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The Wheat Ribosomal DNA Spacer Region: Its Structure and Variation in Populations and Among Species

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Summary. The wheat rDNA clone pTA250 was examined in detail to provide a restriction enzyme map and the nucleotide sequence of two of the eleven, 130 bp repeating units found within the spacer region. The 130 bp units showed some sequence heterogeneity. The sequence difference between the two 130 bp units analysed (130.6 and 130.8) was at 7 positions and could be detected as a 4 °C shift in Tm when heterologous and homologous hybrids were compared. This corresponded to a 1.2% change in nucleotide sequence per ΔTm of 1 °C. The sensitivity of the Tm analysis using cloned sequences facilitated the analysis of small sequence variations in the spacer region of different *Triticum aestivum* cultivars and natural populations of *T. turgidum* ssp. *dicoccoides* (referred to as *T. dicoccoides*). In addition spacer length variation was assayed by restriction enzyme digestion and hybridization with spacer sequence probes.

Extensive polymorphism was observed for the spacer region in various cultivars of *T. aestivum,* although within each cultivar the rDNA clusters were homogeneous and could be assigned to particular chromosomes. Within natural populations of *T. dicoccoides* polymorphism was also observed but, once again, within any one individual the rDNA clusters appeared to be homogeneous. The polymorphism, at the sequence level (assayed by Tm analysis), was not so great as to prevent the use of spacer sequence variation as a probe for evolutionary relationships. The length variation as assayed by restriction enzyme digestion did not appear to be as useful in this regard, since its range of variation was extensive even within populations of a species.

Key words: Wheat – rDNA – Sequence – Populations – Variation – Spacer region – *Triticum*

Introduction

The spacer regions between ribosomal RNA coding regions (rDNA) show extensive sequence divergence relative to the conserved coding regions (for a review see Federoff 1979). These spacer regions are, therefore, potentially useful as a marker for the evolutionary divergence between species. We have examined this possibility by characterizing the wheat rDNA spacer and its relationship to the respective DNA regions in other members of the tribe *Triticeae.* The evolutionary relationships between members of the *Triticeae* have been extensively analysed by using many morphological and cytogenetic criteria and the tribe thus provides a useful system for studying the molecular evolution of nucleic acid sequences.

The rDNA region of wheat has been studied at a molecular level (Flavell and Smith 1974; Flavell and O'Dell 1976; Appels et al. 1980; Gerlach and Bedbrook 1979) and the cloning of the entire 9 kb unit by Gerlach and Bedbrook provided the starting material for the present study. The plasmid pTA250 carries the shortest of the known length variants of rDNA in *Triticum aestivum* cv. Chinese Spring (Gerlach and Bedbrook 1979), inserted into the EcoRI site of the plasmid vector pACY184. Digestion of pTA250 with EcoRI releases the complete wheat rDNA unit in a form which is, as far as can be ascertained, the same as that found in the original wheat DNA. A variant ca. 150 bp longer than the rDNA in pTA250 has also been cloned (pTA71) by Gerlach and Bedbrook (1979), while the longest length variant found in wheat (ca. 450 bp longer than pTA250) has not yet been isolated.

In this study we have defined the molecular structure of the spacer region of the rDNA in pTA250, and examined polymorphism of these nucleotide sequences in populations of several *Triticum* species as well as variation in the sequences among selected species of the tribe *Triticeae.*

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Materials and Methods

Plants

The cytogenetic stocks of *Triticum aestivum* cv. Chinese Spring nullisomic 1B-tetrasomic 1D (N1BT1D), N6BT6D and N5DT5B were produced by E.R. Sears (1953) and supplied by K. Shepherd (Waite Institute, Adelaide, South-Australia). Sears also developed (Sears 1953) and supplied intercultivar disomic substitutions Hope 1A for Chinese Spring 1A [H1A(CS1A)], H1B(CS1B), H5D(CS5D), H6B(CS6B), Timstein 1B for Chinese Spring 1B [T1B(CS1B)], T5D(CS5D) and T6B(CS6B). The intercultivar disomic substitutions Cheyenne 1A for Chinese Spring 1A[CH1A(CS1A)], CH1B(CS1B), CH5D(CS5D) and CH6B(CS6B) were developed and supplied by R. Morris (Morris et al. 1966).

Populations of plants of *Triticum turgidum* L.em. Morris et Sears ssp. *dicoccoides* (referred to as *T. dicoccoides*) were collected in Syria by S. K. Jana, Crop Science Department, University of Saskatchewan, Saskatoon, Canada. Population IZ was collected 2 km from Izra, population 25, 25 km N of Izra and population 35, 35 km N-W of Aleppo (all populations from Syria). The individual spikes were collected randomly every 10 m. Additional material of this species was provided by G. Wains, Department of Plant Science, University of California, Riverside, USA. These accessions were collected by B. L. Johnson, from the same department. The regions covered are: 4 accessions from Turkey, 5 from Lebanon, 5 from Israel and 1 from W Iran.

An accession of *Elytrigia elongata* (Host.) Holub originally came from L. B. Stebbins, Genetics Department, University of California, Davis (USA).

DNA Isolations

Individual seedlings (4–10 cm), germinated aseptically from surface sterilized (2% bleach, 10 min) seeds, were crushed in a mortar and pestle in liquid nitrogen in the presence of a small quantity of acid-washed fine sand (BDH) to aid cell breakage; for good yields of DNA (20–50 μg) it was essential to produce a very fine homogenate. 1 ml of isolation buffer (0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris-HCl, pH 7.0) was added to the homogenate and the resulting ice slurry further homogenized. As the slurry melted 0.1 ml of 5% SDS and 0.1 ml of proteinase K (a 0.2 mg/ml stock solution in H_2O, Boehringer) were added and the homogenization continued. Following transfer of the homogenate to a 15 ml corex centrifuge tube, the mortar was washed with 0.5 ml of isolation buffer and the pooled solutions incubated at 37 °C for 3 h. The homogenate was extracted with phenol-chloroform (1 : 1 v/v) and DNA recovered from the aqueous phase by ethanol precipitation at −80 °C in the presence of 0.2 M sodium acetate pH 5.5. The DNA was redissolved in 0.5 ml of TE (0.01 M Tris-HCl pH 8.0, 0.001 MEDTA) and incubated with 10 μg/ml RNAase A (Sigma). The DNA was ethanol precipitated in the presence of 0.2 M sodium acetate pH 5.5 (after adding SDS to a final concentration of 0.1%), redissolved in H_2O, precipitated once more and dissolved in TE. DNA prepared in this way was suitable for digestion by restriction enzymes.

Subcloning of DNA Segments from pTA250 and pTA71

Digestion of pTA250 with a number of restriction enzymes indicated that the enzymes Hinf, Dde and Taq I were useful markers within the rDNA region. A number of DNA fragments were subcloned by excising the fragment from agarose gels (Weinand et al. 1979; for a survey of techniques used in recombinant DNA work see Wu, 1979) filling in the ends with DNA polymerase I (Goodman 1979) and ligating synthetic Bam linkers (Collaborative Research, Inc.) to the ends of the molecules. The fragments were then digested with Bam HI, excess synthetic linkers removed by passage through a gel filtration column (Biorad A150) and ligated to Bam digested pBR322. Positive selection of clones carrying insertions in the tetracycline gene of pBR322 was carried out using the fusaric acid procedure described by Maloy and Nunn (1981). Taq I fragments were also inserted directly into the Cla I site of pBR322 and selected in a similar way.

DNA Mapping Procedures

(a) Utilizing End-labelled Restriction Fragments

The "fill-in" reaction using DNA polymerase I (Klenow fragment, Boehringer) was used as described by Goodman (1979). Double digestion or partial digestion of ^{32}P-end labelled restriction fragments followed by electrophoresis in 6% acrylamide gels or 2% agarose, and autoradiography was used to establish the order of restriction enzyme sites relative to each other.

(b) DNA Sequencing

Cloned fragments were excised from the vector pBR322 using Bam HI (since synthetic Bam HI linkers were used in the cloning) and the 3′-ends end-labelled by the "fill-in" reaction as described above. The 5′-ends were labelled by dephosphorylating the DNA with bacterial alkaline phosphatase (Shinagawa and Padmanabhan 1979; J. Langridge pers. comm.), extracting with phenol-chloroform and then phosphorylating with γ-^{32}P-dATP and 5′ phosphokinase as described by Maxam and Gilbert (1977). The sequencing strategy and reactions were those described by Maxam and Gilbert (1977).

(c) Cross-hybridization Reactions

Restriction fragments were electroeluted from agarose gels and nick-translated using ^{32}P-deoxynucleotide triphosphates, DNA polymerase I and DNAase (Rigby et al. 1977). The single-strand length of the ^{32}P-probe was increased from 50–100 nucleotides to approximately 300 nucleotides by treatment with T4 ligase. The ^{32}P-probe was then hybridized to restriction enzyme digests of plasmid which had been transferred to "Gene-screen" (New England Nuclear); the gene-screen filter was prehybridized in the solution used for hybridization (without probe) for 1–3 h. Hybridization was carried out in 3×SSC/50% formamide at 37 °C for 10–12 h in the presence of 5×Denhardt's solution (Denhardt 1966), 0.1% SDS and 30–50 μg of unlabelled pBR322 (Hae III digested). The filters were washed in 2×SSC/0.1% SDS at 65 °C.

Genomic DNA's were digested with restriction enzymes, electrophoresed in 1%–2% agarose gels and analysed by hybridization as described above.

Melting Point Determinations

Plant DNA (5 μg) was denatured in 0.2 mls 0.5 M NaOH (37 °C, 10 min) and cooled on ice. Cold neutralizer solution (1.5 ml, 0.5 M Tris-HCl pH 7, 0.5 M NaCl) was added and the mixture immediately loaded onto 25 mm discs of nitro-cellulose (Sartorius) by filtration. The DNA was fixed onto the filters at 80 °C/2 h under vacuum. The filters were then prehybridized for 1–2 h in hybridization mix without ^{32}P-probe and then hybridized and washed as described above. To

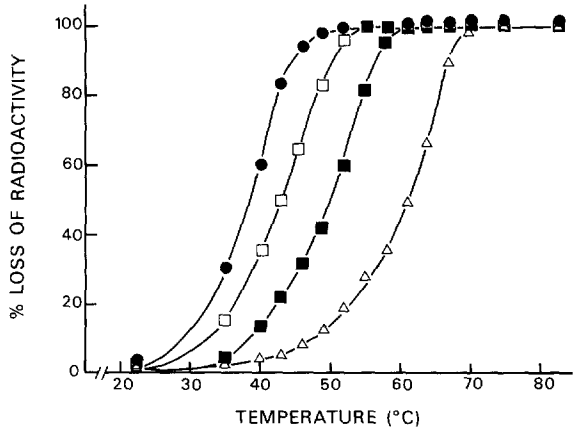

Fig. 1. Effect of formamide on the Tm of hybrids formed between a ^{32}P probe and DNA immobilized on a nitro-cellulose filter. The concentrations used were 50%, 65%, 75%, 84% (△–△, ■–■, □–□, ●–●, respectively) and the DNA immobilized was Chiese Spring DNA. The ^{32}P probe was the "130" bp probe. The same result was obtained using the "750" bp probe

determine the melting point (Tm) of the hybridized probe the filters were serially transferred to 0.75 ml aliquots of 3× SSC/50% formamide (in scintillation vials) at the required temperature. Filters were held at the appropriate temperature for 5–8 min and then transferred to the next vial. Up to twenty melting point determinations were carried out at the same time. To determine the radioactivity released at the various temperatures 0.35 ml of H_2O and 9 ml of Triton-X based scintillation fluid were added to the vials.

The DNA immobilized on nitro-cellulose and the radioactive probe hybridized to it, as used in this study, behaved as normal DNA duplexes as judged by the sigmoidal release of radioactivity (cumulative release is calculated) upon heating and the sensitivity of the melting point to formamide (Fig. 1); from the data shown in Fig. 1 we estimate 1% of formamide lowers the Tm by 0.73 °C which is in good agreement with the 0.72 °C estimated by McConaughy et al. (1969). Since the Tm is very sensitive to the formamide concentration, an internal standard (Chinese Spring wheat DNA in this study) is essential in each set of analyses.

Results

(i) Structure of Wheat rDNA in pTA250

The plasmid pTA250 (Gerlach and Bedbrook 1979) was studied in detail to determine its map (Fig. 2). Seventeen segments from the wheat rDNA in this plasmid were subcloned in pBR322. Segments which were critical to work described here were pTA250.1 (4.4 kb, Bam/Eco fragment), pTA250.2 (3.6 kb Bam fragment containing the coding sequences), pTA250.4 (2.7 kb Taq fragment entirely from within the spacer region pTA250.3 (0.9 kb, Bam/Eco fragment from the 26S rRNA gene), pTA250.10 (1.0 kb Taq fragment from the 18S rRNA gene), pTA250.11 (0.5 kb Taq fragment from the 26S rRNA gene), pTA250.15 (0.75 kb Hha fragment from the spacer region), pTA250.16 (0.65 kb Hha fragment from the spacer region), pTA250.17 (0.65 kb Hinf fragment containing 0.3 kb of the 18S rRNA gene).

The significant feature revealed by the restriction enzyme mapping is the 130 bp tandemly repeating unit in the spacer region. The partial digestions shown in Fig. 3 illustrate this structure with respect to both the Hae III and Hha I sites. The 750 bp Hha I fragment (pTA250.15) contains only 100 bp which cross-hybrid-

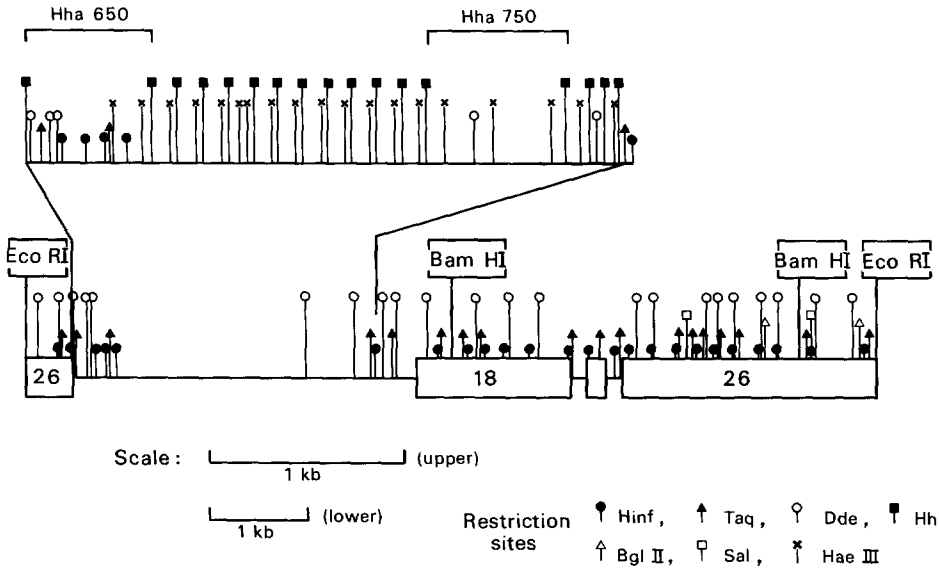

Fig. 2. Restriction enzyme map of pTA250. In the lower map the positions of the restriction enzyme sites are ±50–100 bp while in the upper map they are ± 10 – 50 bp. In the upper map the Hae III sites were not determined in the Hha 650 region

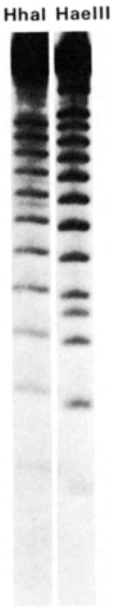

Fig. 3. Partial digestion of the spacer region to reveal the "130" bp repeating unit defined by Hae III and Hha I. The 250.4 sequence was labelled at its Hinf site and partial digestion products electrophoresed on 2% agarose and visualized by autoradiography

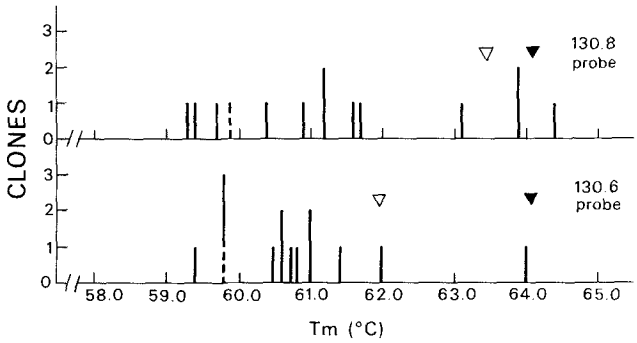

Fig. 4. Summary of the Tm's of hybrids formed between the 130.6 unit and the rest of the "130" bp units (lower panel) and the 130.8 unit and the rest of the "130" bp units (upper panel). The height of the lines at given Tms indicates the number of "130" bp units in this particular category; the broken line indicates the position of the 130.8 sequence in the lower panel and the 130.6 sequence in the upper panel. The open arrow indicates the Tm of the respective sequence with Chinese Spring DNA and the closed arrow the Tm of the homologous hybrid (i. e. the respective sequence hybridized to itself)

ize with the 130 bp units and provided a non-repeated sequence from the spacer region. The "130" and "750" bp sequences as released by Hha I digestion of the spacer region in pTA250.4 were used in this, and the following, papers (Dvořák and Appels 1982; Appels and Dvořák 1982) to investigate their polymorphism in natural populations and their divergence during the evolution of the *Triticeae*.

The 130 bp Hha I repeated units from pTA250.4 were subcloned and a population of 14 clones compared by determining the melting points (Tm's) of cross-hybridized molecules (Fig. 4). The variability in the Tm's indicated that the nucleotide sequences of the "130" bp repeats are not identical. The maximum difference (4 °C) occurred between 130.6 and 130.8. The 130.8 unit appeared to be more representative of the "130" bp repeats in pTA250 since a larger proportion of the population of clones analysed yielded smaller ΔTms (relative to the homologous reaction) when cross-hybridized with the 130.8 sequence than with the 130.6 sequence. The Tm's measured when the 130.6 and 130.8 sequence were hybridized with genomic wheat DNA (indicated in Fig. 4), indicate that the 130.8 sequence forms a hybrid which is more closely related to the homologous hybrid than does the 130.6 sequence. This is consistent with the 130.8 sequence representing a major type of variant of the "130" bp units. The 130.6 sequence is in the group of 130 bp units which are at the lower extreme of the distribution assayed by the 130.8 sequence in Fig. 4; from the size of population of 130 bp units examined we calculate that the probability of the 130.6 sequence representing

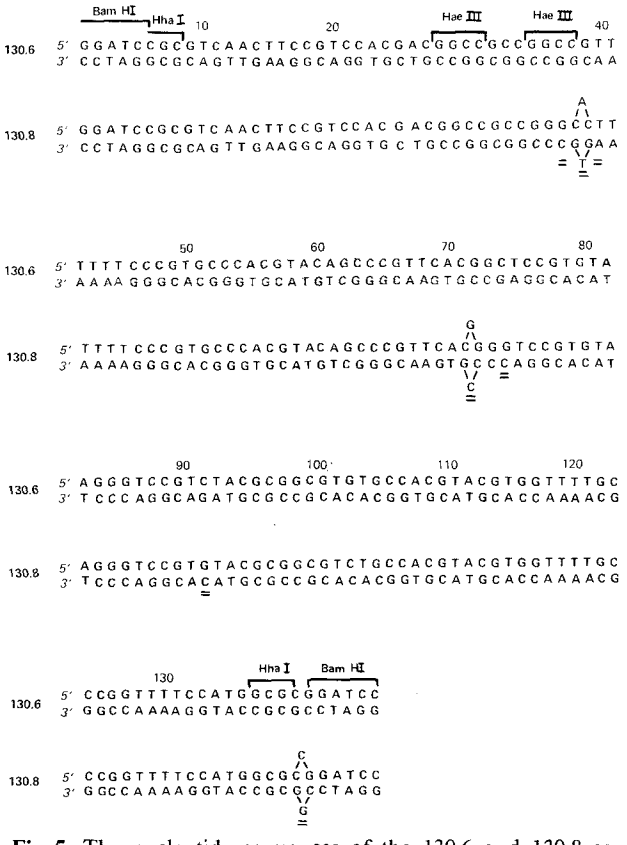

Fig. 5. The nucleotide sequences of the 130.6 and 130.8 sequences. The Bam HI (derived from the addition of synthetic linkers). Hha I (defining the "130"bp unit) and the Hae III sites are indicated. The position of differences between 130.6 and 130.8 sequences is indicated (=). The difference at position 37 results in the absence of a Hae III site in 130.8 relative to 130.6

a variant maximally different from the predominant sequence (represented by the 130.8 sequence) is 0.74 (calculated using the binominal $\left(\frac{1}{11}+\frac{10}{11}\right)^{14}$).

The nucleotide sequences of the 130.6 and 130.8 clones were determined since they appeared to represent sequences near the extremes of the variation among the "130" bp units of the spacer region. The two sequences differed in six positions (Fig. 5), two of which are deletions of a nucleotide in 130.6 relative to 130.8. Both sequences have four tandem duplications, 5–10 nucleotides long (positions 27–32 and 34–39, 46–50 and 63–67, 72–82 and 84–94, 116–122 and 127–131). The synthetic Bam HI linkers introduced one additional difference between the sequences in the cloned form, resulting in a total of 7 base pair differences per 142 nucleotides. Since the ΔTm between the sequences was 4 °C, there is 1.2% nucleotide mismatch per 1 °C of ΔTm. This agrees well with the figure of 1.5% mismatch per 1 °C determined in other systems (Laird and McCarthy 1968).

(ii) Genomic Structure and Distribution of the rDNA Spacer Sequences

The "130" and "750" sequences are confined to the rDNA region of the chromosome and do not occur in large blocks independent of the rRNA genes (contradictory conclusions have been published for animals; compare Grummt et al. 1979; Miesfeld et al. 1981). The evidence for this was derived from two observations. First, restriction enzyme digests of *T. aestivum* genomic DNA, probed with either of the sequences did not show any DNA fragments which could not be clearly assigned to an rDNA cluster. Bam HI digestion (results not shown) showed that the spacer sequences are located in the 5.4 kb and 9.0 kb bands as expected from restriction enzyme maps of genomic rDNA (Appels et al. 1980) and cloned repeating units of rDNA (Gerlach and Bedbrook 1979; Fig. 2). Hae III digestion of Chinese Spring wheat DNA (Fig. 6) revealed hybridization patterns with the "130" bp and "750" bp sequences consistent with the map in Fig. 2; the ladder of fragments hybridizing to the "130" bp sequence has a periodicity of 130 bp and suggests the existence of sequence heterogeneity leading to occasional inactivation of the Hae III site, in Chinese Spring wheat rDNA. Even within pTA250, heterogeneity for the Hae III site is evident since the fourth 130 bp unit in the tandem array has an extra Hae III site (Fig. 3). Sequencing of the 130.6 and 130.8 units clearly showed that a Hae III site in 130.8 has been lost (relative to 130.6) by mutation (Fig. 5). Secondly, in situ hybridization to root tip chromosomes showed the expected,

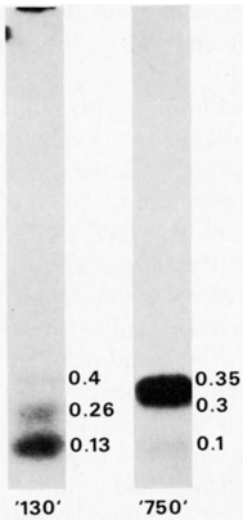

Fig. 6. Hae III digests of Chinese Spring DNA (electrophoresed in 2% agarose) hybridized with either the "130" bp or "750" bp probes. The sizes of the bands were determined relative to a Hae III digest of pBR322

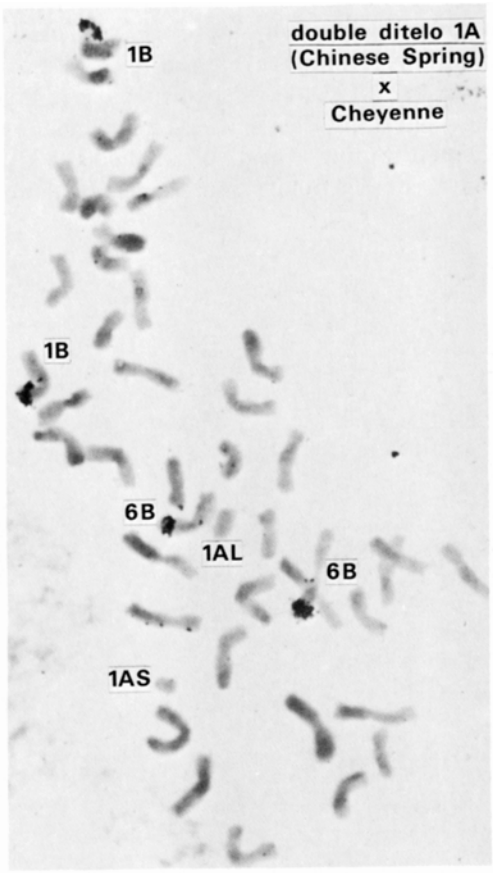

Fig. 7. Chromosomal locations of the "130" bp sequence. The "130" bp DNA as defined by Hha I was ligated and then used as a template for RNA polymerase to produce ^{3}H-cRNA. Hybridization to root-tip chromosomes was carried out as previously described (Appels et al. 1980). The cross shown was analysed to assay for a possible major site of the spacer sequence in chromosome 1A from either Chinese Spring or Cheyenne

major, locations of both "130" bp and "750" bp sequences on the short arms of 1B and 6B ("130" bp probe only shown in Fig. 7).

The studies of Gerlach and Bedbrook (1979) indicated that at least three length variants of the rDNA region exist within the Chinese Spring genotype. The plasmid pTA250 contains the shortest variant while the plasmid pTA71 contains a 9 kb insert which is ca. 150 bp longer than that in pTA250. The longest known variant, 450 bp longer than pTA250, was not cloned. We have analysed pTA71 in sufficient detail to show that the length difference between pTA71 and pTA250 resides in the spacer DNA segment bounded by the 0.65 and 0.75 kb Hha I fragments (see map in Fig. 2). The difference is thus located in the region which contains the "130" bp repeating sequences and is due to the presence of an extra repeat in pTA71. The length variants in pTA250 and pTA71 originate from chromosome 6B. This is most clearly seen in Taq I digests of the DNA from N1BT1D and N6BT6D stocks, which are deficient of chromosomes 1B and 6B respectively (Fig. 8a), hybridized with one of the spacer sequences. As can be seen from the map in Fig. 2 Taq I digestion can assay the central 2.7–3.1 kb of spacer regions in the genomic arrays of rDNA. The smallest fragments observed in Chinese Spring wheat, 2.7 kb and 2.8 kb, originate almost entirely from chromosome 6B; these are the Taq I fragments corresponding to those recovered from pTA250 and pTA71 respectively. The larger, 3.1 kb fragment in Chinese Spring wheat originates from chromosome 1B but this chromosome (and/or 5D) also contains detectable levels of the 2.7 kb and 2.8 kb fragments characterizing 6B (Fig. 8a). In Taq I digestions of Chinese Spring wheat DNA minor bands are also observed, the most obvious one being a 1.8 kb band, and this can be assigned to the rDNA region of 5D (Fig. 8a). A large proportion of the rDNA on 5D (with respect to the spacer region) is probably accounted for by the 1.8 kb band since only 5–10% of the total rDNA in Chinese Spring wheat is located on this chromosome (Flavell and O'Dell 1976; Appels et al. 1980). We cannot rule out the possibility, however, that some of the 2.7, 2.8 or 3.1 kb bands are also located within the 5D rDNA repeat units, particularly in view of the length heterogeneity of the Taq I rDNA spacer fragments among *T. tauschii* (D genome donor) accessions (see for example Fig. 8b).

The differences in the lengths observed in the rDNA spacer regions of chromosomes 1B and 6B is not associated with substantial sequence heterogeneity since the hybrid molecules between genomic DNA of N6BT6D and the "130" bp sequence differ by 1 °C from those involving N1BT1D. The sequence data discussed earlier suggests that this corresponds to only

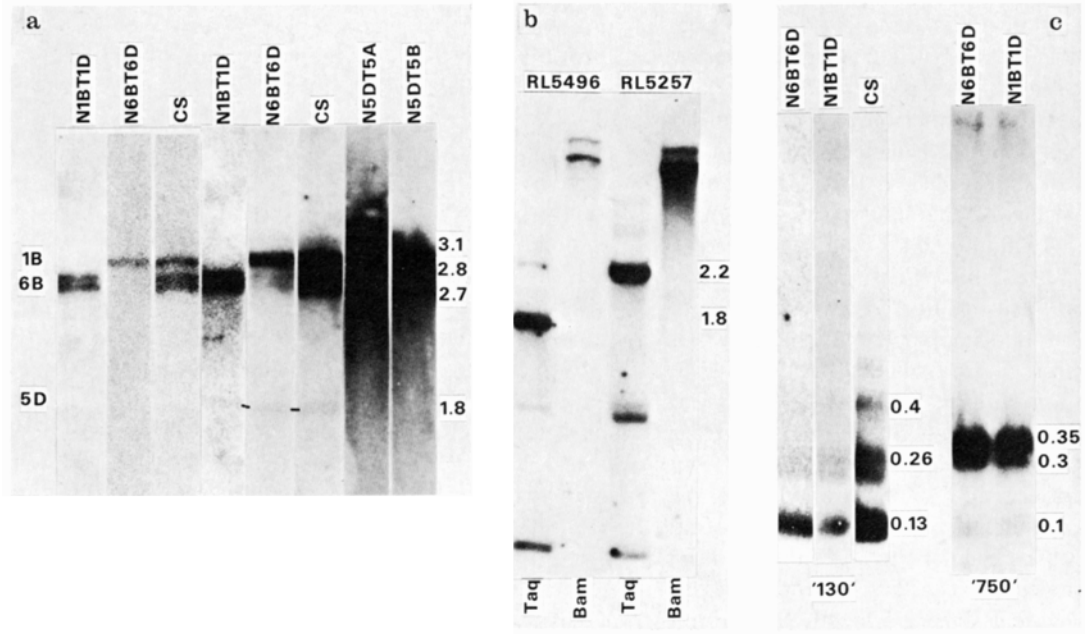

Fig. 8a – c. **a** Assignment of various spacer sequence bands (defined by Taq I digestion of genomic DNA's) to specific chromosomes using the DNA from appropriate nulli-tetra stocks of Chinese Spring. The spacer sequence used as a probe was the "750" bp sequence. The right-most five lanes are long exposures to allow visualization of the 1.8 kb band (if it is present). **b** Bam and Taq I digests of two *T. tauschii* (D genome donor) accessions hybridized with "750" bp probe. The lengths of the Bam fragments were not distinguishable. **c** Comparison of Hae III digests of N1BT1D and N6BT6D genomic DNA's, hybridized with either the "130" bp or "750" bp probes

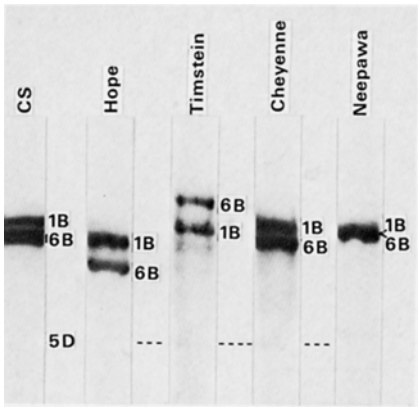

Fig. 9. Variation in length of the major Taq I rDNA spacer segments among *T. aestivum* cultivars. The assignments of particular bands to specific chromosomes is based on the analysis of the respective inter-cultivar substitution lines detailed in the Materials and Methods.

an average of 1.2% difference in nucleotide sequence between the sequences located in chromosomes 1B and 6B. The structure of the spacer regions in 1B and 6B are identical as judged from the hybridization of the "130" bp and "750" bp probes to Hae III digests of the N1BT1D and N6BT6D DNA's (Fig. 8c). The length differences between 1B and 6B of Chinese Spring can therefore be most readily accounted for by a change in the number of "130" bp units in the spacer region as was the case for the length difference between pTA250 and pTA71. This is substantiated by the fact that Bam HI/Eco RI digestion of Chinese Spring DNA shows the size variants expected for 1B and 6B (Gerlach and Bedbrook 1979). Thus to attribute the differences in the Taq I fragments (Fig. 8a) to mutations inactivating one of the Taq I sites would also require corresponding changes in the Bam HI and EcoRI sites, a rather unlikely alternative.

To assay the extent of variation in the length of rDNA spacers within *T. aestivum,* the DNA's of several cultivars (Hope, Cheyenne, Timstein, and Neepawa) were digested with Taq I and probed with the "750" bp spacer sequence from Chinese Spring (Fig. 9). The cultivars Hope, Cheyenne and Timstein in particular were chosen because their chromosomes have been individually substituted into Chinese Spring, replacing their homologues. This enabled us to assign most of the length variants to chromosomes in these cultivars. The cultivars Hope and Timstein originated from hybridization of *T. aestivum* with *T. turgidum* s.l. (McFadden 1930; Watson and Stewart 1956) and it is thus not certain that the rDNA chromosomes in these cultivars originate from *T. aestivum.* None of the 1B and 6B chromosomes assayed in the five cultivars had the same Taq I fragment lengths (Fig. 9). Length variation also occurred among the 5D chromosomes although all cultivars had the 1.8 kb fragment in common (Fig. 9).

Sequence variation in the spacer region was detectable among the cultivars using Tm analysis and the "130" bp probe. The ΔTm's relative to the Chinese Spring standard ranged from 0.5 °C for Timstein to 1.8 °C for Neepawa, reflecting an average difference of 0.6% and 2.2% respectively at the nucleotide sequence level. As detailed in the following section ΔTm's of 0.9 °C and greater are statistically significant.

(iii) The rDNA Spacer as a Probe for the Detection of Variation Between Species and Polymorphism Within Natural Populations

The outer limit of sequence divergence which can be assayed with the rDNA spacer probes is shown by the "130" bp probe hybridized to rye DNA. The melting profile is broad, with a Tm 17.5 °C below that of Chinese Spring wheat DNA (Fig. 10a). This ΔTm suggests at least 21% sequence divergence between the rDNA spacer regions of wheat and rye DNA. The estimate is, however, a minimum estimate since the Tm of the hybrid formed between the "130" bp and rye DNA is only 8.5 °C above the hybridization temperature (37 °C) and thus a significant proportion of very poorly matched hybrids are probably not assayed. Consistent with this interpretation is the observation that the amount of "130" bp sequence hybridized per µg of DNA is much less when rye genomic DNA is compared to wheat genomic DNA (inset Fig. 10a). The "750" bp sequence does not show as high a level of sequence divergence as the "130" bp sequence (Fig. 10b).

In considering the degree of sequence divergence between the rye and wheat rDNA spacer regions it is useful to consider, in parallel, the (lack of) divergence observed in other sequences. The 18S and 26S rRNA genes show a high degree of conservation (Fig. 10c); minor mismatch observed in the early part of the melting curve is very likely contributed by the transcribed spacer region between the 18S and 26S rRNA genes which is present in the probe utilized. A probe for a heterochromatic sequence also shows a relatively high degree of conservation (Fig. 10d). Although this comparison is limited it clearly emphasises the high level of sequence divergence which occurs in the rDNA spacer region.

While the wheat-rye comparison is close to the outer limit of sequence differences in the rDNA spacer that can be assayed by Tm we were also interested in examining the minimum amount of nucleotide sequence differences which could be assayed. The analysis of *T. aestivum* cultivars in the preceding section demonstrated that detectable differences existed within

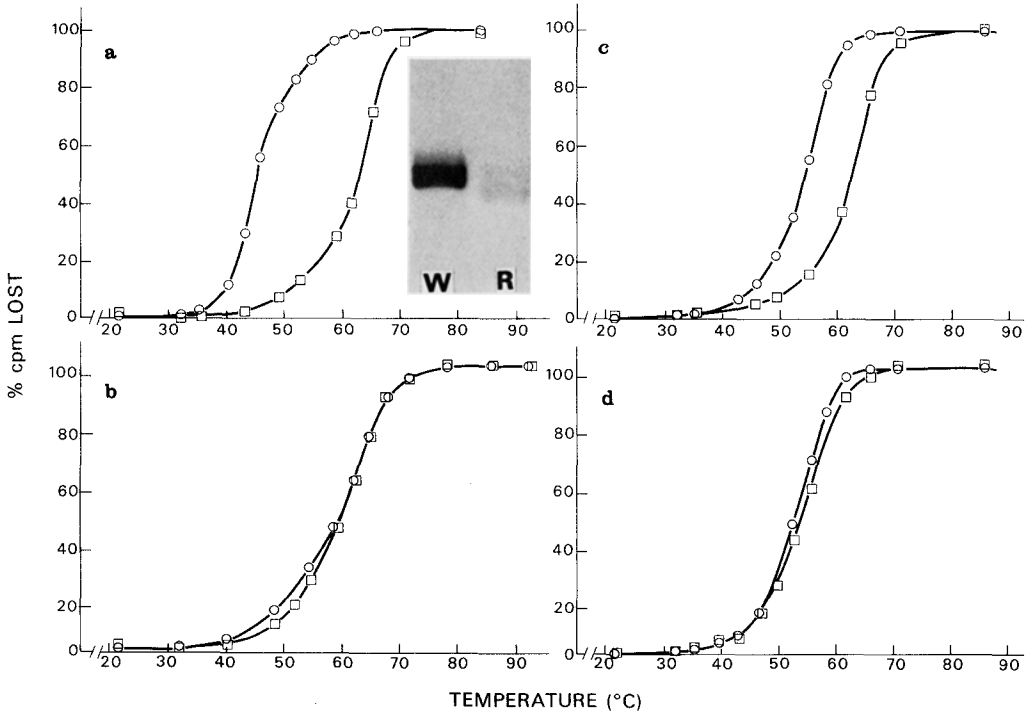

Fig. 10a – d. Tm curves of specific probes from the genomic DNA's of Chinese Spring (□ – □) and *Secale cereale* (cv. Imperial) (○ – ○). **a** "130" bp probe. Inset shows the hybridization of the same probe to Taq I digests of Chinese Spring (W) and rye (R) DNA's. **b** "750" bp probe. **c** Insert from pTA250.2. The probe from this insert hybridizes to 18S, 26S rRNA genes as well as the transcribed spacer region between these two genes. **d** A cloned rye heterochromatic sequence (pSc119; Bedbrook et al. 1980). This sequence hybridizes to heterochromatic regions of non-homologous chromosomes in both wheat and rye

this species. A detailed analysis was therefore carried out on natural populations of *T. dicoccoides; T. dicoccoides* is a tetraploid wheat which is phylogenetically very closely related to *T. aestivum* (see the adjoining paper by Dvořák & Appels 1982 for a fuller discussion). The most extensive analysis was carried out on a population called IZ, which originated from Syria. The DNA from 19 individuals was isolated and analyzed with respect to the Tm of the hybrid molecules involving the "130" bp and "750" bp probes. In addition, the variation in lengths of fragments having sequences homologous to these probes in restriction enzyme digests of genomic DNA's of these individuals was investigated. The Tm analysis (Fig. 11a) indicated that for the "130" bp probe most individuals had a Tm 1 °C below Chinese Spring wheat and suggests and average 1.2% sequence divergence between individuals of the IZ population and Chinese Spring wheat.

Since *T. dicoccoides,* like *T. aestivum,* is a self-pollinating species most of the siblings in a progeny should have the same genotype. This attribute was exploited in determining the minimum number of nucleotide differences that can be reliably detected by Tm analysis. Three individuals IZ5, IZ6 and IZ12, representing the range of the variation in Tm's in the IZ population were chosen on the basis of the data in Figure 11a. Nucleotide sequences of IZ12 appeared to be the most different from the "130" bp nucleotide sequences of Chinese Spring, whereas those of IZ6 and IZ5 were intermediate and least differentiated, respectively. DNA's were isolated from siblings of each individual and the Tm analysis with the "130" bp probe repeated (Fig. 11b). The analysis demonstrated that despite potential sources of variation such as DNA preparation and the experimental determination of Tm, the differences between IZ5 and IZ12, and IZ6 and IZ12 were statistically significant ($P=0.01$); the difference between IZ6 and IZ5 was nonsignificant ($P=0.1$).

To determine to what extent the variation observed in the IZ population is representative of the species, DNA was isolated from two individuals of 17 accessions collected throughout the distribution of *T. dicoccoides.* The ΔTm's relative to the Chinese Spring standard (0→2.2 °C) fell into the range characterizing the IZ population, and therefore it appears that this population is representative of the species.

An analogous study was carried out using the "750" bp spacer sequence. The range of ΔTm variation in the IZ population was very similar to that observed for the "130" bp nucleotide sequences (Fig. 11c),

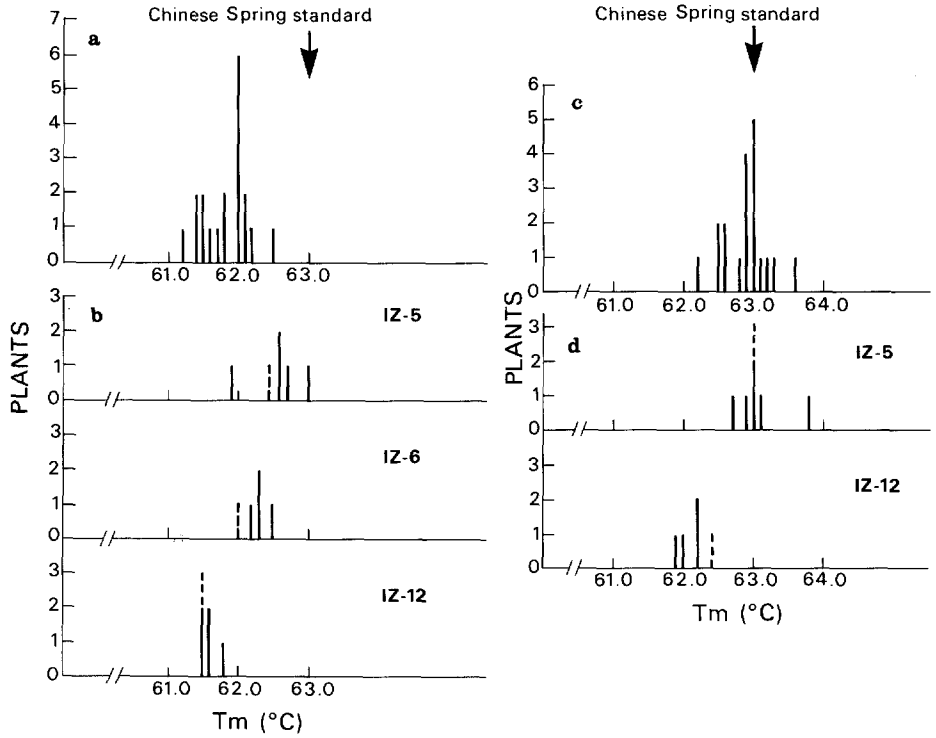

Fig. 11 a – d. Tm analyses of "130" bp and "750" bp probes hybridized to genomic DNA's from individuals of a population of *T. dicoccoides* (IZ). **a** Summary of Tm's of the "130" bp probe hybridized to the IZ population. **b** Summary of Tm's of the "130" bp probe hybridized to siblings of IZ5, IZ6 and IZ12 (position of original measurement from Fig. 11a is indicated as a broken line). **c** Summary of Tm's of the "750" bp probe hybridized to individuals of the IZ population. **d** Summary of Tm's of the "750" bp probe hybridized to the siblings of IZ5 and IZ12 with the position of the original measurements from Fig. 11c indicated as a broken line

amounting to a polymorphism of 1.2% of nucleotides. In contrast to the "130" bp sequences, however, the mean Tm of the IZ population with respect to the "750" bp sequences was the same as that of the Chinese Spring standard; the Chinese Spring standard, it should be recalled, is a *single* DNA preparation used throughout this study as an internal standard for Tm analyses. Differences between two individuals, IZ5 and IZ12, from which sibling DNA was analysed were statistically significant ($P=0.01$) as was the case for the "130" bp sequence (Fig. 11 d).

The IZ population appears to be in linkage disequilibrium for the "130" bp and "750" bp sequences. This is evident from the correlation of $r=0.524$ ($P=0.05$) between ΔTm's (relative to the Chinese Spring standard) of "130" bp hybrid molecules and "750" bp hybrid molecules in individual plants. This result is striking particularly in view of the fact that the sequences occur on two chromosomes which almost certainly assort at random; this indicates that most of the variation observed may be contributed by only one of the chromosomes.

The genomic DNA's of the 19 IZ population plants were also examined by digestion with Taq I and probing the digestion products with a spacer sequence ("750" bp).

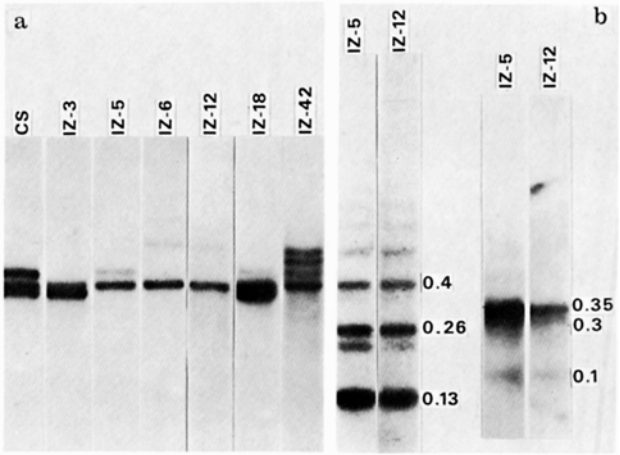

Fig. 12 a and b. Restriction enzyme digests of genomic DNA's from individuals of the IZ population of *T. dicoccoides*. **a** Taq I digests hybridized with the "750" bp probe to visualize the major spacer segment. **b** Hae III digests hybridized with either the "750" bp or "130" bp probes. Only the IZ5 and IZ12 individuals are presented since they were in the classes of individuals maximally different in Tm (particularly with the "130" bp probe). No variation in the bands was observed in the analysis of the entire population available. The numbers indicate lengths in kb. The double band seen most clearly at 0.26 kb is also seen in Chinese Spring (Fig. 8 c) and is most likely due to the presence of "130" bp units with an additional Hae III site (Fig. 2, 3)

Considerable polymorphism in the spacer length occurred among the individuals (Fig. 12a). The most frequent variants were those occurring in Chinese Spring, namely, the Taq I fragments 2.7, 2.8 and 3.1 kb long. The 2.7 kb band occurred together with the 2.8 kb band in 11 individuals, 2.8 kb + 3.1 kb in 4 individuals, 2.8 kb + 4.2 kb + 5.7 kb in 2 individuals, 2.7 kb + 2.8 kb + 3.6 kb and 2.8 kb + 3.1 kb + 4.2 kb + 5.0 kb each in a single individual. Similar analyses were carried out on DNA from 4 individuals of the populations designated #25 and #35 (from Syria) as well as from individuals derived from accessions of *T. dicoccoides* collected throughout the entire geographic distribution of this species. The new length variants observed in the latter populations were 2.0 kb, 2.3 kb and 2.5 kb long. Thus a total of 10 spacer length variants were detected among 42 individuals examined. While some variants, such as those in Chinese Spring, differ in length equivalent to 1 to 3 "130" bp units, other (rare) variants have much longer spacers, requiring "130" bp arrays 2 or 3 times longer than in pTA250 to explain the length of the spacer.

Examination of the *T. dicoccoides* populations by Hae III digestion of the genomic DNA's (and probing the digestion products with the "750" bp spacer sequence) showed a much more constant picture. No variation occurred in the length of the three major bands (0.35 kb, 0.3 kb and 0.1 kb long) assayed, within the populations examined above (Fig. 12b). Some additional bands occur and these most likely reflect inactivation of Hae III sites in some of the rDNA repeated units, as was discussed earlier with respect to the "130" bp sequence (see also Fig. 12b). An extensive analysis using the "130" bp sequence as a probe was

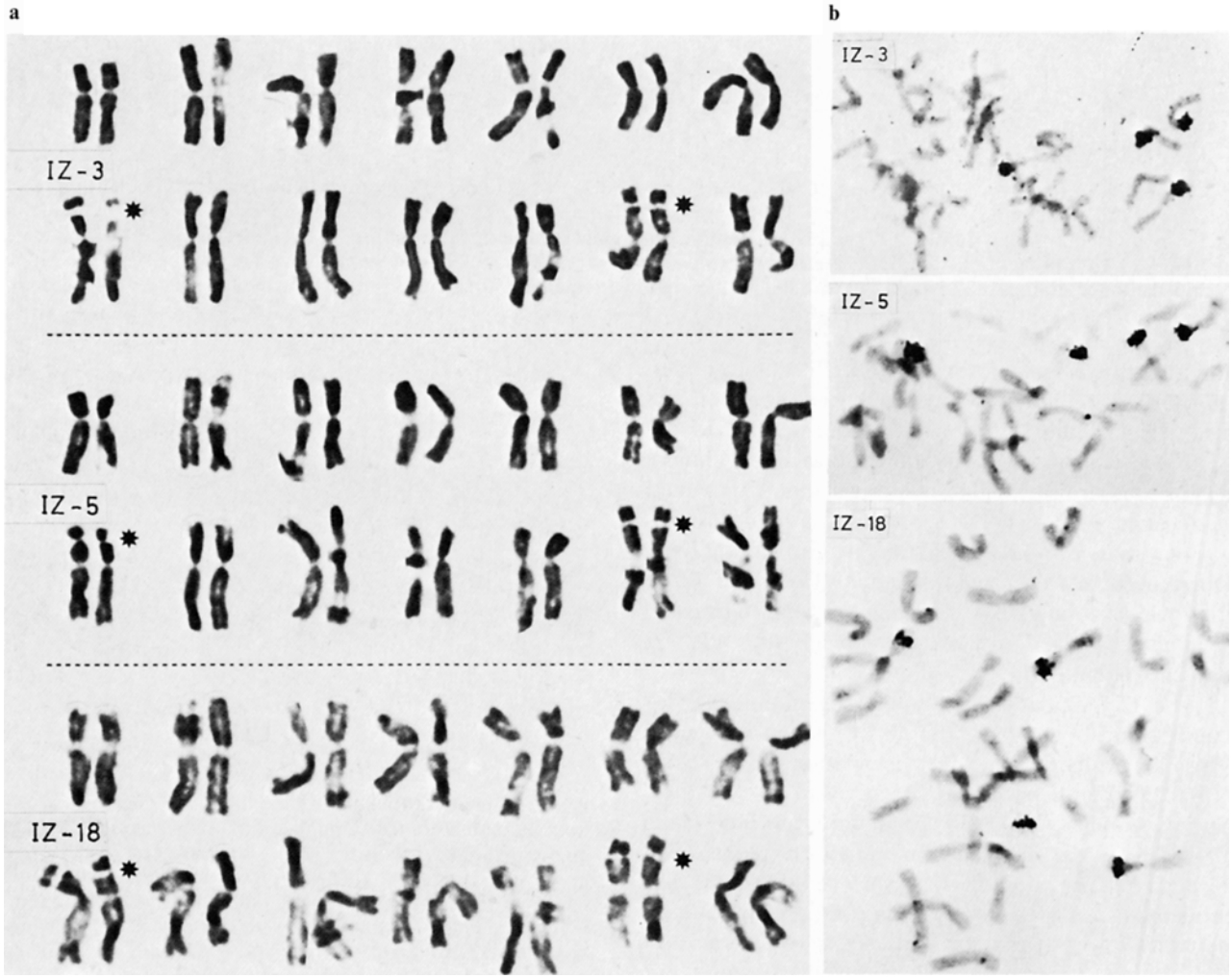

Fig. 13a and b. Chromosome analysis of individuals of the IZ population of *T. dicoccoides*. **a** Feulgen stained chromosomes. Chromosomes were arranged according to their best fit to the Chinese Spring karyotype. Note the chromosomes with secondary constrictions have the arm ratio characteristic of 1B and 6B in Chinese Spring (marked with asterisk). **b** In situ hybridization with the "130" bp probe to prove that only two major rDNA sites are present in the individuals examined. The individuals IZ-3 and IZ-18 are in the same Tm class as IZ-12. No plant of IZ-12 was available for this analysis

not carried out since examination of different species from within the *Triticeae* showed relatively little variation in the length of this sequence (see adjoining paper by Dvořák and Appels 1982).

In considering the origin of the above sequence polymorphism within, for example, the IZ population it was important to rule out the possibility that the population was not polymorphic for the number of chromosomes on which major sites of rDNA were located. Analysis of Feulgen stained root-tip chromosomes from the individuals (Fig. 13 a) showed only two pairs of chromosomes with secondary constrictions and these had the arm ratios expected for chromosomes 1B and 6B. In situ hybridization with a ^{3}H-cRNA probe synthesized from the "130" bp sequence confirmed that only two major locations for the sequence exists in the IZ individuals (Fig. 13 b). The sequence variation assayed in Figures 11 and 12 can therefore be assigned to the rDNA locations on chromosomes 1B and/or 6B.

Discussion

The rDNA regions of many plants and animals have been extensively studied (reviewed in Federoff 1979; Long and Dawid 1980) and in animals the spacer region has been shown to contain tandem arrays of a 200–400 bp unit (Rae et al. 1981; Renkawits-Pohl et al. 1981; Schäffer et al. 1981). Our finding of a "130" bp repeating unit in the wheat rDNA spacer indicates that plants share this internal repeated sequence feature of the spacer region. The variation in length observed within *T. aestivum* and *T. dicoccoides* for the overall rDNA repeat unit is usually, but not necessarily always, due to differing numbers of the spacer repeated sequence, as is the case for animal species (references quoted above). The "130" bp repeating units in wheat rDNA are closely related to each other in sequence as exemplified by completely sequencing two of the "130" bp units. The 7 base pair differences between the two "130" bp units which were sequenced could be readily measured by determining the Tm of cross-hybridization between units. A 1 °C ΔTm (comparing the heterologous and homologous hybrids) corresponded to a 1.2% base pair change. The sensitivity of the Tm analysis for detecting sequence change in the spacer region allowed us to examine a wide range of species within the *Triticeae* in this and the accompanying papers (Dvořák and Appels 1982; Appels and Dvořák 1982). The combination of Tm analysis using cloned probes, sequence information and restriction enzyme analyses as used in these papers allowed a detailed analysis of the nature of sequence change in many species and individuals.

The sequence variation observed among "130" bp units (ranging from 1–6 base pair changes, i.e. 0.7–4.6% change) is important for understanding the sequence variation seen in the rDNA spacer region within an individual as well as among individuals. This variation within "130" bp units of the cloned rDNA unit in pTA250 was 2–3 times greater than the sequence variation assayed between the spacer regions of different arrays of genomic rDNA units. Thus the rDNA regions of chromosomes 1B and 6B differed only by 1.2%, different cultivars of *T. aestivum* by a maximum of 2.2% and the extremes of a *T. dicoccoides* population also by 2.2%. Much of this sequence variation can therefore be accounted for as the result of different "130" bp units being present in different concentrations in a given array of rDNA units. Mechanisms such as unequal exchanges (Smith 1976) could result in changing concentrations of "130" bp units. This mechanism could also account for the length heterogeneity which results from different numbers of "130" bp units. Superimposed on this, however, is the aquisition of variation due to new base-pair changes since sequence variation between individuals of the *T. dicoccoides* population was also observed in the non-repeated spacer, "750" bp, sequence.

The examination of different *T. aestivum* cultivars and individuals of *T. dicoccoides* with respect to length variation showed that in any one individual the population of rDNA was uniform. The apparently rapid "fixation" of a particular length variant among the thousand or more rDNA units of an individual must indicate that an extremely efficient conversion-type mechanism exists. This is discussed further in the adjoining papers.

The clear chromosomal partitioning of rDNA spacer length variants in the *T. aestivum* cultivars examined is in contrast to the lack of partitioning which is generally considered to be the case in animals (Federoff 1979; Long and Dawid 1980). Even though the repeating units within spacer regions show sequence variation, the length of the rDNA unit containing the spacer is extremely uniform over long tandem arrays of these units. An early study of the inheritance of rDNA spacer length variants in *Xenopus* (Reeder et al. 1976) indicated a significant (2/50) occurrence of new spacer length variants in F_1 progeny. A study of this phenomenon in an inbreeding plant species using the length variants of, for example, different 6B chromosomes should provide new information on the rates at which a sequence can spread throughout a repeated gene family in an individual.

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