	86.5[b], 92, 97.5	3	70, 75, 81.5[b], 87	4
L_2	86, 95	2	67.5, 73, 77.5[b], 82[b]	4

[a] Determined from the differential plot after reconstruction from the contributing components
[b] Major components

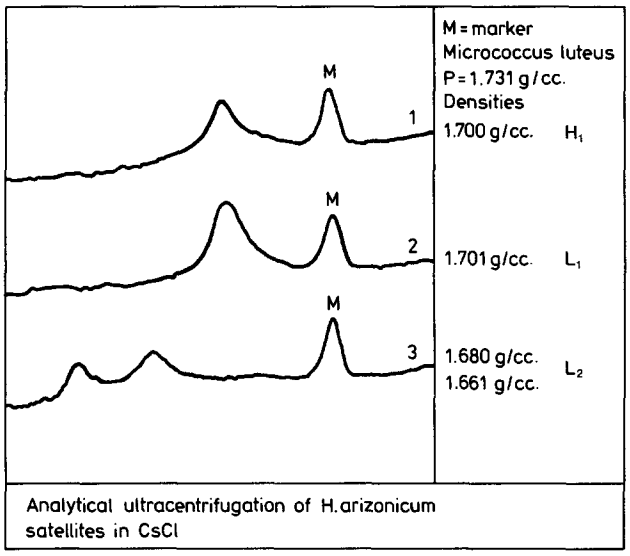

Fig. 2. UV absorbance profiles of *H. arizonicum* satellites in analytical CsCl gradients (neutral). Densities of the components are shown *on the right.* M refers to marker DNA (*Micrococcus luteus,* P = 1.731 g/cm³)

on the heavier side; the other light satellite (L_2) showed two peaks (Fig. 3). Buoyant densities of the reassociated satellites are listed in Table 1.

Thermal dissociation

The melting temperatures of the satellites are presented in Table 2. The same considerations which were followed for *H. vulgare* satellites (Chakrabarti and Subrahmanyam 1986) were used when determining Tm's from the differential plots. The native heavy satellite (H_1) displayed a biphasic transition, indicating at least two components (Fig. 4 a) but the differential plot showed three components with Tm's at 82.5 °C, 87 °C and 91 °C respectively (Fig. 4 b). The seemingly simple denaturation profile of the reassociated H_1 turned out to be complex on differential analysis and had possibly four components, the principal one with a Tm of 75 °C. Thermal denaturation of the L_1 satellite also showed a multicomponent pattern (Fig. 5). By contrast, the native L_2 DNA gave a 'nearly perfect' thermal transition profile (Tm = 86 °C) with the possibility of a minor

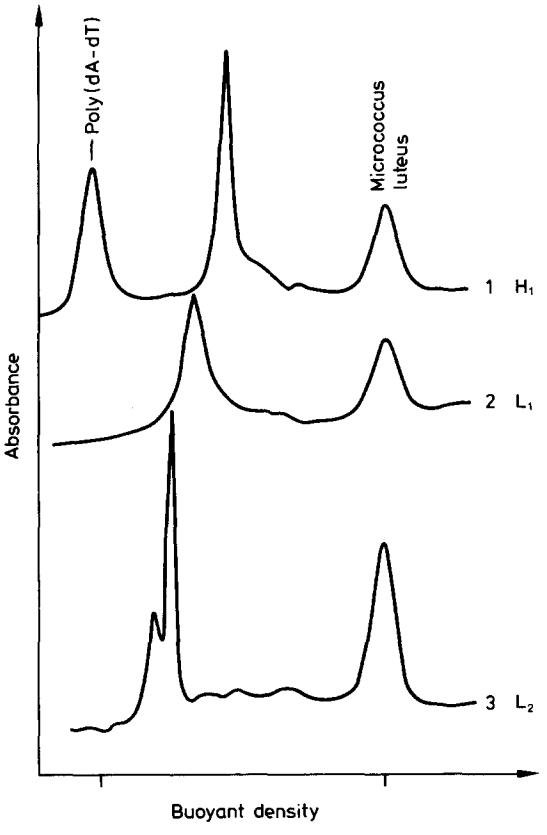

Fig. 3. UV absorbance profiles of reassociated (Cot 3) satellite DNAs of *H. arizonicum* in analytical CsCl gradients (neutral). *Micrococcus luteus* DNA (p = 1.731 g/cm³), or poly (dA–dT) (P = 1.680 g/cm³), or both, were used as reference marker(s)

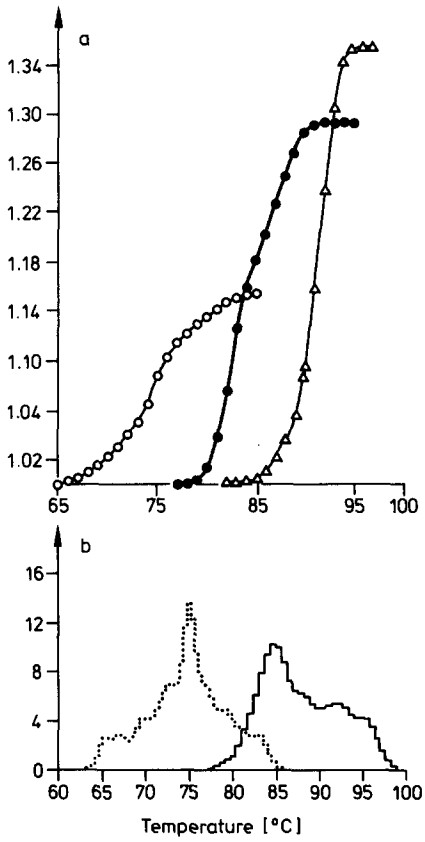

Fig. 4. a Thermal denaturaion profiles of native (●—●) and reassociated (○—○) H_1 satellite DNA of *H. arizonicum,* and of native *E. coli* DNA (△—△); **b** Differential analysis of denaturation data for native (*solid line*) and reassociated (*dotted line*) H_1 satellite DNA

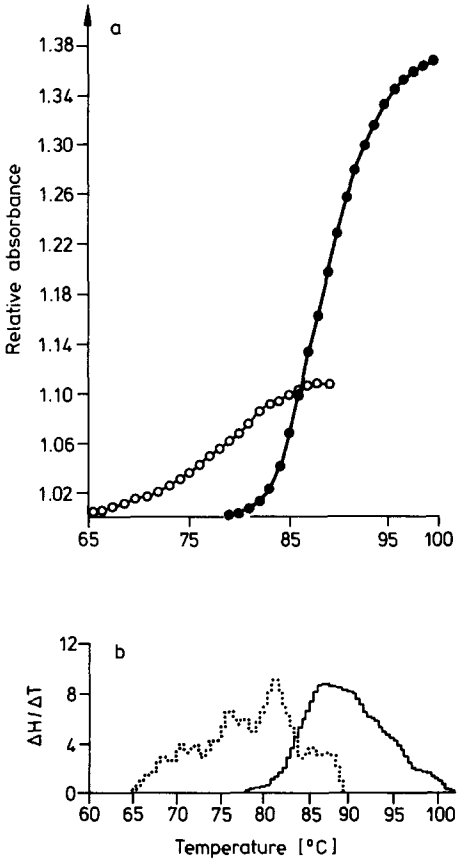

Fig. 5. **a** Thermal denaturation profiles of native (●—●) and reassociated (○—○) L_1 satellite DNA of *H. arizonicum;* **b** Differential analysis of Fig. 5a; native DNA (*solid line*); reassociated DNA (*dotted line*)

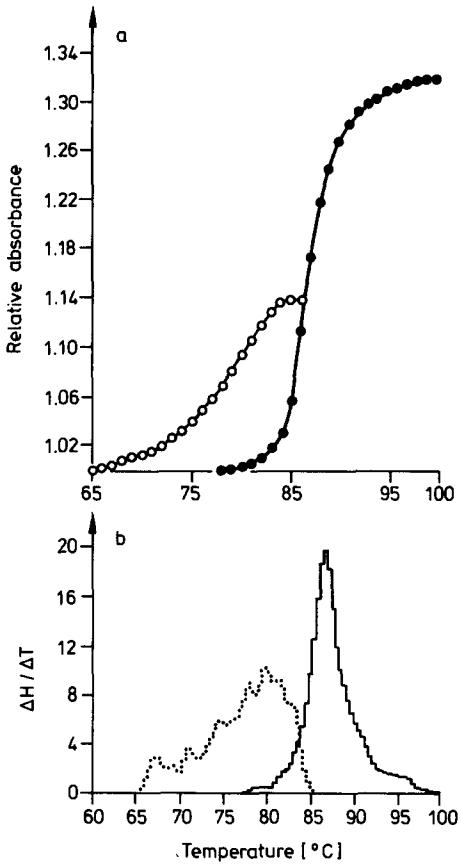

Fig. 6. **a** Thermal denaturation profiles of native (●—●) and reassociated (○—○) L_2 satellite DNA of *H. arizonicum;* **b** Differential analysis of Fig. 6a; native DNA, *solid line;* reassociated *dotted line* DNA

component at Tm 95 °C. Reassociated L_2 was, however, complex, with probably four components having Tm's of about 67.5 °C, 73 °C, 77.5 °C and 82 °C respectively (Fig. 6).

In situ hybridization

Radioactive complementary RNA (^{3}H-cRNA) synthesized from each of the satellites was hybridized with chromosomes of *H. arizonicum.* For convenience of chromosome spread, trihaploid plants (n=3x=21) of *H. arizonicum* obtained via selective elimination of *H. vulgare* chromosomes following hybridization (Subrahmanyam 1980) were used. The same in situ hybridization procedure (Chakrabarti and Subrahmanyam 1986) was used but only the heavy satellite hybridized well with the chromosomes (Fig. 7). Light satellites, on the other hand, did not show any hybridization. As in the case of the light satellites of *H. vulgare* (Chakrabarti and Subrahmanyam 1986) no further attempts were made to optimize the steps involved in the hybridization procedure. While it was not possible to carry out complete karyotypic analyses, some of the chromosomes had little or none of the heavy satellite. One third of the complement became labelled and the label was found around the centromeric regions.

Discussion

Density gradient analysis revealed that the light satellites are more complex in their base composition than the heavy ones. Native and reassociated heavy satellites formed a single band in a CsCl gradient and the buoyant density of the latter is not much different from that of the native DNA (Table 1), indicating very little sequence mismatch. Each satellite was purified as a single component in a preparative Ag^+/Cs_2SO_4 gradient, yet the L_2 satellite revealed two components upon analytical ultracentrifugation in CsCl gradient (Table 1). A similar observation was reported for the light satellite of rye (Appels et al. 1978). The L_1 of *H. arizonicum* is different from the L_2 satellite in

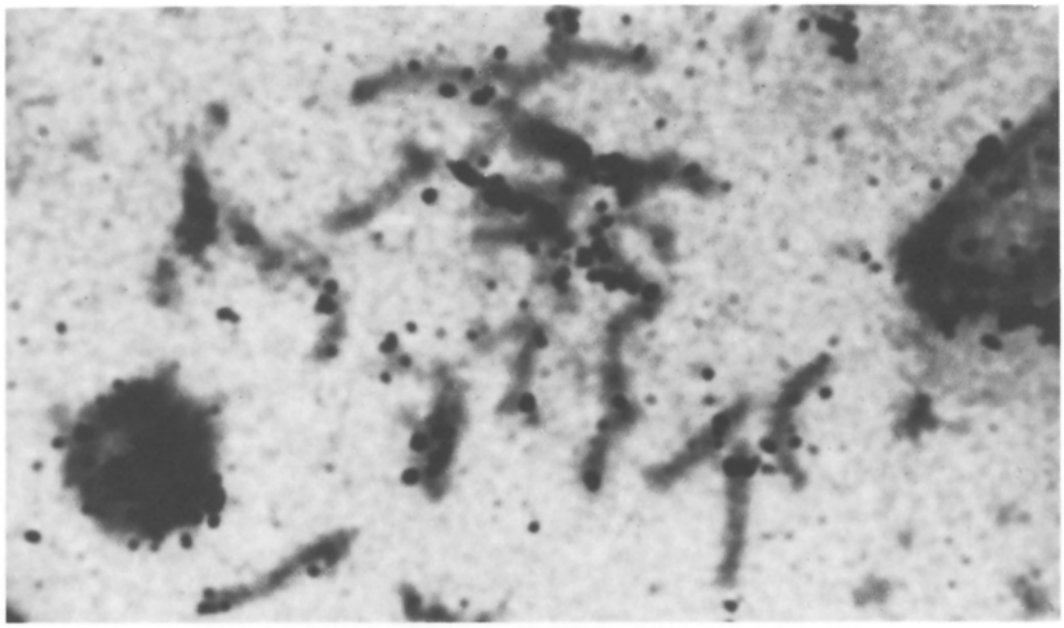

Fig. 7. Distribution of HI^2 sequences of *H. arizonicum* in the nuclei and mitotic chromosomes of the same species (3n = 21). ^3H-cRNA (6×10^4 cpm/μl) was used for in situ hybridization

showing a single peak in a CsCl density gradient profile.

The complicated nature of the L_2 satellite is apparent from the buoyant densities of reassociated components which are much heavier than those of their respective native DNAs except that the density of reassociated L_1 DNA of *H. arizonicum* is slightly lower (0.003 g cm^{-3}) than that of its native DNA. This latter observation is very unusual in the sense that even if one obtains perfect reassociation the density should be the same as that of the native form, not lower.

At an alkaline pH (9.2 was 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 DNAs then heavy satellites, as a whole, must be different from the light satellites in their base composition. This is because these two types of satellites band in quite different densities in a Ag^+/Cs_2SO_4 gradient and are separated by the main band. The buoyant density of the heavy satellite is the same as that of the main band DNA, consequently G+C content determined from buoyant density is also very close. With the exception of the L_1 satellite of *H. arizonicum*, light satellites have density components (in CsCl) which appear to be A+T-rich. Moreover, the G+C content obtained for each satellite by using the established procedures (buoyant density and Tm) is 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) made similar observations in barley and wheat and suggested that satellite DNA may be conformationally different from main band DNA.

The purified 1.701 g/cm^3 component of the Cot 0.02 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 (Chakrabarti and Subrahmanyam 1986). This could only be explained by assuming that the base compositions of the individual components are similar enough to allow cross hybridization. The L_2 DNA of *H. arizonicum* consists of two density classes and unlike both the L_1 and L_2 of *H. vulgare* (Chakrabarti and Subrahmanyam 1985) these classes retain their identity after renaturation.

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*

species also fall into these two classes. The use of differential analysis in the present work reveals that the situation is often more complicated than the thermal transition profile suggests (Chakrabarti and Subrahmanyam 1986).

The physical properties of the *H. vulgare* H_3 satellite and of the *H. arizonicum* H_1 satellite are very similar. Both satellites are similar in respect of the buoyant densities of native and reassociated DNAs, the biphasic melting profiles, the Tm of two thermal components and the Tm of the major reassociated component. The only significant difference appears to be the occurrence of an additional thermal component (Tm = 87 °C) in the H_1 of *H. arizonicum*. It is reasonable, therefore, to suggest that the base composition of the two similar thermal components are also similar in these two species.

There is other evidence which suggests that each species has its own characteristic sequences. In *H. vulgare,* for example, in addition to H_3, there are two more heavy satellites, the last two being undetectable in *H. arizonicum*. Similarly, there are differences in the components of the light satellites.

Attempts to hybridize ^{3}H-RNA complementary to the heavy satellite with the chromosomes were successful. The procedures for cRNA synthesis and in situ hybridization were the same for heavy and light satellites, yet no hybridization was observed between ^{3}H-cRNA of light satellites and chromosomes. The simplest explanation for failure of hybridization is that the light satellites do not contain highly repeated DNA as has been suggested for the light satellite of rye (Appels et al. 1978). Since binding of Ag^+ to DNA is specific at an alkaline pH (Filipski et al. 1973), the banding 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. Composition and conformation may not be mutually exclusive, since the former may influence the latter. A third explanation for the failure of hybridization of the light satellites may be that these sequences are highly interspersed among other non-repeated DNA.

In *H. vulgare,* 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). These similarities of the densities may be coincidental, but one can not rule out the possibility that they are contributed by organelle DNA since the source of the DNA was leaf tissue.

In situ hybridization studies using ^{3}H-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 localized at the heterochromatic parts of the chromosomes which are again distal (Timmis et al. 1975).

Radioactive RNA complementary to the H_3 satellite of *H. vulgare* (Chakrabarti and Subrahmanyam 1986) and the H_1 satellite of *H. arizonicum* 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 contain the sequence on other parts. Some chromosomes (2/3) of *H. arizonicum* do not contain detectable amounts of its H_1 satellite sequences indicating that only one genome out of the three carries H_1 sequences as was also evident from the dilution effect seen in the proportion of this satellite at the hexaploid level compared to its diploid progenitor *H. californicum* (Chakrabarti and Subrahmanyam 1985).

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