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Localisation of foldback DNA sequences in nuclei and chromosomes of Scilla, Secale, and of mouse

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ABSTRACT

Foldback DNA, prepared from mouse and Scilla sibirica main band DNA, and from rye (Secale cereale) total DNA, was characterised by denaturation, renaturation, and electron microscopy. ^{3}H-cRNA of this DNA was hybridised in situ to nuclei and chromosomes of the respective species.

There is no universal labelling pattern among the three species. In mouse, highly repetitive foldback DNA is present in the whole chromatin including the satellite DNA-containing regions. In Scilla sibirica, on the contrary, the highly repetitive foldback sequences are excluded from the satellite DNA loci and are arranged in clusters in the remaining chromatin. In rye, there is a clear preferential labelling of the chromocenters in the interphase nuclei as well as metaphase chromosomes, indicating that highly repetitive foldback DNA is preferentially located among other highly repetitive sequences.

INTRODUCTION

Extremely fast renaturing DNA fractions consisting of inverted repeated sequences have been found in many organisms and have so far been studied by biochemical characterisation and by electron microscopy. However, there are as yet no data available as to the localisation of these sequences in the cell nuclei (exception: human foldback DNA, 1) though in situ hybridisation experiments should provide answers to some questions:

a) Is part of the inverted repeated DNA higher repetitive? Since foldback DNA consists of a considerable amount of "unlooped hairpins" which are not accessible to single strand specific nucleases, it is not possible to elucidate this problem with denaturation-renaturation measurements in solution

or on hydroxyapatite. However, to show significant labelling over the chromatin after *in situ* hybridisation, the sequences must be repetitive to a considerable degree or, at least, very similar to each other (2).

b) Where in the genome is highly repetitive foldback DNA localised? Is it evenly distributed or is it located in distinct regions of the nuclei? Are there similarities in the localisation of foldback DNA among different organisms? And, for satellite DNA-containing genomes: Is highly repetitive foldback DNA present in the satellite DNA-containing regions as well?

In the present study, ^{3}H-RNA, complementary to foldback fractions prepared from main band DNA of mouse and *Scilla sibirica*, and of total DNA from rye, was hybridised *in situ* to cell nuclei and chromosomes. Since it is to be supposed that denatured foldback DNA reassociates very fast in the chromatin too, denaturation and hybridisation were carried out in a cRNA-SSC-formamide mixture (SSC: 0.15 M NaCl, 0.015 M Na_3-citrate) without intermediate interruption. Squashed preparations and semithin sections are shown. The data were compared with the results of *in situ* hybridisation with radioactive satellite cRNAs, carried out in parallel.

MATERIALS AND METHODS

Materials

White mice, bulbs of *Scilla sibirica*, and rye seeds (*Secale cereale*) were obtained commercially. Radioactive nucleoside triphosphates (^{3}H-ATP, 17.3 Ci/mmol; ^{3}H-CTP, 27 Ci/mmol; ^{3}H-GTP, 14 Ci/mmol; ^{3}H-UTP, 40 Ci/mmol) were from Amersham Buchler, Amersham, England. RNA polymerase was from Boehringer, Mannheim (Germany), and S_1-nuclease was from Sigma Chemicals.

Scilla bulbs were grown on wet sand or soil in the cold (5-10°C) for 1-2 months. Rye was grown either on wet filter paper at ca. 23°C for 3 days or in soil in the garden until flowering.

DNA extraction and fractionation

Plant leaves and mouse livers were homogenised in a lysis medium consisting of 0.5 M NaCl, 40 mM Na_2-EDTA, 0.7% sodium dodecyl sulphate (SDS). The homogenate was heated to 60°C for

10 min, cooled and extracted twice with an equal volume of a mixture of chloroform/isoamyl alcohol/phenol (24:1:25 v/v), and three times with diethyl ether to remove the phenol. $CaCl_2$ was added to the remaining supernatant to bind the EDTA. The solution was buffered by addition of sodium phosphate buffer of pH 6.8 (NaP; equal amounts of NaH_2PO_4 and Na_2HPO_4) to a final molarity of 0.18 M, and passed through a column of cellulose powder. The effluent was then chromatographed on granulated hydroxyapatite (3) at room temperature by successive elutions with 0.18 M NaP and 0.5 M NaP. The DNA fractions were concentrated and dialysed against 10 mM Na_2SO_4 (Scilla sibirica, mouse) or 0.1 M NaP (rye) respectively.

Scilla and mouse DNA were fractionated into main band and satellite DNA in Ag^+-Cs_2SO_4 gradients. The Ag^+/DNA molar ratio was 0.26 for Scilla and 0.27 for mouse DNA. Care was taken that the main band fractions were free of satellite DNA. Therefore, the main band fractions used in this study were incomplete since the intermediate fractions between main band and satellite DNA were added to the satellite fractions. The mouse satellite DNA was further purified twice by centrifugation in CsCl density gradients.

Preparation of the fast renaturing DNA

a) The main band fractions of Scilla sibirica and mouse and the total rye DNA were dialysed against 0.1 M NaP, denatured for 10 min at 105°C, cooled for 30 sec to 65°C, and fractionated on hydroxyapatite at 65°C into single and double stranded DNA (C_0t = 0.1 - 0.2).

b) The double stranded fraction thus obtained was dialysed against 0.1 M NaP, denatured again for 7 min at 105°C, renatured to $C_0t = 10^{-4}$, diluted quickly with ice-cold 3 mM NaP, and dialysed immediately against ice-cold 30 mM Na-acetate, 10 mM NaCl, pH 4.6. After addition of S_1-nuclease, the mixture was incubated at 40°C for 1 h. The remaining double stranded DNA, consisting of the total inverted repeated DNA, was purified on hydroxyapatite and dialysed extensively against NaP or Tris-HCl, pH 7.9. The effectiveness of the S_1-nuclease treatment under the described conditions was tested by comparison of the remelting curves of non-treated and S_1-nuclease-treated renatured DNAs.

Thermal denaturation and renaturation kinetics; preparation of the highly repetitive fractions

a) The total inverted repeated DNA, deprived of loops (see above) was denatured in 0.12 M NaP at a heating rate of 0.5°C/min in a Gilford spectrophotometer with thermocuvette and automatic recorder.

Renaturation was carried out in 0.1 M NaP at 52°C (mouse) or 61°C (Scilla) or 58°C (rye) after denaturation for 7 min at 105°C. Thereafter the samples were dialysed against 30 mM Na-acetate, 10 mM NaCl, pH 4.6, and treated again with S_1-nuclease.

b) Satellite fractions of Scilla and mouse (in 0.1 M NaP) were denatured, renatured to C_0t = ca. 0.2 at 25°C below the respective T_m and treated with S_1-nuclease as described before.

Synthesis of cRNA

The synthesis of ^{3}H-cRNA was carried out as described previously (4), with four radioactive triphosphates (mean specific radioactivity: 24.5 Ci/mmol). The following fractions were used as template DNA: foldback DNA from Scilla and mouse main band, and from rye total DNA, mouse and Scilla satellite DNA, and "ordinary" repetitive DNA from rye. To prevent the effect of a possible remaining slight contamination of the foldback fractions with satellite DNA, non-radioactive satellite-cRNA was prepared also and used as carrier RNA with these fractions (this means a dilution of the radioactive with non-radioactive cRNA of at least 1:200). Soluble yeast RNA was used as carrier RNA for the other ^{3}H-cRNAs. After elution from the Sephadex column the cRNAs were dried and dissolved again to a concentration of 2/ug/ml radioactive cRNA in 2xSSC/formamide (sufficient formamide to decrease the T_m to 30°C above room temperature).

Preparation of the tissues and in situ hybridisation

Plant root tips were pretreated for 4 h with 0.25% colchicine. Then, root tips, rye anthers, and mouse testes were fixed in ethanol/glacial acetic acid (3:1 v/v) for 1 h, and incubated in 45% acetic acid over night at 4°C.

a) Part of the material was squashed onto slides and air dried. The slides were treated with 50/ug/ml RNAse for 2 h at room temperature, washed several times in SSC, dehydrated with

75% and 96% ethanol, and air dried. 3-4 µl of the ^{3}H-cRNA/formamide mixture was put onto the slides, covered with a coverslip and heated in moist chambers at 70°C for 15 min to denature the DNA. Then the slides were quickly transferred onto metal plates in moist chambers, kept at room temperature, and allowed to hybridise for 35-40 min. Thereafter the slides were washed 3 x 10 min in 2 x SSC, followed by treatment with RNAse (50 µg/ml) for 60 min at 37°C. The slides were washed 3 x 30 min in 2 x SSC, passed through 75% and 96% ethanol, and air dried. The covering of the slides with Ilford L4 emulsion, exposure at 4°C, and development were done by standard methods.

b) Small pieces of fixed tissue were incubated in SSC for 2 x 30 min; treated with 100 µg/ml RNAse at room temperature for 2 h, dehydrated and hydrated again through an alcohol series to inactivate the RNAse, and infiltrated for 2 x 30 min with 2 x SSC containing the same concentration of formamide as the cRNA solution used for the in situ hybridisation. Then this solution was sucked off, ^{3}H-cRNA solution was added and allowed to stand for 30 min at room temperature, followed by heating for 15 min at 70°C, and hybridisation for 45 min at room temperature. The denaturation and hybridisation were performed in tightly covered small vials. After the hybridisation the samples were washed with 2 x SSC three times for each 30 min, dehydrated through an alcohol series and embedded in epoxy resin according to Spurr (5). Sections of 1 µ thickness were cut, spread onto glass slides, covered with liquid Ilford L4 emulsion and treated further by conventional methods.

The results show that the formamide method used in this study is comparable to the method of Gall and Pardue (6), usually employed. The chromosomal structure is still reasonably well preserved. The observation of Singh et al. (7) that longer times of exposure are necessary after denaturation with formamide cannot be confirmed here because the exposure times were at least equal or even shorter than in standard hybridisations (see also 8). Hybridisation to fixed tissue pieces was also successful though the cRNA did not infiltrate the tissue beyond about two cell layers. However, this method has the advantage that the three-dimensional arrangement of the repeti-

tive DNA fractions can be shown on sections.

DNA spreading and electron microscopy

Scilla and mouse main band DNA and rye total DNA was denatured for 10 min, renatured to $C_0t = 10^{-4}$, and spread according to the method of Davis and Hyman (9). Isolated foldback DNA was spread at room temperature without formamide according to the method of Delain and Brack (10). Dow Latex (Serva; diameter 0.312/u ± 0.002) served as size marker. After spreading, the grids with the DNA were rotary shadowed with platinum and viewed in a Zeiss EM 10 electron microscope. The micrographs were enlarged using a projecting apparatus. Looped and unlooped pins with clearly visible stems were measured with a slide rule caliper and counted.

RESULTS

Electron microscopy

Figs. 1 - 3 show some examples of inverted repeated DNA. Among the looped hairpins sometimes double or triple loops can be found, especially in Scilla DNA. However, the relative amount of unlooped hairpins is different and seems to be species-specific. Mouse foldback DNA is known to consist of ca. 40% palindromes (11), in rye, the unlooped hairpins amount to roughly 50%, and in Scilla to 78% (other data: 20% in Drosophila and rat, 12, 13; 55% in hamster, 14; 36% in man, 15; 50% in Physarum, 16; 80% in wheat, 17). Length measurements on DNA of both plants revealed a similar size range of the double stranded regions, from about 30 up to 180 base pairs though the frequency distribution of the pin lengths varied. Looped hairpins had, under the experimental conditions used here, a frequency peak at a size of ca. 60-90 base pairs and were only rarely or never found in larger size-classes. The unlooped pins, on the contrary, showed a broader size distribution with lengths up to 150 base pairs (Fig. 4). In both plants, the foldback sequences were found to be clustered on certain strands. DNA sonicated to ca. 2000 base pairs (the fraction used for the gross isolation of foldback DNA) showed only unlooped hairpins and pins with very small loops (see also 18). Figs. 1a-3a show isolated hairpins of larger sizes. Occasional-

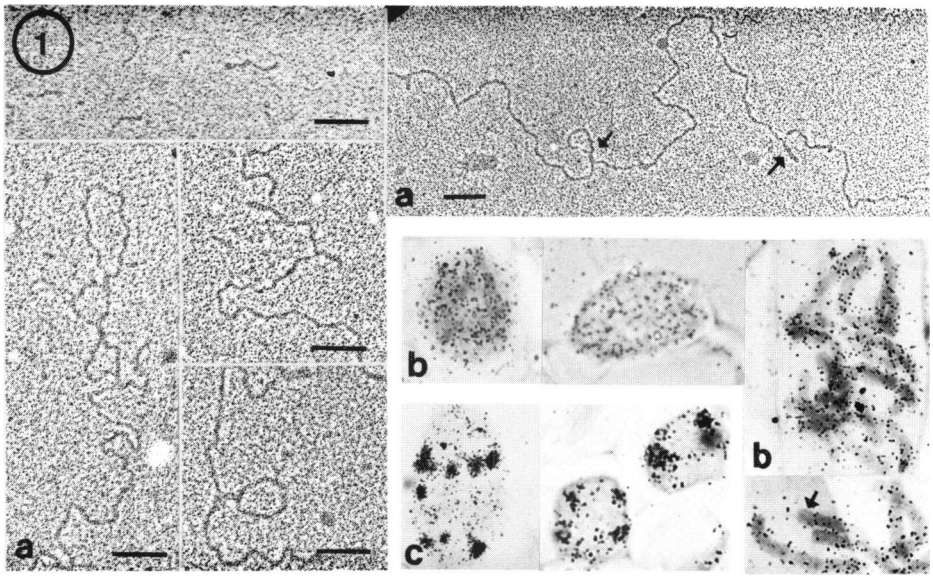

Figure 1

Scilla sibirica. a) Upper left: Isolated foldback DNA (double stranded pins). Upper right: Single strand with a looped and an unlooped hairpin (arrows). Left: Various forms of looped and unlooped hairpins. Bars represent 0.1/u.

b) Radioactive foldback cRNA hybridised in situ to nuclei and chromosomes. Left: Squashed interphase nucleus (exposure: 3 weeks). Middle: Semi-thin section of an interphase nucleus (exposure: 4 weeks). Right: Mitotic chromosomes (note that some of the chromosome termini are not covered with silver grains (arrow, exposure: 4 weeks).

c) Satellite cRNA, hybridised to interphase nuclei. Left: Squashed preparation (exposure: 2 weeks). Right: Semi-thin section (exposure: 3 weeks).

ly, a longer DNA molecule was found, perhaps some contaminating satellite DNA. However, there were too few of them to affect the in situ hybridisation pattern, and the ^{3}H-cRNA was diluted with non-radioactive satellite cRNA in any case.

Denaturation and renaturation experiments

Fig. 5 represents the denaturation kinetics and the differentiation $\Delta T/\Delta t$ of S_1-nuclease-treated isolated foldback DNA. The melting points (T_m=80.5°C for mouse; T_m=89.5°C for Scilla; T_m=83°C for rye DNA, in SSC) were equal or slightly lower than the T_m of the respective main band or total DNAs. The melting range was similar (mouse, rye) or somewhat broader

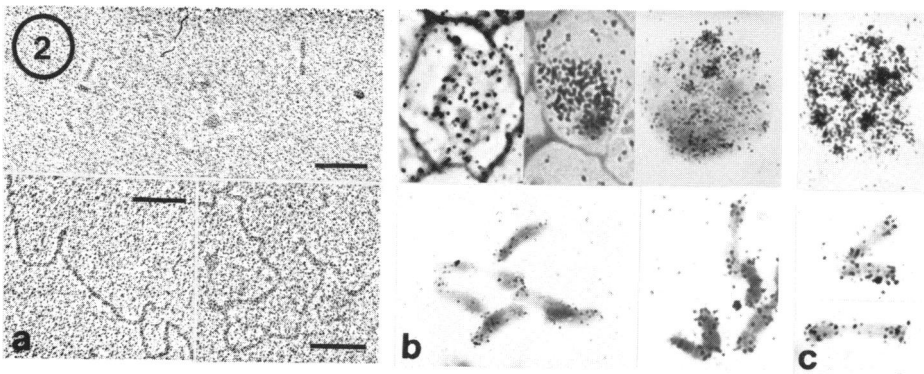

Figure 2
Secale cereale (rye). a) Upper part: Isolated foldback hairpins. Lower part: Looped and unlooped hairpins. Bars represent 0.1 u.
b) Radioactive foldback cRNA, hybridised in situ to nuclei and chromosomes. Upper row, left and middle: Semi-thin sections (exposure: 3 and 5 weeks, respectively). Right: Squashed preparation (exposure: 3 weeks). Lower row, left: Meiotic metaphase chromosomes (exposure: 4 weeks). Right: Mitotic metaphase chromosomes (exposure: 4 weeks).
c) "Ordinary" repetitive cRNA, hybridised to an interphase nucleus and mitotic metaphase chromosomes. Squashed preparations (exposure: 2 weeks for the interphase nucleus, 3 weeks for the chromosomes).

(Scilla) than that of the respective main band or total DNAs (not shown here), with a preference for lower melting DNA components. Thereby the mouse foldback DNA fraction seems to be of more homogeneous composition than the fractions from plants. The two DNA components found in rye were yet detectable in the melting curves of total DNA though with inverse proportions.

Even after destruction of the loops with S_1-nuclease a large number (60-80% of the total foldback DNA) renatured still very fast (Fig. 6). However, this was to be expected in a fraction consisting mostly of unlooped hairpins.

Localisation of the foldback DNA fractions

All hybridisation results show that a considerable part of the isolated foldback DNA must be either highly repetitive or that these sequences must be similar to each other.

In mouse, the label is distributed over the entire chromatin. In interphase and early prophase nuclei (Fig. 3b) condensed chromatin areas, where also the satellite DNA is located

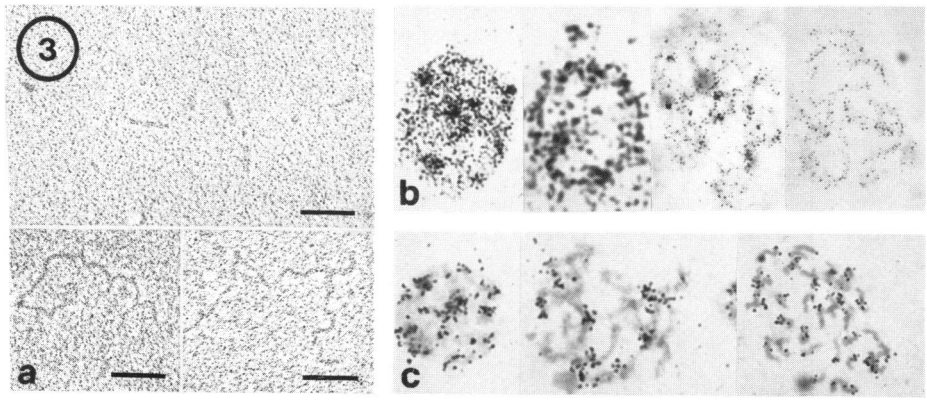

Figure 3

Mouse. a) Upper part: Isolated hairpins. Lower part: Unlooped hairpins on DNA single strands. Bars represent 0.1 µ.

b) Foldback cRNA, hybridised to nuclei and chromosomes. From left to right: Interphase nucleus, squashed preparation (exposure: 3 weeks); interphase nucleus, semi-thin section (exposure: 4 weeks); meiotic prophase, squashed preparation (exposure: 3 weeks); meiotic late prophase, squashed preparation (exposure: 4 weeks).

c) Satellite cRNA, hybridised to nuclei and chromosomes. Left: Interphase nucleus, squashed preparation (exposure: 5 days). Middle: Prophase, squashed preparation (exposure: 5 days; note that the chromosomes are attached to each other in the satellite DNA-containing centromeric retions, see "Discussion"). Right: Metaphase (exposure: 5 days).

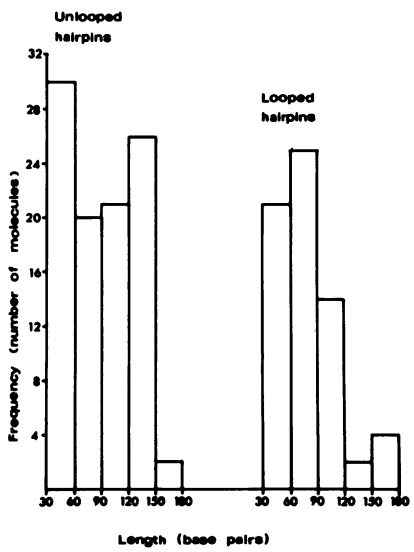

Figure 4

Length distribution of looped and unlooped hairpins in Scilla sibirica main band DNA (168 molecules were measured).

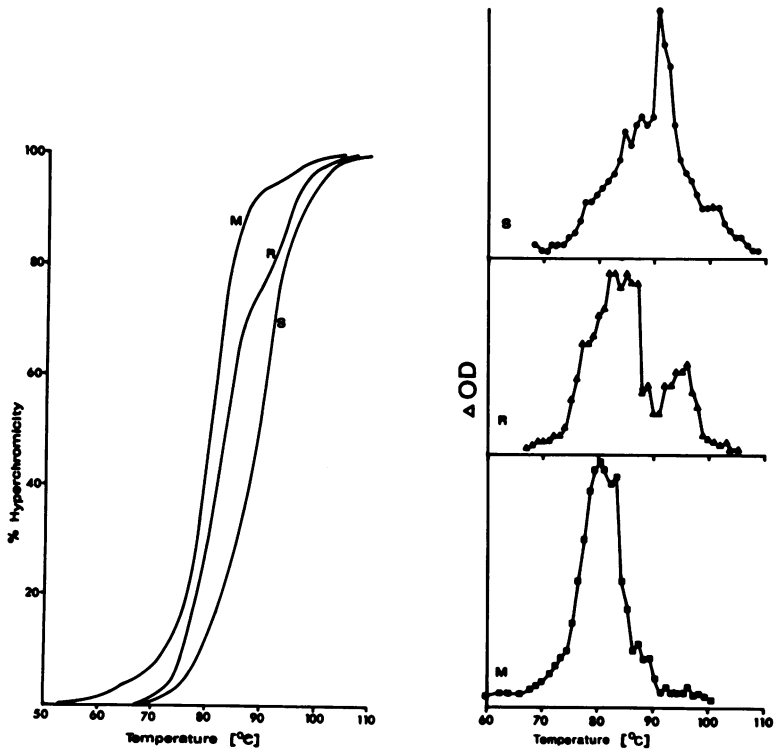

Figure 5
Melting profiles and differentiation $\Delta T/\Delta t$ of S_1-nuclease-treated isolated foldback DNA (M=mouse, R=rye, S=Scilla).

(Fig. 3c), seem to be preferentially labelled. However, this difference disappears in condensed chromosomes, indicating that its cause is only the packing densities of the chromatin. Since an effect of satellite cRNA was ruled out (Materials and Methods), it shows that the repetitive foldback sequences are evenly distributed in the genome, not excepting the centromeric, satellite DNA-containing areas.

In rye which has no satellite DNA, the appearance of the interphase nuclei was much the same as in mouse (Fig. 2b). Contrary to what was found in the case of mouse, these sequences seem to be truly clustered there as to be seen in the late prophase or metaphase chromosomes where the grains appear first at the termini of the chromosomes (known to contain the chromocentric chromatin as revealed by C-banding techniques (19, 20).

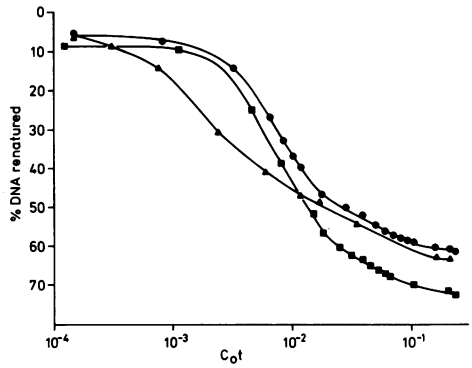

Figure 6 Renaturation of foldback DNA from mouse (■), Scilla (●), and rye (▲) in 0.12 M NaP

After longer exposure, however, grains appear along the chromosome arms too and are also more evenly distributed in interphase nuclei (21). On the whole, the labelling pattern is similar to the pattern obtained after in situ hybridisation with "ordinary" highly repetitive cRNA (Fig. 2c; 22).

In interphase nuclei of Scilla sibirica the label is always slightly concentrated near the chromocenters though not directly over them (Fig. 1b), as is the case after hybridisation with satellite cRNA (Fig. 1c). Here, the repetitive foldback DNA is probably excluded from satellite DNA-containing areas. Hybridisation to metaphase chromosomes shows that the repetitive foldback sequences are not evenly distributed over the rest of the chromatin but are clustered in several regions (Fig. 1b).

DISCUSSION

The results of the in situ hybridisation suggest that a great part of the isolated inverted repeated DNA sequences is highly repetitive. Since DNA of a length of ca. 2000 base pairs was used in this study, the repetitive foldback sequences isolated and hybridised are found among the hairpins without or with only very small loops. It was not ascertained whether the highly repetitive sequences were found among the longer or the short pins. The relatively short times of exposure, however, suggest that various kinds of pins contribute

to the hybridisation results, although the same grain density was never achieved as after hybridisation with satellite cRNAs.

There are several hypotheses published concerning the function of foldback sequences in the genome. A function as recognition sites for DNA polymerases (23, see, however, 24) could only be valid for very short pins. It is quite possible that these sequences contribute to the in situ hybridisation pattern since they are present in the eukaryotic genome in great number, and it is to be expected that they have identical base sequences. However, they are certainly not necessarily - at least not in great number - located at the chromosome termini as postulated by Cavalier-Smith (23) since some of the Scilla chromosome ends do not show silver grains. And, whilst it is easily understandable that in this case these sequences are found scattered over the whole chromosomes as is the case in mouse, it seems peculiar that they are clustered in plant chromosomes. So, it is most likely that the hybridisation pattern does not result solely from sequences with a function in DNA replication.

Other hypotheses are: A function as template for double stranded RNA (e.g. 25, 26), as transcription termination site (27), a function in the attachment of chromosomes to each other (23) or no function at all (15). Nothing concerning the first two of the hypotheses can be concluded from the in situ hybridisation experiments. The chromosome attachment, however, sometimes observed in meiotic or mitotic prophases (Figs. 2b, 3c, ref. 28) is probably better explained by an effect of "ordinary" highly repetitive sequences, since mouse, rye, and Scilla chromosomes are attached in the chromocentric regions containing the satellite DNA or highly repetitive DNA, respectively, which especially in Scilla is located in regions different from the regions of highly repetitive foldback DNA. According to the results shown here (species-specific arrangement of the inverted repeated sequences, no common hybridisation pattern) it seems most likely that highly repetitive foldback DNA derives from "evolutionary accidents" (see also 15) as do other highly repetitive DNAs. Whether they are related to the "ordinary" repeated sequences, seems, however, doubtful. At least,

they are definitely not inverted repeated forms of satellite DNA, as shown in Scilla and mouse.

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