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Heterochromatin and Highly Repeated DNA Sequences in Rye (*Secale cereale*)

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Abstract. *Secale cereale* DNA, of mean fragment length 500 bp, was fractionated by hydroxylapatite chromatography to allow recovery of a very rapidly renaturing fraction (C_0t 0–0.02). This DNA fraction was shown to contain several families of highly repeated sequence DNA. Two highly repeated families were purified; (1) a fraction which renatured to a density of 1.701 g/cc and comprised 2–4% of the total genome, and (2) polypyrimidine tract DNA which comprised 0.1% of the total genome. The 1.701 g/cc DNA consisted of short sequence repeat units (5–50 bp long) tandemly repeated in blocks 30 kb long, while a portion of the polypyrimidine tract DNA behaved as part of a much larger block of tandemly repeated sequences. The chromosomal location of these sequences was determined by the *in situ* hybridisation of radioactive, complementary RNA to root tip mitotic chromosomes and showed the 1.701 g/cc sequences to be largely limited to the telomeric blocks of heterochromatin, accounting for 25–50% of the DNA present in these parts of the chromosomes. The polypyrimidine tracts were distributed at interstitial locations with 20–30% of the sequences at three well defined sites. The combined distributions of the 1.701 g/cc DNA sequences and polypyrimidine tracts effectively "individualised" each rye chromosome thus providing a sensitive means of identifying these chromosomes. The B chromosomes present in *Secale cereale* cv. Unevita, did not show defined locations for the sequences analysed. – The data are discussed in terms of the structure of the rye genome and the generality of the observed genomic arrangement of highly repeated sequence DNA.

Introduction

Despite extensive analyses of highly repeated sequence DNA in animals, relatively little is known about this class of DNA in plants. General properties of highly repeated sequence DNA in animal chromosomes (for a review see Appels and Peacock, 1978) which may also apply to plant chromosomes, are: (a) the sequence repeats are arranged in long, tandem arrays, (b) these arrays of sequences are grouped in heterochromatin around centromeres and at other

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chromosomal sites, (c) the distribution of the arrays of different sequences show a chromosome specific pattern i.e. each chromosome has its own "fingerprint", (d) highly repeated sequences show a considerable degree of sequence conservation on an evolutionary time scale, although marked quantitative changes are common even between species of the same genus.

The aim of the present study was to assess whether some or all of the above generalities apply to plant chromosomes. Plants do contain repeated DNA sequences (Britten and Kohne, 1968; Bendich and McCarthy, 1970) although buoyant density satellites in CsCl gradients are not obvious in many plant species (Ingle et al., 1973). In the case of melon, a satellite has been purified by CsCl centrifugation and fractionated, in Ag^+/Cs_2SO_4 gradients, into two kinetically complex components (Sinclair et al., 1975), one of which has a 600 nucleotide unit, tandemly repeated many times (Bendich and Taylor, 1977). In the cereals (wheat, rye, barley and oats) approximately 10% of the DNA is recovered as a very fast renaturing fraction (Ranjekar et al., 1974, 1976; Smith and Flavell, 1977) and satellite DNA has been reported in Ag^+/Cs_2SO_4 gradient analyses of wheat and barley (Ranjekar et al., 1976; Huguet et al., 1975). Chromosomal locations of highly repeated sequences have been established in *Scilla sibirica* where satellite sequences from a Ag^+/Cs_2SO_4 gradient were shown by *in situ* hybridisation to occur in heterochromatin regions of the chromosomes. In contrast to this, the correlation between highly repeated sequence DNA and heterochromatin did not appear to hold in *Secale cereale* as judged from in situ analysis of meiotic chromosomes (Timmis et al., 1975). In this case the sequences were thought to be distributed throughout the genome and not restricted to the particularly well defined, telomeric heterochromatin found in this plant.

Since the above observation represents a major departure from the widely applicable synonymity of highly repeated sequence DNA and heterochromatin we have chosen to study rye in detail. Ranjekar et al. (1974) showed several buoyant density components in a fraction of rye DNA renaturing with a C_0t of 0–0.01 (10–12% of the genome). The predominant component was a well defined species at 1.702 g/cc in a CsCl gradient. Smith and Flavell (1977) considered this class of DNA to consist mainly of palindromic sequences capable of "fold-back" renaturation and suggested that simple sequences present in thousands of copies may not exist within the rye genome, contrasting with the situation in animal genomes. Smith and Flavell suggested that 4% of the rye genome consists of palindromic sequences which are distributed in clusters through at least 30% of the genome, as judged from the increased yield of "very rapidly reannealing DNA" as the molecular weight of DNA segments was increased. Our experiments have not been addressed to the question of palindromic sequences but our data do show that highly repeated DNA sequences are present in rye and that the properties of these sequences, including heterochromatin localization, parallel those of animal genomes.

Material and Methods

DNA Isolations. 10 g batches of seed of *Secale cereale* cv. Unevita were surface sterilised in 1% Na hypochlorite and germinated in sterile vermiculite at 25° C. After 3–4 d shoots were removed,

washed 4 × in distilled water, dried and frozen in liquid nitrogen. The frozen shoots were blended in a Waring Blendor for three periods of 1 min after which buffer (0.1 M EDTA, 0.15 M NaCl, pH 7.0) was added and the blending cycle repeated. The mixture was incubated at 60° C for 15 min after sodium lauryl sulphate was added to 0.5%. Protease (Sigma) was then added to a final concentration of 100 µg/ml and incubation continued at 45° C for 45 min. The preparation was extracted with an equal volume of phenol saturated with TE buffer (0.001 M EDTA, 0.01 M Tris-HCl pH 8.4). Addition of ethanol to the aqueous phase allowed DNA to be wound onto a glass rod (Marmur, 1961). The DNA was dissolved in cold 5 M $NaClO_4$ and ethanol precipitated as before. A second cycle of $NaClO_4$ extraction and ethanol precipitation was generally carried out before DNA was used for further experiments. DNA was dissolved in TE buffer for storage at 4° C.

Hydroxylapatite Chromatography. Fast renaturing DNA fractions were isolated by means of a continuous column loading system in which denatured DNA in 0.12 M sodium phosphate pH 7.0 was renatured to the required C_0t and loaded onto a 2 cm hydroxylapatite column of diameter 4 cm. Usually 5–10 mg quantities of DNA were processed for the large scale preparation of C_0t fractions. Hydroxylapatite was prepared according to the procedure described by Levin (1962). DNA of mean length 500 nucleotides was produced by 3 passages of DNA through a French press at 16,000 psi.

Isolation of Polypyrimidine Tracts. Polypyrimidine tracts were prepared from 20 mg of rye DNA following the procedure of Birnboim et al. (1975). The polypyrimidine tract preparation showed a high absorbance at 320 nm compared to normal DNA which meant that estimates of concentration of nucleotides in solution were approximate. Since the preparation *per se* provided an adequate template for *Escherichia coli* RNA polymerase the absorbance at 320 nm was not investigated further.

Assays for Simple Sequence DNA. DNA sequences were assayed only by hybridisation of cRNA probes (Peacock et al., 1973) to DNA fixed to nitrocellulose filters (Birnstiel et al., 1972). Hybridisation reactions, in 3 × SSC, 50% formamide, were heated above the T_m of cRNA-DNA hybrids for 5 min to ensure denaturation of both cRNA and DNA sequences. Where preheating did not substantially change the *amount* of cRNA hybridized it was omitted in later experiments. Saturation analyses to determine the fraction of the DNA complementary to the cRNA were carried out on nitro-cellulose filters with corrections being made for loading efficiencies and losses of DNA from the filter. Data were analysed as suggested by Bishop et al. (1969).

The T_m of RNA-DNA hybrids was determined by transferring the filter, at 3 min intervals, into 0.6 ml aliquots of 3 × SSC, 50% formamide at increasing temperatures. Released radioactivity was measured by adding 0.3 ml H_2O and 9.0 ml of Triton X 100 based scintillation fluid.

In all T_m determinations the hybrid between *Drosophila melanogaster* DNA and cRNA to the satellite of density 1.705 g/cc (Brutlag et al., 1976) was also melted in parallel to provide an internal standard. The T_m of this hybrid was standardised to 61° C – corrections to T_m values using this internal standard ranged from 0–3° C. The condition chosen for hybridisation of any isolated rye sequence was T_m minus 10–15° C with a 3 h incubation time.

The predominance of the oligonucleotides ApGp and ApApGp in rye polypurine cRNA (copied from polypyrimidine tract DNA) prompted a test for cross-reactivity with the *D. melanogaster* satellite of density 1.705 g/cc. cRNA to this satellite is composed largely of tandem repeats of ApGpApApGp (Brutlag and Peacock, 1975; Endow et al., 1975; Birnboim et al., 1975; Endow, 1977). Cross-reaction does occur as judged from the competition of 1.705 cRNA hybridization by rye polypyrimidine tracts (Fig. 1a). The cRNA-DNA hybrid has a T_m of 46° C (Fig. 1b). The low T_m of this hybrid indicates considerable mismatching (15–30% relative to the homologous *D. melanogaster* hybrid using the data of Blumenfeld, 1973), but the *D. melanogaster* cRNA, which was readily prepared, did serve as a useful probe for rye polypyrimidine tracts.

Preparative gradients were analysed for the distribution of defined sequences following the procedures of Birnstiel (1972). Generally the DNA in 25 µl aliquots from the gradient fractions were loaded on 25 mm Sartorius nitro-cellulose filters (cat. no. SM11336). The filters were handled in bulk in the hybridization experiments.

Labelled DNA was prepared by the nick-translation procedure described by Rigby et al. (1977).

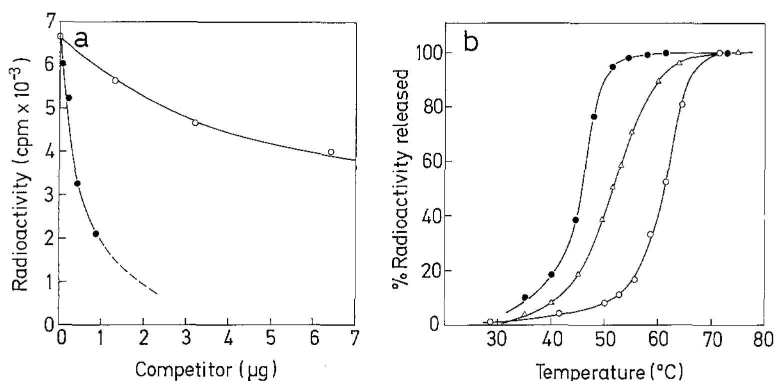

Fig. 1a and b. Cross-hybridisation of *Drosophila melanogaster* 1.705 g/cc satellite to rye polypyrimidine tracts. **a** Competition for the sites on unfractionated rye DNA (immobilised on nitrocellulose filters) to which *D. melanogaster* 1.705 g/cc satellite ^{3}H-cRNA hybridises by added rye polypyrimidine tracts (o——o) and unfractionated DNA (o——o). Hybridisation was carried out as described in the methods except that preheating of the reaction mix to 80° C/3 min was employed to ensure denaturation of double stranded DNA in solution (to allow it to compete effectively for ^{3}H-cRNA). The curves shown could in theory be used to estimate the level of polypyrimidine tracts in unfractionated DNA – this was not done because estimates of the concentration of polypyrimidine tract DNA were only approximate (see Materials and Methods). **b** Melting curves for the hybrids formed between *D. melanogaster* 1.705 g/cc satellite H^3-cRNA and unfractionated rye DNA (●---●). Also shown are the curves for rye polypyrimidine tract H^3-cRNA hybridised to unfractionated rye DNA (Δ---Δ) and 1.705 g/cc satellite H^3-cRNA hybridised to *D. melanogaster* DNA (o---o). The latter hybrid was always used as an internal standard for T_m analyses and was set at 61° C

Buoyant Density and Sedimentation Centrifugation. In titrations with Ag^+ (Corneo et al., 1968) aliquots of a stock solution of the cation were added to DNA dissolved in a solution of 1 part sodium borate buffer (10 mM, pH 9.2) and 1 part TE. Before preparative gradients were set up an analytical centrifugation was used to check that the correct titration point had been reached. Densities in neutral CsCl gradients were calculated assuming a linear gradient and using *Micrococcus luteus* as an internal standard (1.731 g/cc, Szybalski and Szybalski, 1971). Sedimentation velocity analyses were carried out according to the methods described by Studier (1965).

In situ Hybridisation. In situ hybridisations were carried out in 3 × SSC, 50% formamide, 10–15° C below the T_m of the RNA-DNA hybrid formed. Usually 2–3 h were allowed for the hybridisation reaction. Root tips for chromosome preparations were obtained by 2° C treatment (20 h) of seeds germinated for 16 h, followed by a 16 h incubation at 27° and 4 h in 0.1% colchicine at 27° C. Meristematic cells were dissected and squashed in 45% acetic acid following fixation in ethanol: acetic acid (3:1). Coverslips were removed after freezing the slide in liquid N_2 and in situ hybridization carried out as described in Peacock et al. (1978).

To determine the T_m of hybrids formed in situ duplicate slides were incubated in 3 × SSC, 50% formamide at various temperatures for 5 min. The slides were then washed with 2 × SSC and RNase treated as described (Peacock et al., 1978). The % loss of radioactivity was computed from grain count data. In each experiment the T_m of RNA-DNA hybrids formed on nitro-cellulose filters was determined in the same 3 × SSC/50% formamide solution to provide an internal control.

The in situ hybridisation patterns were determined in *Secale cereale* cv. Imperial and *Secale cereale* cv. Unevita. The rye addition lines used in this study involve the individual chromosomes of Imperial rye added to Chinese Spring wheat. The chromosome designations A to G follow those used by Sears which equate as follows A = 5R, B = 2R, C = 7R/4R, D = 4R/7R, E = 1R, F = 6R and G = 3R.

Chemical Analysis of cRNA. Base compositions and analysis of RNAse A and T1 RNAse digestion products were carried out using standard procedures (Brownlee, 1972; Griffin, 1972; Brutlag and Peacock, 1975). The ^{32}P nucleoside triphosphates used to label the cRNA for this type of analysis were prepared as described by Symons (1974).

Results and Discussion

Buoyant Density Analysis of Renatured DNA Fractions

A rapidly renaturing DNA fraction (C_0t 0–0.02) was isolated from rye DNA by hydroxylapatite chromatography at 78° C in 0.12 M sodium phosphate pH 7.0. This temperature was chosen because the melting curve of unfractionated rye DNA showed no hyperchromic shift before 79–80° C, the main transition being at 87° C. No major satellite sequences should be missing from the C_0t 0.02 fraction due to inadequate renaturing conditions, since analyses of *Drosophila melanogaster* satellite DNA (Brutlag et al., 1977) have shown that under the conditions used here simple sequences can renature at T_m −9° C.

A preparative buoyant density profile of C_0t 0.02 DNA is shown in Figure 2 (see Fig. 4a for an analytical trace) along with the analytical traces of the subfractions of this gradient. The density of the predominant species (1.701 g/cc) agrees well with that found by Ranjekar et al. (1974). Additional components at densities 1.691 g/cc, 1.704 g/cc and 1.720 g/cc are evident.

Electron microscopy showed that the hypersharp nature of the 1.701 g/cc DNA is due to aggregate formation, a characteristic of renatured DNA where the repeating sequence is shorter than the fragment length (i.e., <500 nucleotides in this case). The 1.701 g/cc peak is visible in buoyant density analysis after only 5 h centrifugation confirming the presence of high molecular weight aggregates. This result implies the presence of tandemly repeated simple sequences. We have therefore purified the 1.701 g/cc DNA by buoyant density centrifugation and analysed it in more detail. Digestions of ^{32}P cRNA to this DNA with RNase A and T1 RNase show relatively simple patterns when the oligonucleotides are separated by two-dimensional chromatography (Fig. 3a). In RNase A oligonucleotides, 53% of the radioactivity is found in the mono- and di-nucleotides C_P, A_PC_P, G_PC_P, U_P, U_PA_P and G_PU_P. The summary in Table 1 suggests that the 1.701 g/cc DNA contains a simple sequence repeat. Since we did not have separated single strands, we did not attempt a more detailed analysis of the sequence. Renaturation analysis in $0.1 \times SSC$ as described by Brutlag et al. (1977), using the 1.705 g/cc *Drosophila melanogaster* satellite as a standard (complexity 5 bp), shows 2–3 major components with a complexity in the range of 5 to 50 base pairs (Fig. 3b). The renaturation analysis shows very little evidence for "fold-back" DNA since the 30% instantaneous drop in absorbance at 260 nm is explicable solely in terms of base stacking phenomena (Bendich and Anderson, 1977).

The yield of the C_0t 0.02 DNA varied in a number of experiments with concomitant changes in the buoyant density species recovered. An extreme example of the variation in buoyant density profiles is shown in Fig. 4 where

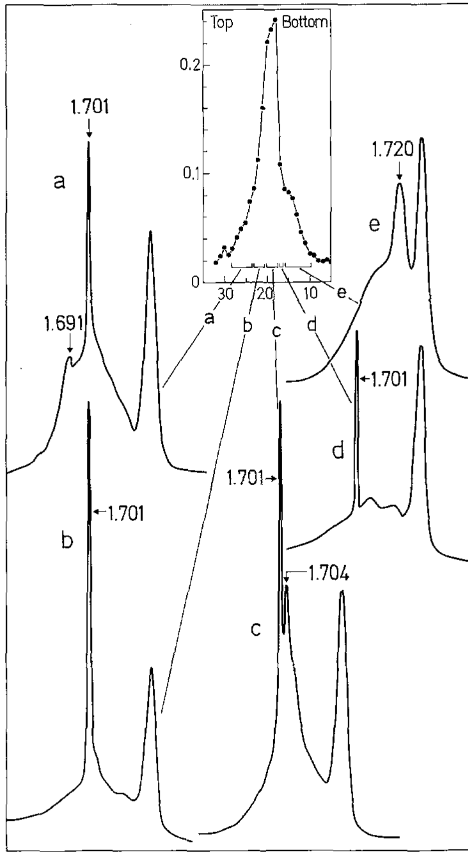

Fig. 2. Buoyant Density components present in rye C_0t 0.02 DNA. The C_0t 0.02 DNA, isolated from unfractionated rye DNA, sheared to a mean length of 500 nucleotides, by hydroxylapatite chromatography, was centrifuged to equilibrium in a preparative CsCl gradient. Fractions were collected from the bottom of the tube and various parts of the fractionated DNA analysed in neutral CsCl gradients centrifuged to equilibrium in a model E ultracentrifuge. The central panel in the figure presents the preparative gradient (Vertical axis denoting absorbance at 260 nm, horizontal axis denoting fraction number) with a, b, c, d and e representing model E analyses of the fractions pooled as shown. In each model E buoyant density profile the right most peak is marker DNA, density 1.731 g/cc

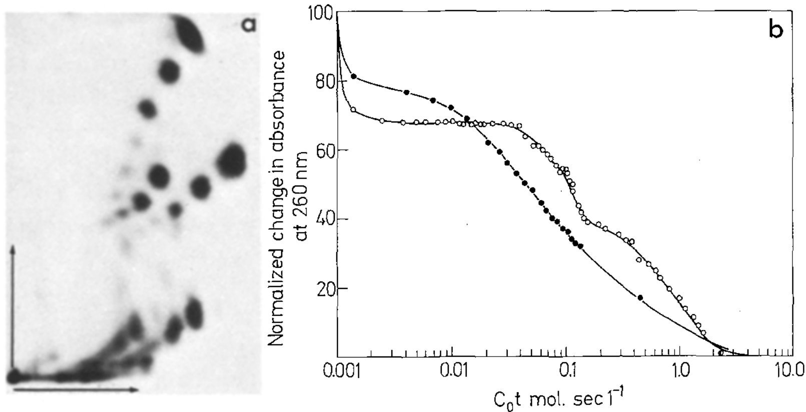

Fig. 3a and b. Sequences present in 1.701 g/cc DNA. **a** The ^{32}P-labelled cRNA prepared using 1.701 g/cc DNA as template was digested with RNase A (enzyme: substrate = 1:100) and the oligonucleotides separated on polyethyleneimine impregnated cellulose plates as described by Griffin (1972). Autoradiography was used to detect the oligonucleotides. **b** The renaturation of 1.701 g/cc DNA (o——o) was carried out in 0.1 × SSC at 40° C using *Drosophila melanogaster* satellite of density 1.705 g/cc as a standard (●——●) (see Brutlag et al., 1977). Jacketted cuvettes were used to ensure a rapid transition (15–30 seconds) from the denaturing temperature to the renaturing temperature

Table 1. Major oligonucleotides recovered from RNase A digestion of RNA complementary to 1.701 g/cc DNA

C_P	C_PA_P	$C_PA_PA_P$	C_PG_P	$C_PA_PG_P$	U_P	U_PA_P	$U_PA_PA_P$	G_PU_P
14.2	5.6	3.1	5.4	3.0	4.4	6.6	2.7	6.8

The numbers indicate the % of radioactivity on the chromatogram shown in Fig. 2 which corresponded to the respective oligonucleotides

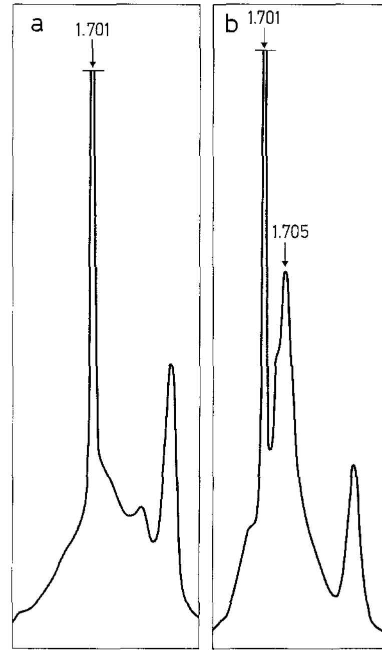

Fig. 4a and b. Buoyant density profiles of C_0t 0.02 DNA. Unfractionated rye DNA, sheared to a mean length of 500 nucleotides, was used to provide the C_0t 0.02 fractions after hydroxylapatite chromatography. DNA samples were analysed in neutral CsCl with an internal marker of density 1.731 g/cc (the right most component in each profile). **a**, and **b** are two C_0t 0.02 DNA fractions to show an extreme in the variation of buoyant density species recovered with the appearance of a species at 1.705 g/cc in **b**.

the main difference between the profiles in Fig. 4a and Fig. 4b is in the content of a DNA species at 1.705 g/cc. This variance cannot be completely accounted for by variation in DNA segment lengths or in actual C_0t values, and in part must be attributed to the hydroxylapatite method per se (Britten et al., 1974).

Segment Length of the 1.701 g/cc DNA Sequences

The hypersharp peak at 1.701 g/cc was reproducibly a major buoyant density component (2–4% of genome) of the C_0t 0.02 fraction, when DNA of fragment size 500 nucleotides was used for the isolation. With fragment size of 3350 nucleotides the 1.701 g/cc species was difficult to discern (Fig. 5). The presence of 1.701 g/cc sequences could however be monitored with a cRNA probe. This analysis showed some 1.701 g/ccc sequences to be present at higher densities, implying that 1.701 g/cc repeats are linked to sequences which do not renature

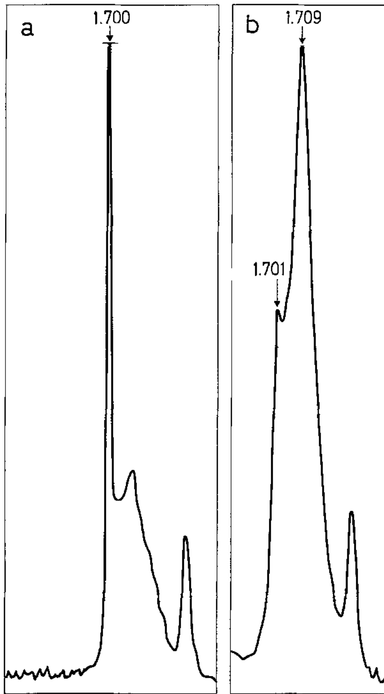

Fig. 5a and b. Effect of length on the nature of C_0t 0.02 DNA. Samples were analysed in the model E ultracentrifuge using an internal marker at 1.731 g/cc (the right most component in each profile). **a** C_0t 0.02 DNA fraction isolated from DNA with a mean single strand length of 485 nucleotides. The proportion of the genome recovered as this fraction was 10.6%. **b** C_0t 0.02 DNA fraction isolated from DNA with a mean single strand length of 3360 nucleotides. The proportion of the genome recovered as this fraction was 17.6%

at a C_0t of 0.02. The genome distribution of 1.701 g/cc repeat was further characterized in an experiment in which the C_0t 0.02 DNA from short and long fragment DNA was prepared in the presence of ^{32}P or ^{3}H-labelled nick-translated 1.701 g/cc DNA, the ratio of added 1.701 g/cc DNA: 1.701 g/cc DNA expected to be recovered, being approximately 1:50. The two C_0t 0.02 DNA preparations were then mixed, centrifuged to equilibrium in CsCl and the distribution of ^{32}P and ^{3}H radioactivity determined (Fig. 6). In the long fragment DNA, 89% of 1.701 g/cc sequences band at 1.701 g/cc with only a small fraction showing any density shift. This suggests these sequences are arranged in large blocks in vivo. The amount of 1.701 g/cc sequences which do show a density shift allows an estimate of the mean size of the in vivo blocks to be made (using the formula $m=n/(1\text{-}f)$, see legend to Fig. 6). The estimate, although very approximate, is 30,000 nucleotides. We conclude that long segments of simple sequences, tandemly repeated, do exist in rye.

The substantial quantity of DNA recovered at 1.709 g/cc need not be due entirely to linkage to 1.701 g/cc sequences since the other fast renaturing components of the C_0t 0.02 fraction could also contribute to the recovery of molecules with single stranded regions.

Isolation of Polypyrimidine Tract DNA

Since in *Drosophila* long pyrimidine tracts are composed of simple sequence DNA (Birnboim and Sederoff 1975; Birnboim et al., 1975), we isolated polypy-

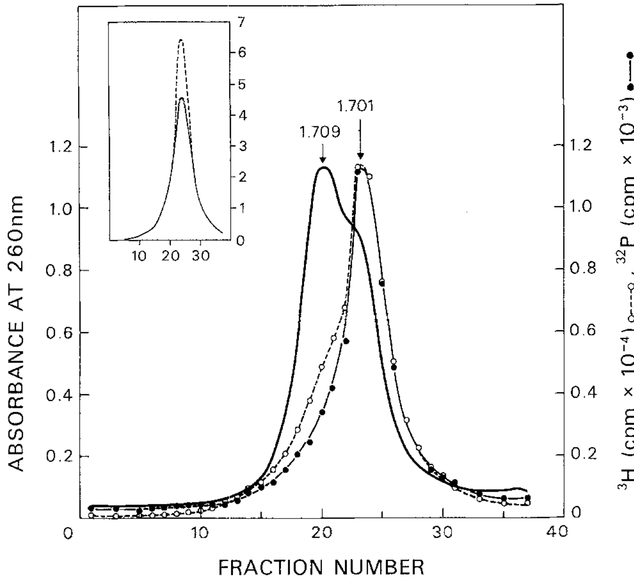

Fig. 6. Distribution of 1.701 g/cc sequences in C_0t 0.02 DNA fractions. Purified 1.701 g/cc DNA was nick-translated using *E. coli* DNA polymerase I and either ^{3}H-labelled nucleoside triphosphates or ^{32}P-labelled nucleoside triphosphates. The ^{3}H-labelled DNA (o---o) was mixed with DNA of mean single strand length 3360 nucleotides prior to using this for the isolation of a C_0t 0.02 fraction while the ^{32}P-labelled DNA (•—•) was mixed with DNA of mean single strand length 485 nucleotides before using this to isolate a C_0t 0.02 fraction. The two C_0t 0.02 fractions were then mixed, centrifuged to equilibrium in CsCl and the distribution of radioactivity determined by acid precipitating fractions from the gradient. The insert shows samples of the nick-translated DNAs simply mixed per se and centrifuged to equilibrium in CsCl (--- ^{3}H-labelled 1.701 g/cc DNA, — ^{32}P-labelled 1.701 g/cc DNA). The fraction of 1.701 g/cc sequences which showed a density shift in long fragment DNA was used to estimate the in vivo size of the blocks of 1.701 g/cc DNA using the formula $m = n/(1\text{-}f)$ (Brutlag et al., 1977); in the equation n = fragment size of DNA fractionated, f = fraction of 1.701 g/cc recovered "pure", m = the extrapolated in vivo size of the blocks of 1.701 g/cc DNA. As emphasized in the text this is an approximate estimate of m

rimidine tracts from rye DNA as an approach to obtaining specific simple sequences.

Base composition analyses of the polypurine cRNA yielded 56.7% AMP and 43.3% GMP (97% of the radioactivity accounted for). T1 ribonuclease digestion resulted in a simple pattern of oligonucleotides. Molar yields of these oligonucleotides (Table 2) show the simple sequence nature of the polypyrimidine tracts in the rye genome.

Polypurine cRNA sedimented as a broad peak near added 5S *E. coli* ribosomal RNA in 5–20% sucrose gradients, indicating a mean length of 50–100 nucleotides. (Assuming random initiation of RNA chains this indicates the DNA template is approximately 100–200 nucleotides long.) Saturation analyses showed that the proportion of total DNA represented by long stretches of pyrimidines in one strand and purines in the other is 0.12%. This is a minimum

Table 2. Composition of RNA complementary to rye polypyrimidine tracts[a]

G_P	A_PG_P	$A_PA_PG_P$	A_P[b]	Origin[c]
7.2%	19.8%	43.9%	5.4%	23.7%

[a] Distribution of radioactivity in nucleotides and oligonucleotides recovered from a T1 RNase digestion. Pancreatic RNase showed no digestion products

[b] Free A_P results from a nonspecific hydrolysis. The figure shown was not reduced by reducing the amount of enzyme used

[c] The radioactivity remaining at the origin was unexpectedly high. This material has not been analysed in detail since sequence studies on a native DNA satellite containing the polypyrimidine tracts are in progress

estimate since some cRNA may have been digested in the RNase procedure even though it was paired with DNA (Bishop et al., 1969).

Polypyrimidine tract DNA sequences were detected in the C_0t 0.02 fraction as a broad peak at approximately 1.704 g/cc as judged from its position relative to the 1.701 g/cc sequences assayed in the same CsCl gradient. The recovery of polypyrimidine tracts was increased 3–4 fold when DNA fragments had a mean length of 3,350 nucleotides instead of 500 nuleotides. This suggests that the tracts are linked to other repeated sequence DNA. We return to this point in the following section.

Distribution of Simple Sequence DNA Within Native DNA Fractionation of DNA in Buoyant Density Gradients

Gradients of Cs_2SO_4 containing Ag^+ result in fractionation of the rye genome, contrary to the report of Ranjekar et al. (1976). The R_f values shown in Fig. 7 are calculated from the ratio Ag^+/DNA-phosphate with the concentrations of DNA-phosphate being calculated from the absorbance at 260 nm. (These values are useful only for comparisons within an experiment – the absolute values vary depending on the DNA preparation and the commercial origin of Cs_2SO_4.) The satellite on the light side of main band in the Ag^+, R_f 0.21 condition has several components when analysed in neutral CsCl (densities 1.706, 1.714, 1.719 g/cc) in addition to main band DNA at 1.701 g/cc, but renaturation analysis indicates it does not contain any major components of highly repeated sequence DNA.

The locations of 1.701 g/cc and polypyrimidine tract sequences were examined in a R_f 0.08 Ag^+/Cs_2SO_4 gradient (Fig. 8). The majority of 1.701 g/cc sequences remained within the main band region with only a small proportion appearing as a satellite. Since we estimated 1.701 g/cc sequences to be organised in arrays approximately 30,000 nucleotides long and the DNA fractionated here was approximately 24,000 nucleotides long (double stranded length), the

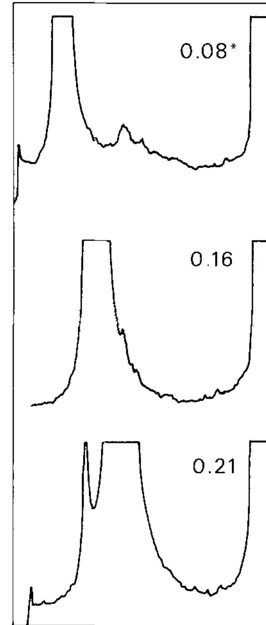

Fig. 7. Fractionation of rye DNA in Ag^+/Cs_2SO_4. Addition of cations to rye DNA were carried out over a wide range of R_f values (Ag^+/DNA-phosphate). Three representative buoyant density profiles are shown (bottom of gradient is on the right-hand side in each panel). – The numbers indicate the R_f value while the asterisk denotes the condition analysed in more detail (see text)

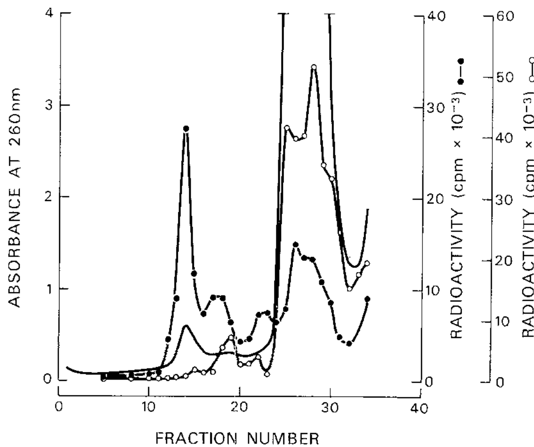

Fig. 8. Distribution of sequences within rye DNA fractionated in a Ag^+/Cs_2SO_4 gradient. The location of 1.701 g/cc sequences (o---o) and polypyrimidine tract sequences (●---●) assayed using *D. melanogaster* 1.705 g/cc satellite ^{3}H-cRNA were determined by filter hybridisation

observation that very few 1.701 g/cc sequences can fractionate away from main band is not surprising. If the 1.701 g/cc sequences were recoverable as a native satellite these numbers suggest that we would expect only 20% of the sequences to be free of adjoining DNA and thus behave as a satellite. In contrast to the behaviour of the 1.701 g/cc sequences, a major proportion of the polypyrimidine tract sequences are drawn out into a satellite (Fig. 8). Since we have

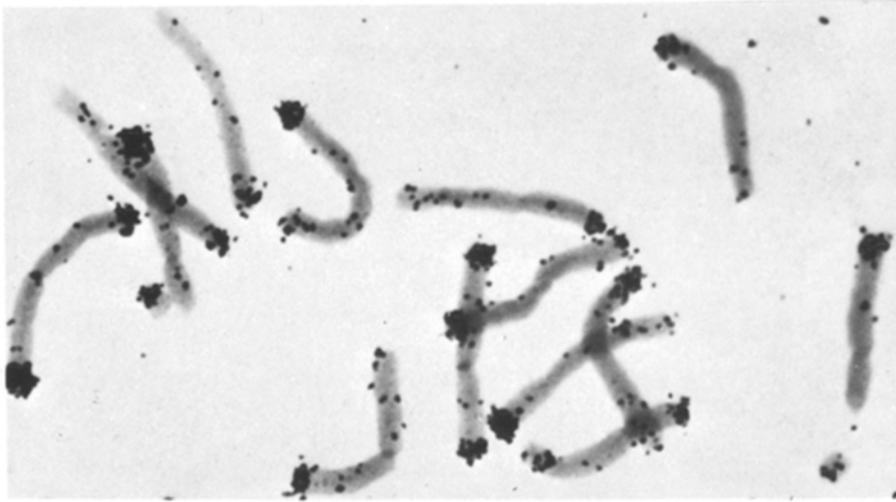

Fig. 9. Chromosomal distribution of 1.701 g/cc DNA sequences. In situ hybridisation using ^{3}H-cRNA synthesised from 1.701 g/cc DNA was carried out to locate complementary sequences in chromosome preparations. Saturation experiments utilising unfractionated DNA immobilised on nitrocellulose filters suggest that the DNA complementary to ^{3}H-cRNA in the final hybrid assayed (i.e., after RNase treatment) represents approximately 0.8% of the genome

estimated polypyrimidine tract sequences to be relatively short (200 nucleotides), the fact that they appear in a satellite must mean they are linked to DNA sequences which are homogeneous enough to behave as a well defined satellite in Ag^+/Cs_2SO_4 gradients. The polypyrimidine tract sequences remaining in main band DNA however may be linked to more complex DNA. The main band DNA polypyrimidine tract sequences do differ from those drawn out into the satellite in that the respective RNA-DNA hybrids in the assay differ in T_m by 1-2° C.

In situ Distribution in Mitotic Chromosomes

a) C_0t *0.02 DNA.* The distribution of 1.701 g/cc sequences was studied by in situ hybridisation using root tip mitotic chromosomes (Fig. 9). Approximately 80% of the grains are localised to the telomeric heterochromatic blocks with the remaining grains distributed over the euchromatic regions. Fig. 10 shows a karyotype constructed from chromosomes in a single metaphase spread. Similar patterns were found in both the Imperial and Unevita cultivars. The distinctive pattern of sequence distribution was analysed further using the Chinese Spring-Imperial rye addition line series in which single pairs of rye chromosomes are present together with 21 pairs of Chinese Spring wheat chromosomes. Fig. 11 is a metaphase spread from "rye add A" in which the unambiguous identification of the rye chromosomes in the background of wheat chromosomes is clearly shown. Similar material was used to identify the rye chromosomes B

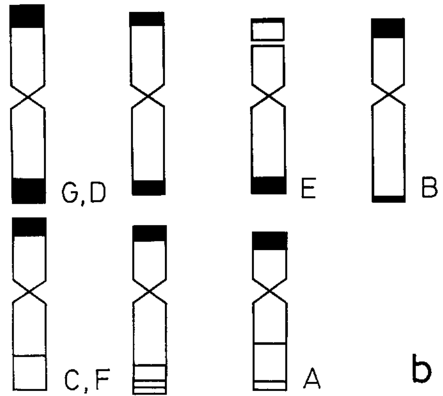

Fig. 10a and b. Karyotype of *Secale cereale* cv. Imperial for the distribution of 1.701 g/cc DNA sequences. **a** Chromosomes from a single metaphase spread. **b** Diagram representing the distribution of grains as determined from a number of experiments involving the rye addition lines shown in Figure 11. The location of interstitial sites are only approximate

through to G (see cut-outs in Fig. 11). The ratios of grains on the telomere of the short arm: telomere of the long arm were; chromosome A, short arm telomere only; chromosome B, 4:1; chromosome C, short arm telomere only; chromosome D, 1:1; chromosome E (nucleolar organiser on short arm), 1:6; chromosome F, short arm telomere only; chromosome G, 1:2. All the rye chromosomes identified in the addition lines could be identified in the normal rye genotype, suggesting that the rye heterochromatin has been stable in the wheat genome background.

The distribution of 1.701 g/cc sequences in interphase nuclei (Fig. 12a) shows a characteristic polarity in arrangement, presumably a consequence of anaphase chromosome movement in the preceding mitotic cycle. Fusion of the telomeres (to form chromocentres) must occur since the number of blocks of grains is

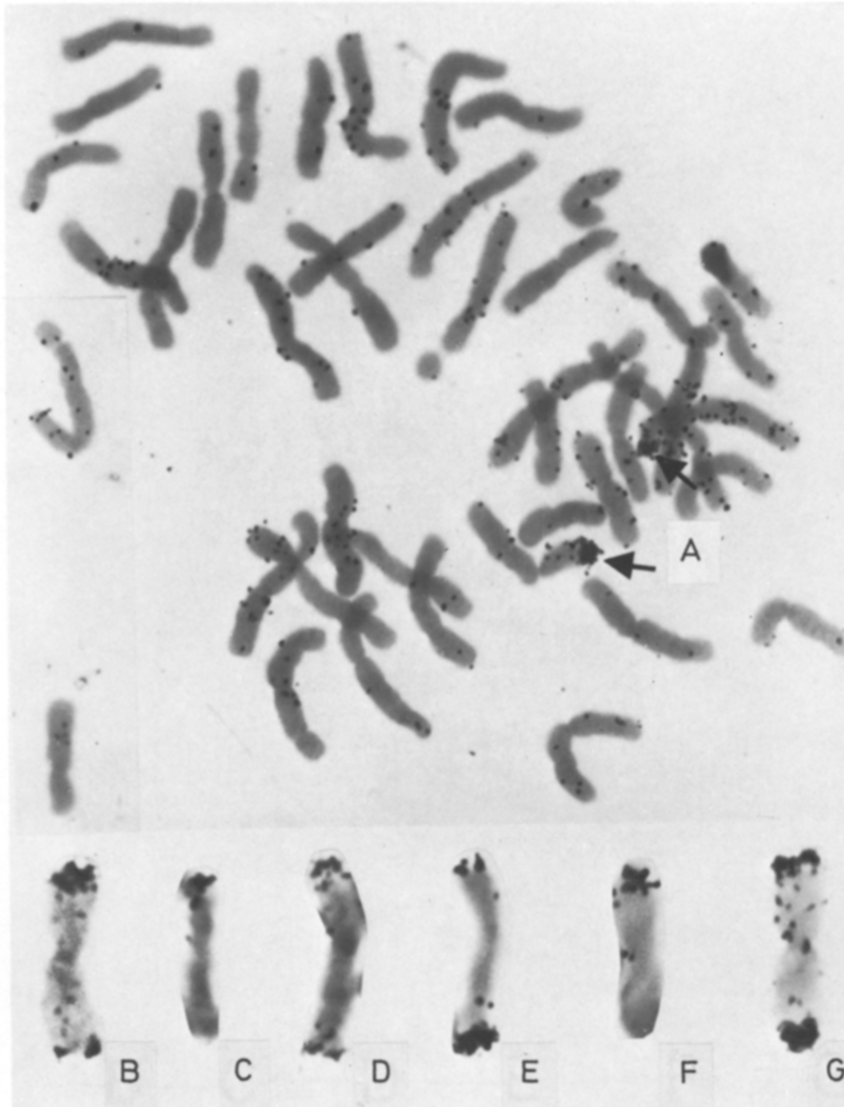

Fig. 11. In situ hybridisation analysis of rye addition lines 1.701 g/cc DNA sequences were assayed in all the rye addition lines A through to G. The respective rye chromosomes from addition lines B→G were cut out as indicated.

The cross-reaction of the 1.701 cRNA with wheat chromosomes is not with exactly the same set of sequences found in rye because T_m analyses of hybrids formed with unfractionated DNA on nitrocellulose filters indicates the hybrid formed with wheat DNA has a $T_m = 50.3°$ C compared with a T_m of 55.3° C with rye DNA

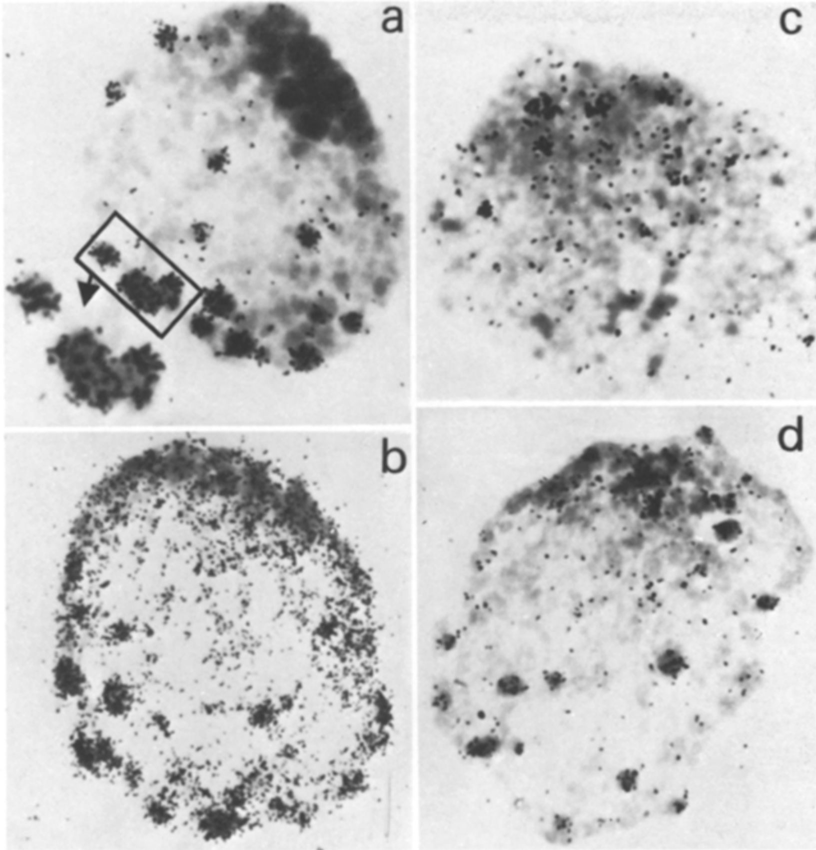

Fig. 12a–d. Distribution of sequences in interphase nuclei. **a** 1.701 g/cc DNA sequences, **b** 1.705 g/cc sequences isolated from C_0t 0–0.02 DNA, **c** polypyrimidine tract sequences, **d** simultaneous assay for both the sequences in **a** and **c** in a double label experiment. The insert in **a** indicates heterochromatic areas where the distribution of 1.701 g/cc DNA sequences is not uniform (discussed in text)

usually less than the number of telomeres labelled in metaphase chromosomes. The distribution of grains over a chromocentre is not uniform (Fig. 12a) suggesting sequence differentiation within each telomeric heterochromatic block.

In contrast to 1.701 g/cc DNA, other DNA species isolated from C_0t 0.02 DNA are not located so predominantly in the large blocks of heterochromatin. Both the 1.705 g/cc species described earlier and the 1.720 g/cc species (see Figs. 4b and 2e respectively) show more extensive location in the euchromatin than the 1.701 sequences. The distribution of 1.705 sequences (purified from C_0t 0.02 DNA by Ag^+/Cs_2SO_4 centrifugation) in an interphase nucleus is shown in Fig. 12b.

b) Polypyrimidine Tract Sequences. The polypyrimidine tract sequences have a distinctive distribution within the chromosome complement (Fig. 13). Figure

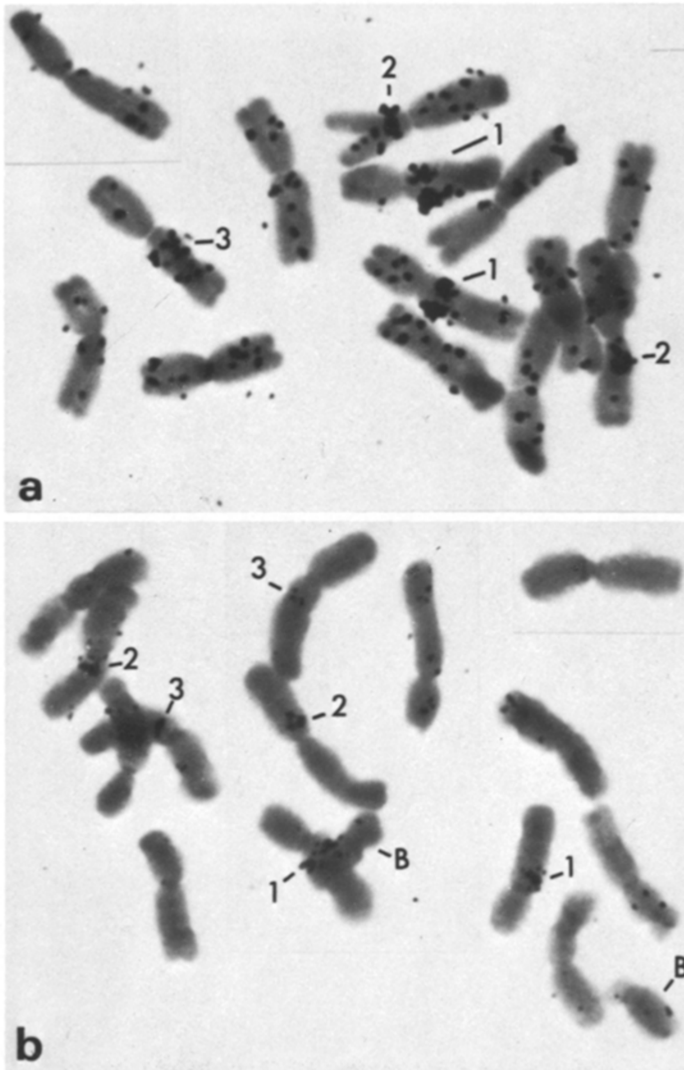

Fig. 13a and b. Chromosomal distribution of polypyrimidine tract sequences. ^{3}H-cRNA synthesised from either rye polypyrimidine tracts (**a**) or *D. melanogaster* 1.705 g/cc satellite (**b**) was utilised for in situ hybridisation

13a shows a metaphase spread of chromosomes hybridised with cRNA synthesised from rye polypyrimidine tracts, while Fig. 13b shows a chromosome preparation hybridised with cRNA from the *D. melanogaster* 1.705 g/cc satellite (the particular root tip used here came from a plant, *Secale cereale* cv. Unevita, which also contained a pair of B chromosomes). Two major interstitial sites (1, 2) were readily recognised while a third site (3) was sometimes evident. The two polypyrimidine tract probes labelled the same major sites. This was

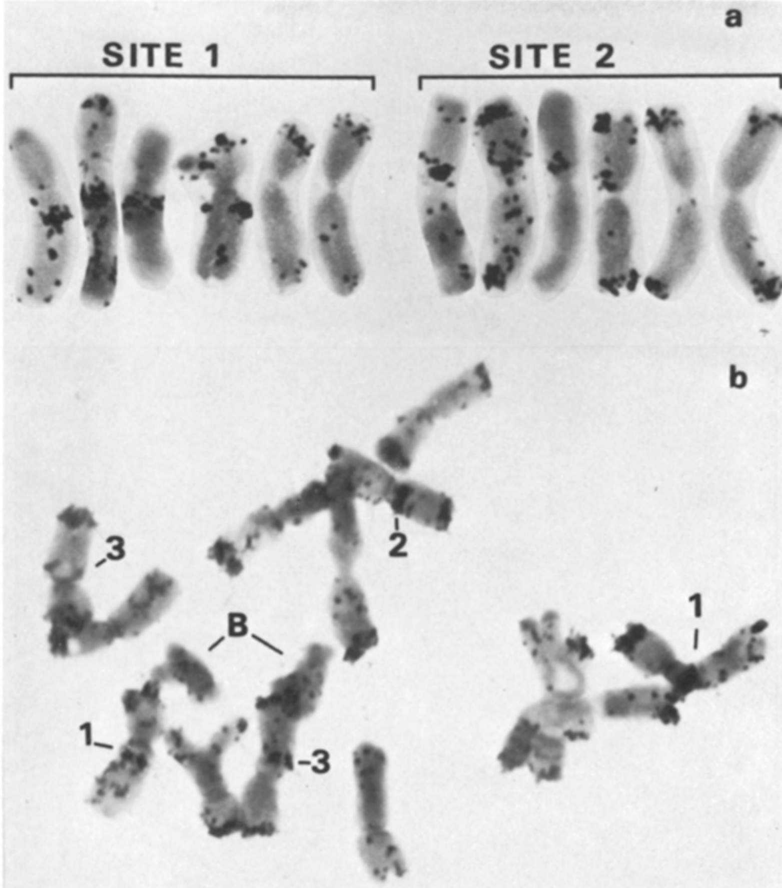

Fig. 14a and b. Double label patterns of rye chromosomes. ^{3}H-cRNA synthesised from 1.701 g/cc DNA to label the telomeres of chromosomes and from polypyrimidine tracts (either rye polypyrimidine tracts or *D. melanogaster* 1.705 g/cc satellite) to label interstitial sites were mixed in a ratio of 1:15 and then used for in situ hybridisation. **a** Comparisons of the sites assayed by ^{3}H-cRNA from rye polypyrimidine tracts and *D. melanogaster* 1.705 g/cc satellite. For each of the sites chromosomes from representative cells hybridised in a number of different ways have been arranged in the order (from left to right), rye polypyrimidine tract ^{3}H-cRNA, rye polypyrimidine tract ^{3}H-cRNA plus 1.701 g/cc ^{3}H-cRNA, *D. melanogaster* 1.705 g/cc ^{3}H-cRNA, *D. melanogaster* 1.705 g/cc ^{3}H-cRNA plus 1.701 g/cc^{3}H-cRNA. The last two chromosomes are two possible chromosomes (labelled with 1.701 g/cc ^{3}H-cRNA) which correspond to the polypyrimidine tract labelled chromosomes. **b** A metaphase spread labelled with 1.701 g/cc ^{3}H-cRNA plus *D. melanogaster* 1.705 g/cc ^{3}H-cRNA

best shown by a double label experiment in which 1.701 g/cc sequences were hybridised along with one or the other of the polypyrimidine tract probes. Figure 14a shows that site 1 for both probes is on the long arm of a chromosome labelled predominantly on the short arm telomere by 1.701 g/cc sequences while site 2 is on the short arm of a chromosome labelled approximately equally on both telomeres by 1.701 g/cc sequences. The two probes show an interesting difference however, in that the *D. melanogaster* 1.705 g/cc satellite cRNA does

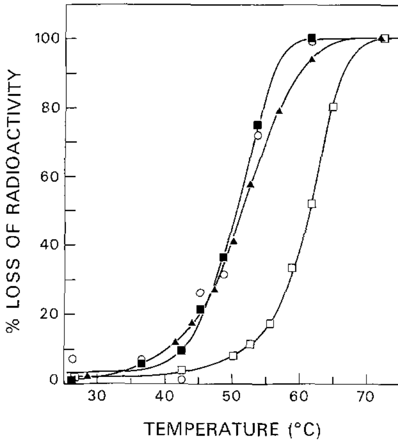

Fig. 15. Melting points of in situ hybrids. The T_m of the hybrids formed at sites 1 (■---■) and 2 (○---○) using rye polypyrimidine tract ^{3}H-cRNA. In the same experiment the T_m of hybrids formed to DNA immobilised on nitrocellulose filters were determined to provide internal controls. ▲---▲ rye polypyrimidine tract ^{3}H-cRNA hybridised to rye DNA, □---□ *D. melanogaster* 1.705 g/cc satellite ^{3}H-cRNA hybridised to *D. melanogaster* DNA (the T_m of this hybrid is standardised to 61° C)

Fig. 16. Double labelling of rye add-G chromosomes. The double labelling was carried out as described in Fig. 14

not show as much general labelling of the chromosomes as the rye polypyrimidine tract cRNA. With the 1.705 cRNA site 3 is distinguishable in a double label experiment, and has been located on the long arm of a chromosome which shows 1.701 g/cc sequences on both telomeres (Fig. 14b). The metaphase chromosome preparation in Fig. 14b also shows two B chromosomes which show no major sites for the sequences under investigation.

Both polypyrimidine tract probes showed that the sequences located at sites 1 and 2 must be very similar, if not identical, to each other because the T_m of hybrids formed at these sites were indistinguishable (Fig. 15).

We attempted to identify the rye chromosomes carrying the major polypyrimidine tract sites using the rye addition lines. This was not possible however, even when a double label experiment (as in Fig. 14) was carried out, because of the much greater content of polypyrimidine tract sequences in wheat chromosomes. Figure 16 shows rye add G in a double label experiment and although the chromosome indicated could be tentatively identified as one of the rye chromosomes (because of its larger size), the identification was ambiguous. Detailed analysis of the chromosomal distribution of polypyrimidine tract sequences in wheat have been reported elsewhere (Gerlach et al., 1978). From the double label experiment shown in Fig. 14b it seems likely that site 1 is on either chromosome C or F, and sites 2 and 3 on either D or G. Sites 2 and 3 must not be located on chromosome E because in chromosome preparations where the secondary constriction was clearly visible no polypyrimidine sites were present on this chromosome.

The distribution of polypyrimidine tract sequences in interphase nuclei (Fig. 12c) indicates the major sites remain condensed and are found in a well defined part of the nucleus which has a characteristic heavy Giemsa stain. In doubly labelled nuclei (Fig. 12d) the polypyrimidine tract sequences are seen to be located in the hemisphere opposite the 1.701 g/cc sequences.

Discussion

The data presented in this paper show that the arrangement and chromosomal distribution of highly repeated sequence DNA in *Secale cereale* is, in general terms, the same as that found in animals. Before we discuss the data in detail, we need to briefly summarise what is known about the rye genome. Cytologically, rye chromosomes have been carefully studied at pachytene of meiosis (Lima-de-Faria, 1952) and more recently by C-banding of root tip mitoses (Gill and Kimber, 1974; Darvey and Gustafson, 1975; Bennett et al., 1977; Singh and Röbbelen, 1977). These studies show that 4 of the 7 chromosomes have heterochromatic regions at both telomeres while the remaining 3 have heterochromatic regions only at the short arm telomeres. In addition, interstitial C-bands are seen on some chromosomes although their reproducibility varies amongst published data. It has been estimated from length measurements that 12% of the genome is in telomere heterochromatin. While this estimate is an approximation due to the variable density of DNA along the chromosome (Heneen and Caspersson, 1973) it is of the same order as the 10% of the genome recovered as

very rapidly renaturing DNA. Ranjekar et al. (1974) estimated this DNA to have a repetition frequency of 6×10^5 to 6×10^6, assuming no "fold-back" DNA sequences are present. Smith and Flavell (1977) have argued that this is not a valid assumption and suggest, by analogy with wheat, that 4% of the genome may consist of DNA capable of forming intra-molecular duplex structures upon renaturation. If the very rapidly renaturing DNA fraction is largely "fold-back" DNA there is no reason, a priori, to expect this to be confined to heterochromatic regions of the genome.

Our data suggest the C_0t 0–0.02 fraction of rye DNA is composed of several simple sequence DNA species. For example, some properties of the 1.701 g/cc component (2–4% of the genome) which indicate that it consists of repeated simple sequences are; the presence of high molecular weight aggregates; the simple oligonucleotides present in RNase A digests of cRNA; and the rate of renaturation under low salt conditions. Other simple sequences are present in the C_0t 0–0.02 fraction in smaller quantities but at present have not been individually purified, except that it was possible to purify simple sequences in the form of polypyrimidine tracts and these were shown to band at approximately 1.704 g/cc in the C_0t 0–0.02 DNA fraction. The fraction of the genome containing this sequence was estimated to be 0.12%. Smith and Flavell (1977) considered the very rapidly reannealing sequences to be clustered. Our data suggest this is correct but also emphasise that this class of DNA cannot be treated as a single group of sequences. Whereas the 1.701 g/cc sequences behave as short sequence repeat units tandemly arranged in blocks of approximately 30 kb, a proportion of the polypyrimidine tracts are located in much larger, homogeneous blocks. The analysis in Figure 8, however, clearly shows the distribution of polypyrimidine tract sequences within the genome to be complex. We have recently found that both 1.701 g/cc sequences and polypyrimidine tracts can be recovered from total rye DNA in defined restriction endonuclease fragments so that DNA-cloning should be readily applicable and should assist in the resolution of the complexities of arrangement of these sequences.

The finding that the rye genome does contain highly repeated sequence DNA organised in long blocks is compatible with the in situ hybridization observation that they are located in heterochromatin. Approximately 80% of the 1.701 g/cc sequences are located in the telomeric blocks of heterochromatin and thus account for 25–50% of the DNA present in this chromatin. At least some of the 1.701 g/cc sequences which are located in the euchromatin may be located at sites corresponding to the interstitial heterochromatic bands. Site 1 of the polypyrimidine tracts appears to correspond to one of the interstitial C-bands. There also appears to be an interstitial C-band on the short arm of a chromosome corresponding to site 2 of the polypyrimidine tracts (Zeller et al., 1977). However the simple sequences also show a dispersed distribution which is not correlated with any C-banding pattern. The lack of complete synonymity of heterochromatin, as defined by C-banding, with highly repeated DNA sequences has been found for animal systems and probably reflects the fact that highly repeated sequence DNA is a prerequisite for the cytological definition of heterochromatin, but is not necessarily the only requirement (Appels and Peacock, 1978). There may, for example, exist requirements for a

certain length of DNA to be composed of highly repeated sequences, tandemly arranged, before the respective chromatin segment is visibly heterochromatic.

The observation that only approximately 25–50% of the DNA in telomeric blocks of heterochromatin could be accounted for by 1.701 g/cc sequences is reflected in the in situ hybridisations. Examination of interphase nuclei showed that in heterochromatic chromocentres, the 1.701 g/cc sequences were not uniformly distributed. Furthermore another DNA species purified from C_0t 0–0.02 DNA, banding at 1.705 g/cc, was also found in the telomeric heterochromatin. These observations must reflect a certain degree of sub-structure within these regions of the chromosomes which possibly correlates to cytological differentiation of the type discussed by Lima-de-Faria (1952) in his description of the chromomeres in rye pachytene chromosomes.

In conclusion, we find that most aspects of the genomic distribution of highly repeated DNA sequences in rye are the same as those found for highly repeated DNA in animal genomes. It thus appears that a general feature of the organisation of this class of DNA is that it occurs in long blocks which serve to "individualise" the heterochromatic regions of chromosomes. The fact that the rye telomeric sequences cross-hybridize only to a limited degree with wheat chromosomes allows rye chromosomes (or translocated segments of chromosomes) to be readily detected in wheat-rye hybrids. This sequence specificity has already proved useful in breeding programmes where in segregating populations of hybrids, involving one rye chromosome, the determination of plants carrying one or two doses of the rye chromosome were made from interphase nuclei hybridised with the 1.701 g/cc probe.

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