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Cloning and characterization of ribosomal RNA genes from wheat and barley

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ABSTRACT

Wheat and barley DNA enriched for ribosomal RNA genes was isolated from actinomycin D-CsCl gradients and used to clone the ribosomal repeating units in the plasmid pAC184. All five chimeric plasmids isolated which contained wheat rDNA and eleven of the thirteen which had barley rDNA were stable and included full length ribosomal repeating units.

Physical maps of all length variants cloned have been constructed using the restriction endonucleases Eco Rl, Bam Hl, Bgl II, Hind III and Sal I. Length variation in the repeat units was attributed to differences in the spacer regions. Comparison of Hae III and Hpa II digestion of cereal rDNAs and the cloned repeats suggests that most methylated cytosines in natural rDNA are in -CpG-. Incomplete methylation occurs at specific Bam Hl sites in barley DNA. Detectable quantities of ribosomal spacer sequences are not present at any genomic locations other than those of the ribosomal RNA gene repeats.

INTRODUCTION

The genes for the 25-28S, 5.8S and 18S cytoplasmic ribosomal RNAs in eukaryotes are generally organized in tandem arrays of units, each of which contains one structural gene for each RNA. These rRNA repeat units contain both transcribed and non-transcribed spacer sequences.

In plant species the number of ribosomal gene repeats is usually in the order of 10^4 per genome, compared with animals where the number is generally 10^2-10^3 (1,2). In cereal plant species the multiplicity ranges from 5,000 to 18,000 (3,4). In wheat, var. Chinese Spring, two major locations for rRNA genes are found on chromosomes 1B and 6B, rye has one major location on chromosome 1R and in barley chromosomes 6 and 7 bear the ribosomal genes (4,5,6,7,8,9).

The locations of Bam Hl and Eco Rl restriction endonuclease sites in cereal ribosomal RNA gene repeats have been investigated (8). The Eco Rl sites define the repeating unit size in wheat, rye and barley since each

repeat has only one Eco Rl target. Wheat rDNA is organised in units of length 9.5 Kb while in barley there are two repeat size classes, 9.5 Kb and 10 Kb. The wheat repeats each contain two Bam Hl recognition sites but analysis of Bam Hl targets in barley ribosomal repeats presented difficulties owing to the heterogeneous size classes of digestion products.

In this paper we report the isolation of fractions of wheat and barley DNA which are highly enriched for ribosomal RNA genes and the use of these to clone full length, stable ribosomal RNA gene repeating units in the plasmid pAC184. Comparative restriction maps of wheat and barley repeats have been determined using the cloned rDNA and the organization of the ribosomal genes and their spacer sequences have been characterised.

MATERIALS AND METHODS

Isolation of DNA enriched for rRNA genes

DNA was isolated from barley, Hordeum vulgare var. Sultan, and wheat, Triticum aestivum var. Chinese Spring. For embryo DNA preparation embryos were homogenised in HB buffer (0.3 M sucrose, 50mM Tris-HCl pH=8.0, 5mM $MgCl_2$) and filtered through three layers of Miracloth. Triton X-100 was added to 1% and the eluate was centrifuged (5', 500 xg). The pellet was resuspended in 40% w/w metrizamide (Nyegaard and Co., Oslo) in HB, layered onto a 40%-50% metrizamide step gradient, centrifuged (20', 32,000 xg) and nuclei isolated from the 40%-50% interface. Nuclei were diluted with HB, pelleted by centrifugation(5', 2000 xg), resuspended in RB buffer (50mM Tris-HCl pH=8.0, 20mM EDTA) and lysed by treatment with $\frac{1}{8}$ volume of 10% Sarkosyl. The lysate was incubated with $\frac{1}{8}$ volume Calbiochem B grade pronase (5 mg/ml, previously autodigested 2 hrs, 37°C) at 60°C for 5 mins and then at 37°C for at least 4 hours. DNA was purified by centrifugation in CsCl (ρ = 1.56 gm/cm^3, 40,000 rpm, MSE 8 x 14 ml rotor, 2 days) containing ~1 mg/ml ethidium bromide. Ethidium bromide was extracted with isoamyl alcohol saturated with 5mM Tris-HCl pH=8.0, 0.25mM EDTA, and the DNA dialysed into 25mM sodium tetraborate pH=9.2 for samples to be centrifuged in actinomycin D-CsCl or 5mM Tris-HCl pH=8.0, 0.25mM EDTA for storage.

The method used to prepare DNA from plant shoots was essentially that of Graham (10).

Actinomycin D-CsCl centrifugation of total DNA was based on that described by Hemleben et al. (11). CsCl was added to DNA in 25mM sodium tetraborate, pH=9.2, to a density of ~1.70 g/cm^3. Actinomycin D (1 mg/ml in

25mM borate) was added to this solution to a final weight actinomycin D:weight DNA ratio of 0.5-1:1 depending on the particular preparation. Samples were centrifuged at 30,000 rpm for 3 days at 3°C (MSE 8x35 ml rotor), and fractions collected from the bottom of the tubes. Fractions were diluted with H_2O, their OD_{260} determined and aliquots of denatured DNA were loaded onto nitrocellulose filters and hybridised with ^{32}P-labelled wheat rRNA to determine the positions of the rDNA (NOTE: We use the term "rDNA" to refer to the nuclear DNA which contain the ribosomal RNA gene repeating units, including the associated spacer regions). DNA was loaded onto filters using the method of Birnstiel (12). Hybridisations were in 3xSSC/50% formamide at 40°C for 20 hrs. After hybridisation the filters were washed in 2xSSC with 0.05% SDS at 60°C for 2 hours, dried and radioactively assayed. rDNA fractions were pooled, actinomycin-D was extracted with isoamyl alcohol, and dialysed against 5mM Tris-HCl, 0.25mM EDTA, pH=8.0.

Preparation of E. coli DNA

HB101 cells grown to late log phase were washed in one half growth volume of 10mM Tris-HCl pH=8.0 1mM EDTA and resuspended in $\frac{1}{60}$ the growth volume of 25% sucrose 0.25 M Tris-HCl pH=8.0. The concentrated cells were incubated at 4° for 10 minutes with Lysozyme (Sigma, 2.3 mg/ml). Sarkosyl (1% v/v) and pronase (1 mg/ml) were added and the lysate incubated at 65°C for 5 mins and 37°C for 4 hours. The DNA was then centrifuged in CsCl-ethidium bromide and CsCl-actinomycin D as for cereal DNA.

Molecular cloning of rDNA and preparation of rDNA containing plasmids

7.5 µg of wheat DNA and 11.6 µg of barley DNA, enriched for ribosomal DNA by actinomycin DNA CsCl density gradients, were digested to completion with 15 units of Eco R1 and heat denatured (65°C, 5'). Each digested DNA was ligated using 20 units of polynucleotide ligase (34) to 0.5 µg of Eco R1 digested pAC184 plasmid DNA (13). Ligation was in 100 µl of 30mM Tris-HCl pH=7.5 10mM $MgCl_2$ 0.2mM ATP (4 hrs, 15°C). The ligation reaction was continued after a 3 fold dilution of the reaction mixture for an additional 5 hrs to circularise ligated fragments. The reaction was terminated by the addition of ammonium acetate to 0.2M, 25 µg of E. coli B tRNA (Sigma) and an equal volume of water saturated phenol. The mixture was homogenised and a further volume of chloroform added. After further homogenisation the aqueous phase was collected and precipitated with 2.5 volumes of ethanol. The resulting precipitate was washed twice in 70% ethanol, dried and redissolved in 20 µl of 30mM $CaCl_2$. 10 µl from each reaction was incubated separately with 200 µl of 50 fold concentrated $CaCl_2$ treated E. coli cells (strain HB101, F^- pro^- gal^-

leu$^-$ str^r recA r_b^- m_b^-) at 4^{o}C for 60 mins. After further incubation at 42^o for 2 minutes the cells were diluted 10 fold in Luria broth and incubated at 37^o for 60 minutes. The cells were then plated on media containing 10 μg/ml tetracycline. Resulting transformants were tested for DNA inserts in the Eco R1 site of pAC184 plasmid by testing growth on media containing 30 μg/ml of chloramphenicol. Chloramphenicol sensitive transformants were hybridised to ^{32}P labelled rRNA using the colony hybridisation method of Grunstein and Hogness (14). Those transformants containing sequences complementary to rRNA, as revealed by autoradiography, were used to prepare 100 ml cultures in broth containing 20 μg/ml of tetracycline. Chloramphenicol (100 μg/ml) was added to the cultures when the cells were in mid-log phase and the cultures incubated at 37^o for 16 hrs. Plasmid DNA was prepared from detergent-lysed cells on CsCl ethidium bromide gradients as described by Clewell (15). Ethidium bromide was removed using isoamyl alcohol and the DNA was dialysed and stored at 4^{o}C in 5mM Tris-HCl pH=8.0, 0.25mM EDTA.

Restriction endonucleases, agarose gels and filter hybridisation

Restriction endonucleases Sal G1, Bam H1, Eco R1 Bgl II, Hind III, Hae III and Hpa II were all prepared by phosphocellulose and hydroxyapatite column chromatography (16). Restriction endonuclease digests were fractionated on agarose (unless otherwise specified), stained with ethidium bromide and photographed as described previously (17). Labelled RNA was hybridised to DNA fragments digested with restriction endonucleases and fractionated on agarose gels using the method of Southern (18).

Preparation of radioactive probes and in situ hybridisation

cRNAs: Complementary RNA transcripts (cRNA) were prepared from 1-2 μg DNA template in a 50 μl reaction mixture containing 400 μM unlabelled nucleoside triphosphates, 40mM Tris-Cl pH=7.9, 10mM $MgCl_2$, 150mM KCl, 1mM DTT and contained 1-2 units E. coli holo RNA polymerase prepared according to Burgess and Jendrisak (19). 20 μCi α-P^{32}-GTP (Radiochemical Centre, Amersham, Cat. No. PB 161) was substituted for unlabelled GTP in the preparation of P^{32} labelled cRNAs. 100 μl each of H^3-CTP, UTP, GTP (Radiochemical Centre, Amersham, Cat. Nos TRK 339, 289, 314) substituted for their corresponding unlabelled nucleoside triphosphate in the preparation of ^{3}H-labelled cRNAs. These radioactive precursors were dried by vacuum dessication before use. Reactions were incubated at 37^{o}C for 2 hours. Following this, the preparation of ^{32}P-labelled cRNAs was continued by addition of 0.1 mg E. coli tRNA, phenol and chloroform-octanol extraction and precipitation from ethanol. The precipitate was washed twice with 75% ethanol in 0.2 M ammonium acetate and

resuspended in 100 µl H_2O. H^3-labelled cRNAs were recovered from the reaction mixture according to Appels et al. (20).

^{32}P-labelled rRNA: Alkaline hydrolysed wheat rRNA was 5'-labelled using γ-^{32}P-ATP and polynucleotide kinase using the method of Maizels (21).

In situ hybridisation: In situ hybridisation was carried out essentially as described by Appels et al. (20).

Physical containment

The recombinant DNA experiments were carried out under Category II containment conditions as defined by GMAG.

RESULTS AND DISCUSSION

Isolation of cereal DNA enriched for ribosomal genes

Actinomycin D-CsCl buoyant density gradients were used to isolate rDNA from total genomic DNA (Fig. 1). The ribosomal RNA genes were located in these gradients at a low density relative to the bulk of the DNA by hybridisation with ^{32}P-rRNA. The hybridisation profile in Fig. 1a was obtained from the centrifugation of 75 µg DNA per gradient. This hybridisation profile is very similar to that shown by Hemleben et al. (11) for a number of plant DNAs in actinomycin D-CsCl. The degree of separation of rDNA from mainband DNA is indicative of relatively high molecular weight DNA since ribosomal genes only resolve fully from plant mainband DNA when its molecular weight is greater than ~10^7 daltons (22). The ribosomal DNA is not fully separated from mainband DNA in the two preparative gradients (Fig. 1b and 1c). This is due to trailing of mainband DNA into the rDNA region which occurs during fractionation from the bottom of these heavily loaded gradients.

The pooled DNA fractions containing sequences complementary to rRNA were approximately 30-fold (wheat) and 70-fold (barley) enriched for ribosomal RNA genes, as estimated from the proportion of the DNA recovered from the pooled regions in Figs. 1b and 1c. This enriched rDNA was used in the cloning experiments.

Eco R1 restriction digests of the enriched rDNA fractions showed distinct bands of the ribosomal repeating units as judged by their size (~10 Kb) and hybridisation with ^{32}P-rRNA after transfer to nitrocellulose filters (Fig. 2). It is striking that whilst total DNA shows a complex series of length products after Eco R1 digestion, the only obvious bands resulting from the enriched DNA contain the ribosomal RNA genes. This illustrates the effectiveness of the actinomycin D-CsCl enrichment technique in fractionating rDNA from other repeated sequences in the genome.

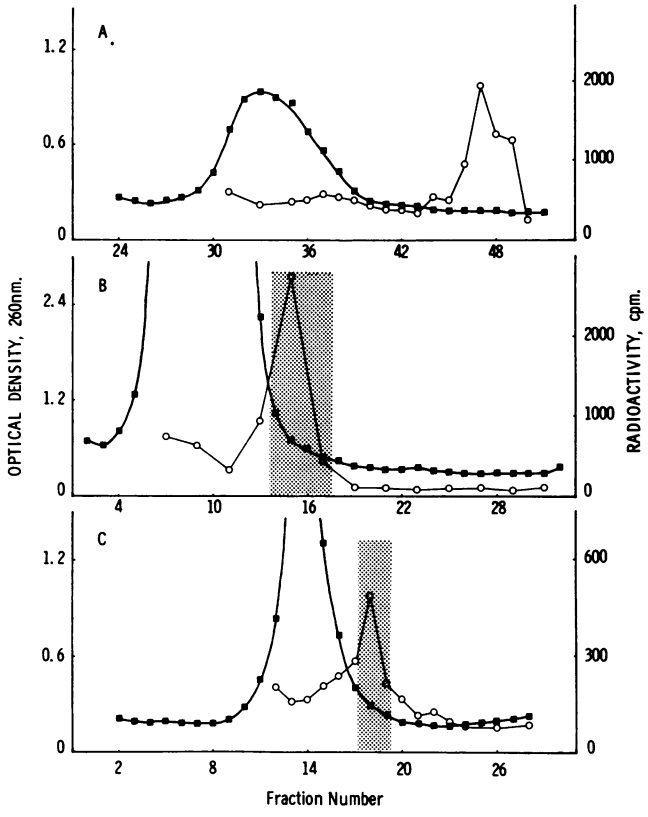

Figure 1. Actinomycin D-CsCl fractionation of cereal ribosomal genes from total DNA. ■——■ : OD_{260nm} profile of gradient. o——o: hybridisation of fractions with ^{32}P-rRNA. (a) barley embryo DNA, 75 µg per gradient. (b) barley shoot DNA 1.2 mg per gradient (c) wheat embryo DNA, 440 µg per gradient. The shaded regions show the fractions which were pooled to form rDNA enriched fractions which were used to clone the ribosomal gene repeats.

The relative cloning efficiency of E. coli and wheat DNA

To verify that the DNA isolated by our preparative techniques would be suitable for molecular cloning we tested its cloning efficiency in experments in which E. coli DNA was included as a comparative control. The cloning efficiencies of wheat DNA which had been purified by CsCl centrifugation were compared with that of DNA which had been additionally purified by actinomycin D-CsCl centrifugation, in order to determine whether the actinomycin D-CsCl step had any effect, either deleterious or advantageous, in preparing the DNA for cloning. Table 1 gives the results.

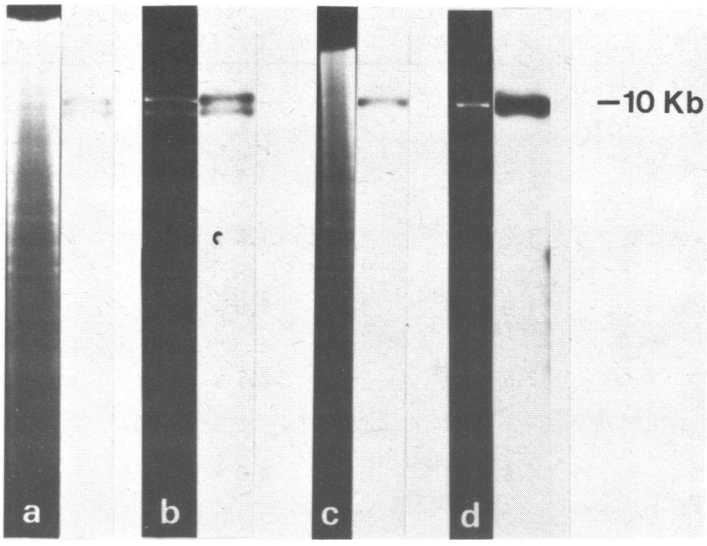

Figure 2. Results of Eco R1 digestion and fractionation on 1% agarose gels of total wheat and barley DNA and DNA enriched for ribosomal genes. Autoradiographs of hybridisations of ^{32}P-rRNA to DNA after transfer to nitrocellulose filters are shown adjacent to each sample. Samples: (a) total barley DNA (b) barley DNA enriched for ribosomal genes (c) total wheat DNA (d) wheat DNA enriched for ribosomal genes.

Untreated vector plasmid DNA gave 10^6 transformants per μg. This number was reduced by 3 orders of magnitude when the plasmid was linearised with Eco R1. Self ligation of the linearised plasmid increased the number to 10^5 transformants per μg. Ligation of E. coli DNA prepared by either method gave the same number of transformants as self ligated pAC184 plasmid DNA (within sampling deviation). The transformation efficiency (number of transformants produced by 1 μg of plasmid) of wheat DNA prepared by either CsCl ethidium bromide gradients or CsCl ethidium bromide followed by actinomycin D CsCl centrifugation was between two and four times lower than the E. coli controls. The frequency of inserts in pAC184 was about the same with E. coli and wheat DNA. The reduction in transformation frequency by ligation to wheat DNA is a consistent feature and we have no simple explanation for this observation. Importantly, however, for our experiments with cloning DNA enriched for rDNA, wheat DNA can be readily inserted in plasmid pAC184 and cloned in E. coli, and purification on actinomycin D-CsCl gradients does not alter its ability to be cloned. We determined the sizes of 61 wheat DNA

DNA sample	Number transformants per μg vector DNA	Frequency transformants per cell	Insertion frequency (%)
No DNA (control)	0	0	-
closed circular pAC184 DNA	1.0×10^{6}	1.0×10^{-3}	-
Eco R1 digested pAC184 DNA	1.2×10^{3}	1.2×10^{-6}	-
Ligated pAC184 DNA	1.0×10^{5}	1.0×10^{-4}	-
pAC184 DNA ligated to wheat DNA 1	2.5×10^{4}	2.5×10^{-5}	11
pAC184 DNA ligated to wheat DNA 2	4.0×10^{4}	4.0×10^{-5}	12
pAC184 DNA ligated to *E. coli* DNA 1	8.5×10^{4}	8.5×10^{-5}	13
pAC184 DNA ligated to *E. coli* DNA 2	1.2×10^{5}	1.2×10^{-4}	12

Table 1. The relative cloning efficiency of *E. coli* and wheat DNA using plasmid pAC184 and *E. coli* strain HB101.

Wheat and *E. coli* sample 1 DNAs were purified by two cycles of CsCl - ethidium bromide centrifugation and sample 2 DNAs were purified by CsCl - ethidium bromide centrifugation and one cycle of CsCl - actinomycin D centrifugation. 10 μg of each of these DNAs were digested to completion with Eco R1 and ligated with 0.2 μg of Eco R1 digested pAC184 plasmid DNA. The ligated DNA was mixed with 0.1 μg of untreated RSF1030 plasmid DNA. RSF1030 is compatible with pAC184 plasmid (33) and provided an internal control which was used to standardise the transformation frequencies of the various DNA samples. The DNA samples were used to transform 10^{9} $CaCl_2$ treated HB101 cells as described in methods. As controls closed circular pAC184 DNA (0.2 μg), Eco R1 digested pAC184 DNA (0.2 μg) and pAC184 DNA digested with Eco R1 and self ligated were also used to transform HB101. The cells were plated on media containing 30 μg/ml ampicillin, to select RSF1030 transformants, on media containing 10 μg/ml tetracycline to select pAC184 transformants, and on non selective media to assay the total number of viable cells per transformation. The number of pAC184 transformants was standardised for each transformation according to the number of RSF1030 transformants. The frequency of transformation was standardised according to the viable cell titre. The fraction of pAC184 transformants containing DNA fragments inserted at the Eco R1 site of the Cm^{R} locus in pAC184 are shown.

inserts chosen at random from these experiments. The range was 14 Kb to <0.1 Kb with a mean of 2.9 Kb. This mean value of the insert sizes is in good agreement with the number average molecular weight of Eco R1 digested wheat DNA (3.2 Kb, data not shown), indicating that there is no gross instability of the wheat DNA sequences inserted into plasmid pAC184.

Cloning the wheat and barley ribosomal gene repeats

Enriched DNA from the actinomycin D-CsCl gradients was digested with Eco R1 and ligated to Eco R1 digested plasmid pAC184 DNA. This was used to transform E. coli strain HB101. Table 2 shows the results. Five clones containing wheat rDNA inserts and 13 clones containing barley rDNA inserts (as judged by hybridisation with radioactive rRNA probe) were obtained. Plasmid DNA was prepared from these clones. The proportion of inserts containing ribosomal genes was consistent with that expected from the number average fraction of the DNA which was full length ribosomal gene repeats (Table 2).

Length of the rDNA inserts

The five wheat rDNA chimeric plasmids all contained 9 Kb inserts; that is, inserts of the same length as the ribosomal gene repeat unit (Fig. 3a) in wheat DNA (Fig. 3a).

Eleven of the thirteen barley rDNA inserts are full length repeats. Two size classes of ribosomal gene repeats are present in barley (8; and Figs. 2b, 3g) and three of the eleven full length inserts are the shorter repeat while the others are the longer repeat (Fig. 3h-m). Plasmid pHV309 contains a full length rRNA gene unit and an additional Eco R1 fragment of length <4 Kb.

Two barley rDNA plasmids, pHV256 and pHV279, did not contain full length repeats. pHV256 plasmid contains part of a ribosomal RNA gene repeat and only part of the vector pAC184, as deduced from its digestion pattern with a number of restriction endonucleases. The reasons for this deletion(s) are not known but it is a stable plasmid. pHV279 has a small deletion within the ribosomal repeat.

In view of previous reports of difficulty in cloning full length ribosomal gene repeating units from plants (various personal communications) both embryo (wheat) and shoot (barley) DNA were used in the cloning experiments to determine whether the source of the DNA might affect the ability to clone full length inserts. Plant embryo DNA is less damaged by nucleases during isolation (23) and we had considered that this might therefore provide higher frequencies of stable inserts in cloning experiments. However, the data (Table 2) indicate that cereal embryo and shoot DNA provide stable inserts at comparable frequencies.

Table 2. Cloning of wheat and barley ribosomal gene repeats.

DNA enriched for ribosomal genes was digested with Eco R1 and cloned in pAC184 DNA as described in Methods.

Source of enriched rDNA	Fraction of* DNA containing ribosomal repeats	Transformation** efficiency	Insertion frequency	Number and proportion containing ribosomal repeats	Designation			
wheat	1.12%	~6 x 10^4	$\frac{274}{5559}$ =4.9%	$\frac{5}{274}$ =1.8%	pTA	71 158 201	250 **269**	
barley	2.01%	2.4 x 10^4	$\frac{366}{3026}$ =12.1%	$\frac{13}{366}$ =3.6%	pHV	29 99 103 107	132 144 158 256	279 287 294 309 361

* This is the number average fraction of the DNA which is full length ribosomal repeat DNA after digestion with Eco R1. It was obtained from quantitative scans of Eco R1 digested DNA products after separation by electrophoresis on 0.8% agarose gels. It underestimates the proportion of ligatable rDNA because not all molecules in the DNA contain two Eco R1 ends required for production of a circular, chimaeric plasmid.

** Number of transformants per µg vector DNA.

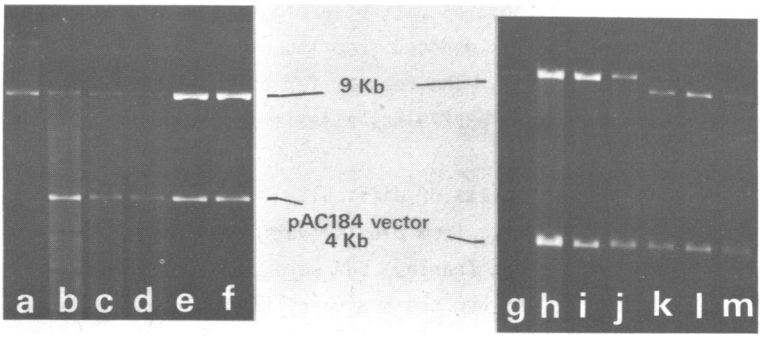

Figure 3. Length of ribosomal gene inserts. DNAs were digested with Eco R1 and electrophoresed on 1% agarose gels. (a) wheat DNA enriched for ribosomal genes by actinomycin D-CsCl centrifugation (b)-(f) plasmids pTA 71, 158, 201, 250, 269 DNAs (g) barley DNA enriched for ribosomal genes (h)-(j) pHV29, 103, 132 DNAs. DNAs from plasmids pHV144, 158, 279, 294, 361 had similar digestion patterns. (k)-(m) pHV99, 107, 287 DNAs.

Bam Hl digestion further defines length variation in cereal ribosomal RNA gene repeats

(a) Wheat

Eco Rl + Bam Hl digestion of the wheat rDNA plasmids gives three rDNA fragments in addition to the two vector fragments (Fig. 4b-f). Two of the fragments, 3.6 and 0.9 Kb long, are identical in length for all five plasmids. The largest rDNA fragment is 4.4 Kb in pTA 158, 201, 250 and 269 and 4.55 Kb in pTA 71. Eco Rl + Bam Hl digests of wheat rDNA also show length variation in the largest fragment (Fig. 4a and inset). The bulk of the fluorescence is in a 4.4 Kb fragment which comigrates with the largest fragment in pTA 158, 201, 250 and 269. Two minor bands of 4.55 Kb and 4.7 Kb are also present. pTA 71 appears representative of the 4.55 Kb class while the 4.7 Kb class has not been cloned. From scans of these gels of natural rDNA we estimate that

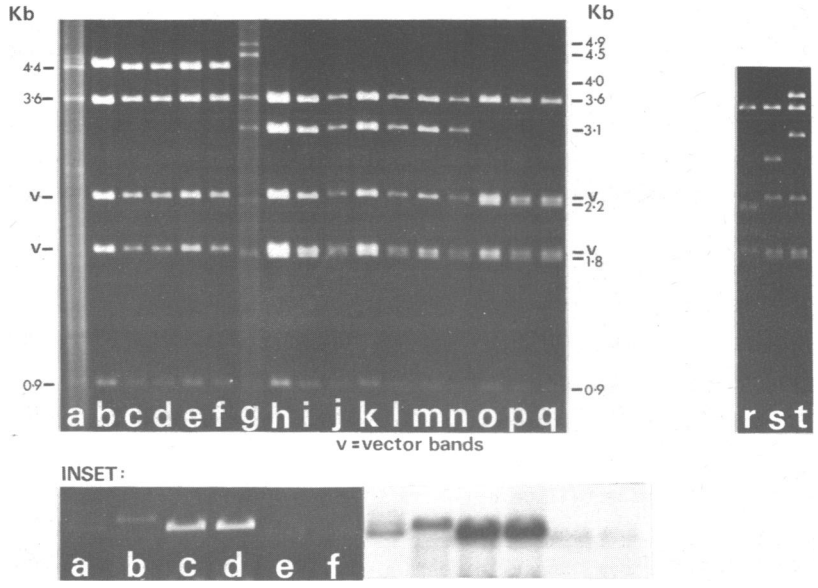

Figure 4. Combined Eco Rl + Bam Hl digestion of wheat and barley native ribosomal DNA and cloned ribosomal repeats. Digestion products were fractionated on 1% agarose gels (a) wheat DNA enriched for ribosomal genes by Act D-CsCl centrifugation (b)-(f) plasmids pTA 71, 158, 201, 250, 269 (g) barley DNA enriched for ribosomal genes (h)-(s) plasmids pHV29, 103, 132, 144, 158, 294, 361, 99, 107, 287, 256, 279, 310. Inset: the longest Eco Rl + Bam Hl digestion fragment from wheat rDNA and cloned wheat rDNA after electrophoresis on 0.8% agarose gel to more clearly resolve the length heterogeneity. Tracks (a)-(f) as in Fig. 4. Autoradiographs show hybridisation with ^{32}P-rRNA after transfer of fragments to nitrocellulose filter.

70% of the wheat ribosomal genes contain the 4.4 Kb fragment while 13% and 16% of the repeats are in the 4.55 Kb and 4.7 Kb fragment classes. From hybridisation with ^{125}I-rRNA Appels et al. (8) demonstrated that the 4.4 Kb fragment is predominantly but not completely from the long spacer of the rDNA repeat. It is likely therefore that the length heterogeneity resides in this region. In summary, wheat ribosomal RNA gene repeats exist in three size classes differing overall by approximately 300 bp. This slight length heterogeneity is not discernible in the Eco Rl digests (Fig. 3) most probably due to poor resolution of high molecular weight DNA in these gels.

(b) Barley

Eco Rl + Bam Hl digestion of the barley clones (Fig. 4h-s) shows that the 3.6 Kb and 0.9 Kb DNA fragments present in digests of wheat clones are also produced by all the barley clones. These fragments contain structural gene sequences and transcribed spacer (8). However, as for wheat, the fragments resulting from long spacer regions are of different lengths in some clones. The longer barley repeat unit as defined by Eco Rl digests produces two fragments of length 3.1 and 1.8 Kb while those produced by the shorter repeat are 2.2 and 1.8 Kb. This result partly explains the complexity of the Bam Hl digest of barley rDNA (Fig. 4g, and as discussed by Appels et al., 8), since it will be at least a composite of the restriction patterns of the cloned repeats.

Bands of 4.9 Kb, 4.5 Kb and 4.0 Kb (weak) which hybridise rRNA are also present in Eco Rl + Bam Hl digests of barley rDNA (Fig. 4g). If these were mutational variants which had lost Bam Hl recognition sites or spacer length variants we would have expected to have detected at least one such example in the cloned barley rDNA. The most likely explanation for these bands is that they are due to methylation of C resides in some of the rDNA repeats. This is discussed in more detail below.

Physical maps of the various rDNA repeats

As discussed above, Eco Rl digests of barley rDNA defined two major repeat length classes for the rDNA in barley and Eco Rl plus Bam Hl digests of wheat rDNA defined 1 major class and 2 minor classes of rDNA repeats in wheat. The large class of barley rDNA repeat is typified by pHV29 and the shorter class by pHV99. The major rDNA repeat in wheat DNA is typified by pTA 250 and the one minor class which we have cloned by pTA 71. Fig. 5 gives schematic diagrams for the physical maps of each of these repeats. The method of mapping is given in the legend to Fig. 5. The major class of repeat in wheat DNA (Fig. 5a) is 8.8 Kb in length. The length of DNA fragments,

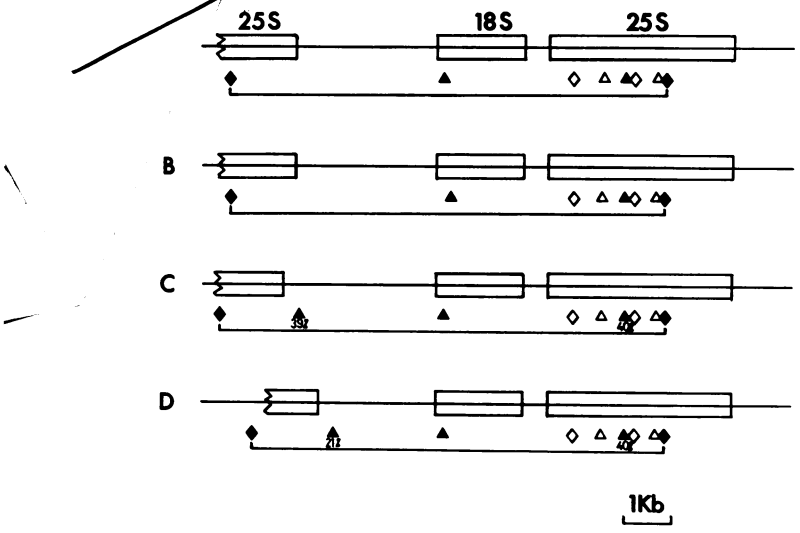

Figure 5. Physical mapping of rDNA repeats of barley and wheat using restriction endonucleases. pTA 250, pTA 71, pHV29, pHV99 and pAC184 plasmid DNAs were each digested with Eco Rl and then digested with the complete set of combinations of Bam I, Bgl II, Hind III and Sal I. The digests were fractionated on 1% agarose gels using λ phage DNA digested with Eco Rl (29) and Hae III (30) and pBR322 DNA digested with Hae III (31) as size markers. The maps were deduced by determining the fate of the various fragments produced by one digest when digested with an additional enzyme. This method gave unambiguous location of the Eco Rl, Sal I and Bgl II sites. None of the rDNA repeats contained sites for Hind III or Pst I. The line joining the Eco Rl sites shows the extent of the cloned fragments in each case. The exact location of the Bam Hl and Eco Rl sites relative to the structural genes are based on the data of Appels et al. (8). The slight differences between lengths of digestion products reported in this paper and those determined by Appels et al. (8) are within expected experimental deviations.

The degree of methylation of three of the Bam Hl sites in the barley repeating units (see text) are shown. No detectable methylation was found at other Bam Hl sites in wheat and barley. The map of pTA 250 is given in (a), pTA 71 in (b), pHV29 in (c), and pHV99 in (d).

◆ sites for Eco Rl, ◇ Sal Gl,
▲ Bam Hl, △ Bgl II.

produced by the various enzymes, from within the coding portion of the repeat are identical in the one minor length class, characterised by pTA 71 (Fig. 5b), and the major length class. However the Bam Hl-Eco Rl fragment which spans the non transcribed spacer sequence is increased in length by 0.15 Kb.

The larger barley repeat (Fig. 5c) is 9.9 Kb and the smaller repeat

(Fig. 5d) is 9.0 Kb. As in wheat the difference in length of the two barley repeats can be attributed to a difference in length of the non transcribed spacer in the two families; since DNA restriction fragments arising from within the coding portions are identical in both repeats. Additionally, these restriction fragments from the structural gene regions are indistinguishable from those from the wheat rDNA suggesting that the rRNA structural gene sequences of wheat and barley are extremely closely related.

Methylation of Cytosine residues in wheat and barley ribosomal DNA

Wheat DNA contains 25% of its cytosine residues methylated (24) and barley DNA 23% (25). We have investigated the distribution of methyl cytosine residues in ribosomal DNA by comparing restriction enzyme digestion of plant rDNA with the rDNA repeats cloned in E. coli. Fig. 6 illustrates an experiment which compares the Hae III and Hpa II fragmentation pattern of rDNA from

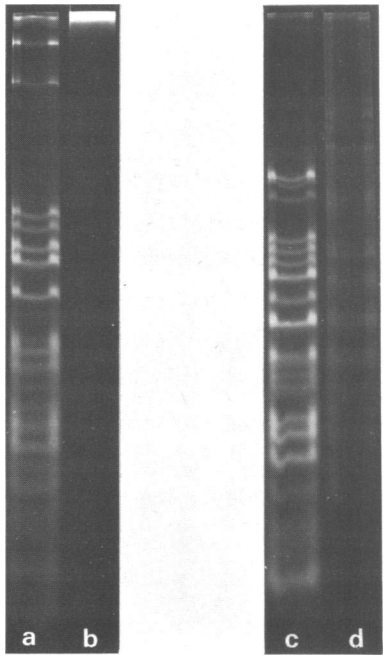

Figure 6. Comparison of Hpa II and Hae III digestion of cloned and native barley ribosomal RNA gene repeats. Digestions were fractionated on composite 6% polyacrylamide, 0.75% agarose gels. (a) pHV99 + pHV103 (i.e. a mixture of both barley rDNA length classes) digested with Eco Rl + Hpa II (b) native barley rDNA isolated by actinomycin D-CsCl centrifugation, and consisting mainly of ribosomal genes, digested with Eco Rl + Hpa II. (c)-(d) same as (a)-(b) except digested with Eco Rl + Hae III.

barley and clones of barley rDNA (pHV99 and pHV103, representing both size classes). Both natural and cloned rDNA are digested by Hae III. Hpa II digestion, on the other hand, gives many fragments when used to cut cloned rDNA but does not fragment natural rDNA at all. We assume that the failure of Hpa II to cut natural rDNA is a consequence of the presence of methylated cytosine residues. Both Hae III and Hpa II recognition sequences are modified by methyl cytosine. Hae III recognises GG$\overset{*}{C}$C and Hpa II recognises C$\overset{*}{C}$GG with the $\overset{*}{C}$ denoting the methylated cytosine causing specific modification of these sites (26). Since rDNA cloned in E. coli does not contain methylated cytosine we suggest that the results of Fig. 6 can be explained if most of the methylated cytosines in plants are in the sequence $\overset{*}{C}$pG. This arrangement for methylated C resides in ribosomal repeats is similar to that described for somatic Xenopus rDNA (27) where ~99% of CpGs contain methylated cytosine, but contrasts with insect ribosomal genes where cytosines are not methylated (35).

The results of Figure 4 suggest that not all methylated cytosines are in the sequence CpG in barley rDNA. As mentioned above and in Fig. 5, the three bands in Bam Hl + Eco Rl digests of barley rDNA of length 4.9, 4.5 and 4.0 Kb most probably arise from cytosine methylation in the Bam recognition sequence GGAT$\overset{*}{C}$C (28). This methylation does not appear to be random for all Bam sites in barley rDNA; those which are modified to produce these fragments have been determined from the lengths of the digestion products and are shown in Fig. 5. The percentage methylation at these sites have been calculated from the molar ratios of the digestion products as determined from scans of the gels.

Wheat rDNA spacer sequences occur only in rDNA

Sequences present in spacer regions of ribosomal RNA gene repeating units are not present in detectable quantities elsewhere in the genome of Mouse since a probe which contains spacer sequences hybridises only to the known ribosomal repeat fragments in digests of total DNA (32). The same is true for wheat rDNA spacers. ^{32}P-cRNA prepared from clone pTA 201, chosen as a representative wheat ribosomal clone, only hybridises to the same fragments as ^{32}P-end labelled rRNA in Eco Rl and Bam digests of total DNA (Fig. 7) when assayed according to the procedures of Southern (18), and to no other discrete size classes. Furthermore, a ^{3}H-cRNA prepared from clone pTA 201 hybridised markedly to only two chromosome pairs in hexaploid wheat (Fig. 8). ^{125}I-rRNA also showed only two major locations on chromosomes 1B and 6B when hybridised to wheat metaphase chromosomes (8).

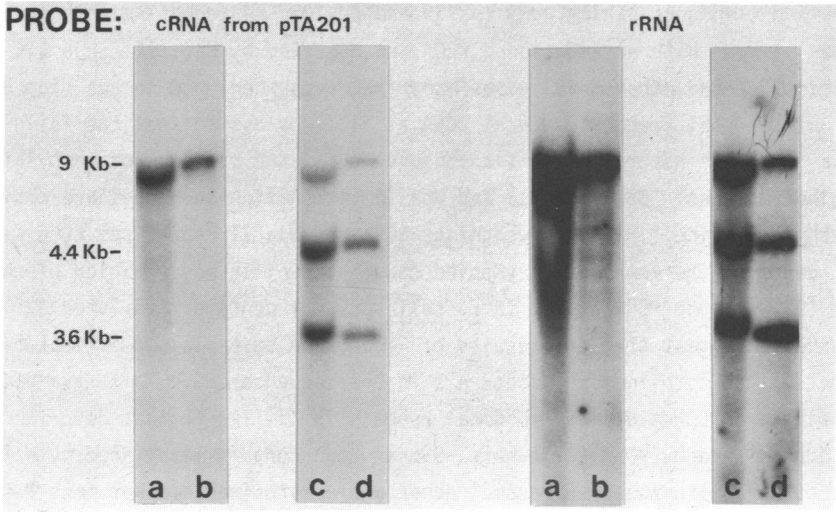

Figure 7. Hybridisation of ^{32}P-rRNA and ^{32}P cRNA from clone pTA 201 to Eco R1 and Bam H1 digested wheat DNA. DNAs were digested, fractionated by electrophoresis on 1% agarose gels, transferred to nitrocellulose filters and hybridised with the radioactive probe. (a) total wheat DNA, Eco R1 digestion (b) wheat DNA enriched for ribosomal genes, Eco R1 (c)-(d) as for (a)-(b) except partial Bam H1 digestion.

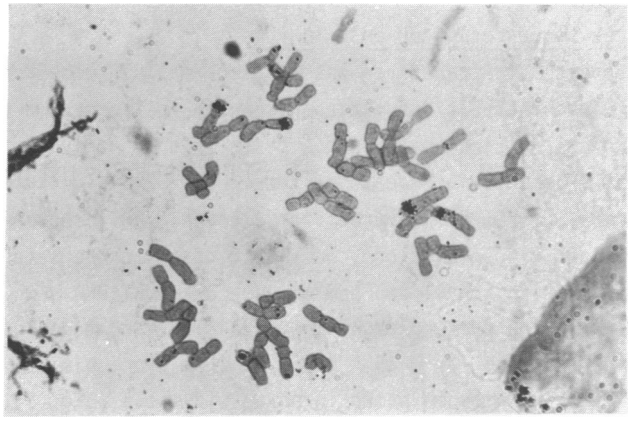

Figure 8. In situ hybridisation of ^{3}H-cRNA from cloned wheat ribosomal gene repeat to a root tip metaphase of hexaploid wheat (var. Chinese Spring, 2n=42).

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REFERENCES

1. Ingle,J. and Sinclair,J.(1972) Nature 235, 30-32
2. Steele Scott,N. and Ingle,J.(1973) Plant Physiol. 51, 677-684
3. Flavell,R.B. and Smith,D.B.(1974) Chromosoma 47, 327-334
4. Liang,G.H., Wang,A.S. and Phillips,R.L.(1977) Can.J.Genet.Cytol. 19, 425-435
5. Flavell,R.B. and Smith,D.B.(1974) Biochem.Genet. 12, 271-279
6. Flavell,R.B. and O'Dell,M.(1976) Heredity 37, 377-385
7. Mohan,J. and Flavell,R.B.(1974) Genetics 76, 33-44
8. Appels,R., Gerlach,W.L., Dennis,E., Swift,H. and Peacock,W.J. Submitted to Chromosoma
9. Nicoloff,H., Anastassova-Kristeva,M., Kunzel,G. and Rieger,R.(1977) Chromosoma 62, 103-109
10. Graham,D.E.(1978) Anal.Biochem. 85, 609-613
11. Hemleben,V., Grierson,D. and Dertmann,H.(1977) Plant Sci.Lett. 9, 129-135
12. Birnstiel,M.L., Sells,B.H. and Purdom,I.F.(1972) J.Mol.Biol. 63, 21-39
13. Chang,A.C.Y. and Cohen,S.N.(1978) J.Bacteriol. 134, 1141-1156
14. Grunstein,M. and Hogness,D.S.(1975) Proc.Natl.Acad.Sci.USA 72, 3961-3965
15. Clewell,D.B.(1972) J.Bact. 110, 667-676
16. Greene,P.J. et al.(1978) Nucleic Acids Res. 5, 2373-2380
17. Bedbrook,J.R. and Bogorad,L.(1976) Proc.Natl.Acad.Sci.USA 12, 4309-4313
18. Southern,E.M.(1975) J.Mol.Biol. 98, 503-517
19. Burgess,R.R. and Jendrisak,J.J.(1975) Biochemistry 14, 4634-4638
20. Appels,R., Driscoll,C. and Peacock,W.J.(1978) Chromosoma 70, 67-89
21. Maizels,N.(1976) Cell 9, 431-438
22. Hemleben,V. and Grierson,D.(1978) Chromosoma 65, 353-358
23. Stern,H.(1968) Meth.Enzym.(1968) 12B, 100-112
24. Spencer and Chargaff(1956) Biochim.Biophys.Acta 19, 256
25. Vanyushin, Kadyrova, Karimov. and Belozerskii(1971) Biokhimya 36, 1251
26. Mann,M.B. and Smith,H.O.(1977) Nucleic Acids Res. 4, 4211-4221
27. Bird,A.P. and Southern,E.M.(1978) J.Mol.Biol. 118, 27-47
28. Hattman,S., Keister,T. and Gottherer,A.(1978) J.Mol.Biol. 124, 701-711
29. Thomas,A.M. and Davis,R.W.(1975) J.Mol.Biol. 91, 315-328
30. Allet,B. and Solem,R.(1974) J.Mol.Biol. 85, 475-484
31. Suttcliffe,J.G.(1978) Nuc.Ac.Res. 5, 2721-2728
32. Grummt,I., Soellner,I. and Scholz,I.(1979) Nuc.Ac.Res. 6, 1351-1369
33. Bedbrook,J.R., Lehrach,H. and Ausubel,F.M.(1979) Nature, in press
34. Panet,A. et al.(1973) Biochemistry 12, 5045-5050
35. Rae,P.M.M. and Steele,R.E.(1979) Nuc.Ac.Res. 6, 2987-2995