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Heterogeneity in cucumber ribosomal DNA

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Summary. Restriction and hybridization analysis of cucumber native ribosomal (r) DNA purified from actinomycin-D/CsCl gradients suggested that the repeat units were heterogeneous in both length and sequence. Several full length rDNA repeat units were cloned and five are described which account for all the EcoR I and Xba I fragments present in native DNA. One of a number of BamH I sites found in the clones is not found in a proportion of native rDNA because of base modification. Restriction maps are described for the representative clones and aligned with R-loop maps obtained from electron microscope analysis of each type of repeat unit hybridized under R-loop conditions to pure 18S and 25S rRNAs. The major heterogeneity is explained by differences in length of the external spacer region and by a proportion of the repeat units showing a restriction fragment length polymorphism on EcoR I digestion. The regions coding for 18S and 25S rRNA are uninterrupted and highly conserved.

Key words: Cucumber – Ribosomal DNA – Heterogeneity – R-loops – Plant gene structure

Introduction

In all eukaryotes, the genes for cytoplasmic ribosomal (r) RNA are present in multiple copies, clustered at one or a few nucleolus organising regions of specific chromosomes. In most organisms normal cell function can be maintained even when the number of copies of these genes is markedly reduced, as in the case of *Xenopus laevis,* which is phenotypically normal even when its rRNA gene number is reduced to 50% by chromosome deletion (Brown and Gurdon 1964). Higher plants show very large numbers of rRNA genes and some species may devote large proportions (up to about 9% in turnip) of their genomes to coding for rRNA (Ingle et al. 1975).

These sorts of observations, together with the presence of major intra-species variation in rRNA gene number in plants (e.g. Domoney and Timmis 1980), suggest that a proportion of these genes are not transcriptionally active. Indeed, calculations (Timmis et al. 1972) suggest that only 300 of the 8,000 rRNA genes present in onion cells need be utilised for production of rRNA in actively growing root tips. Despite the possibility that many rRNA genes remain unused, it appears that the regions of these genes which code for the final rRNA product are highly conserved in plants within the individual, the species and the whole plant kingdom (Ingle et al. 1975). This implies a strong selection on this part of the gene and suggests that all the genes are utilised at some stage of the plant life cycle or, alternatively, that there is a specific homogenizing mechanism for these repeated genes (Dover and Flavell 1984).

The non-transcribed regions and transcribed but unconserved regions of rDNA are, however, more variable. Much information derived from cloned rDNAs indicates an unexpected degree of structural heterogeneity both between and within species. There is at least a five-fold difference in rDNA repeat unit length between soybean (7.8 kbp, Varsanyi-Breiner et al. 1979) and man (43 kbp, Wellauer and Dawid 1979). In plants, repeat unit sizes range from the 7.8 kbp of soybean to 18.5 kbp in *Trillium kamschaticum* (Yakura et al. 1983).

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Length heterogeneity is also present within certain species (Yakura et al. 1983; Gerlach and Bedbrook 1979; Siegel and Kolacz 1983; Waldron et al. 1983) but apparently absent from others (Maggini and Carmona 1981; Goldsbrough and Cullis 1981; Rafalski et al. 1983; Varsanyi-Breiner et al. 1979).

The detailed origin of this rDNA heterogeneity has been examined in relatively few plant species (Yakura et al. 1983; Siegel and Kolacz 1983; Ellis et al. 1984), though it is of particular interest in the highly redundant plant systems because of implications for the control of gene expression and, more generally, for the evolution of rDNA sequences.

In this study we describe the cloning and structural analysis of repeat units representative of the heterogeneous rDNA population of cucumber (*Cucumis sativus*).

Materials and methods

Material

Fruits and seeds of cucumber (*Cucumis sativus,* var. 'Telegraph') were commercially obtained.

Preparation of total plant DNA and partial purification of rDNA

Nuclei were prepared (Matsuda and Siegel 1967) from single cucumber fruits and dispersed and digested for 2 h at 45 °C in 2% SDS, 10 mM EDTA, 20 mM Tris-Cl (pH 7.5), 100 μg/ml proteinase-K (Gross-Bellard et al. 1973). Lysates were phenol/chloroform deproteinised (Scott and Ingle 1973) and high molecular weight DNA ethanol precipitated at room temperature, spooled onto sealed pasteur pipettes and stored in 80% ethanol at −20 °C. DNA was purified by CsCl-ethidium bromide equilibrium centrifugation (Maniatis et al. 1978). Ribosomal DNA was resolved from the majority of the cucumber genome by equilibrium centrifugation in CsCl containing actinomycin-D (Hemleben et al. 1977).

Restriction fragments of rDNA were also obtained by partial digestion of genomic DNA with EcoR I followed by neutral CsCl centrifugation. Gradient fractions enriched for rDNA were located by 0.8% agarose gel electrophoresis (Maniatis et al. 1978) and loaded on to 5 ml, 10–30% sucrose gradients. After centrifugation at 190,000×g for 6 h at 4 °C in a Beckman SW50 rotor, 150 μl fractions were monitored for rDNA fragments by agarose gel electrophoresis, Southern transfer (Southern 1975) and hybridization to rRNA.

Preparation and purification of rRNA

Tissue was ground to a fine powder in liquid nitrogen and further homogenized in 10 mM NaCl, 50 mM Tris-Cl (pH 8.0), 7.5 mM EDTA, 2.4% SDS, 350 μg/ml proteinase K. The homogenate was stirred at 40 °C for 30 min and the supernatant from a 10,000×g spin with Na-perchlorate added to 1.4 M, filtered through a 0.22 μm "Millipore, Millex GS" filter. After a further 5 min at 40 °C, nucleic acids were precipitated at 4 °C for 2 h by the addition of 4 volumes of ethanolic perchlorate reagent (Lizardi and Engelberg 1979). Nucleic acids were recovered by centrifugation, dissolved in a minimum volume of 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.2% SDS and precipitated by addition of 0.6 volumes of isopropanol and incubation for 4 h at −20 °C. Total nucleic acids were digested with RNAase free DNAase I (Tullis and Rubin 1980) and RNA recovered by isopropanol precipitation.

Samples of RNA (400 μg) were loaded onto 10–30% sucrose gradients containing 50 mM Na-acetate (pH 5.0), 1 mM EDTA and 25S and 18S rRNA collected separately after centrifugation at 110,000×g for 22 h at 4 °C in a Beckman SW27 rotor. Pure rRNA fractions were pooled, precipitated and stored in 90% ethanol at −20 °C.

Radioactive labelling of nucleic acids

a) DNA. Restriction fragments of DNA were dephosphorylated and low molecular weight nucleic acid contaminants removed by sucrose gradient centrifugation. Samples were rephosphorylated by bacteriophage T4 polynucleotide kinase in the presence of ^{32}P-ATP (Chaconas and van de Sande 1980). Specific activities of DNA after this procedure varied between 10^5 and 10^6 cpm/μg.

b) RNA. (i) Ribosomal RNA was labelled in vitro (Maizels 1976) by the polynucleotide kinase/γ-^{32}P-ATP reaction after partial hydrolysis for 2 min at 100 °C in 25 mM Tris-Cl (pH 9.0), 5 mM MgCl, 2.5 mM DTT, 5% glycerol. Unincorporated ATP was removed on Sephadex G50 columns to yield rRNA of Sa between 10^6 and 10^7 cpm/μg.

(ii) In vivo labelling of RNA was achieved by growth of artichoke explants in culture containing ^{32}P-orthophosphate (Ingle et al. 1975) and rRNA purified as described above.

Gel electrophoresis

Samples of DNA were electrophoresed on 0.8–1.0% agarose gels as described by Maniatis et al. (1982) and restriction fragment lengths calculated using the best fit parabolic curve to standard fragment mobilities (Duggleby et al. 1981). Denatured RNA was electrophoresed in 1.0% agarose gels containing 5 M urea (Locker 1981).

Cloning of rDNA

Gradient purified EcoR I restriction fragments representing full length repeat units of cucumber were ligated with EcoR I digested, phosphatased pACYC184 (Chang and Cohen 1978). The recombinant molecules were used to transform *E. coli* ED8676 (Murray et al. 1977) by standard procedures (Maniatis et al. 1982) under P1(Cl) containment conditions.

Identification of rDNA

Ribosomal DNA was located in CsCl gradients by filter hybridization of samples with ^{32}P-rRNA. Restriction fragments containing the rRNA transcribed and conserved regions were detected by filter hybridization after Southern transfer (Southern 1975) to nitrocellulose or by "crossed-contact" hybridization (Rozek and Timberlake 1979). Bacterial colonies containing rDNA inserts were identified by colony hybridization to ^{32}P-rRNA (Grunstein and Hogness 1975).

Electron microscopy

R-loops (Thomas et al. 1976) were formed in 20 μl of 70% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 400 μg/ml rRNA and 400 μg/ml cloned, linearised rDNA. The reaction mixture was incubated for 16–20 h at 40 °C and a 2 μl sample diluted 50 fold into a spreading solution containing 50% formamide, 0.1 M NaCl, 10 mM PIPES (pH 6.4), 10 mM EDTA, 50 μg/ml cytochrome-c, 0.5 μg/ml circular double stranded DNA from bacteriophage ΦX174. Nucleic acids were mounted on Parlodion film by standard methods (Davis

et al. 1971). The grids were dehydrated in 90% ethanol and stained in 0.1% acidic phosphotungstick acid (Thomas 1978), viewed in a Phillips 201 electron microscope and photographed at 15,000× magnification. At least 10 molecules of each sample were measured on a 'HIPAD' digitiser (Heuston Instruments) linked to a Sirius microcomputer for statistical determination of molecular lengths. Figures showing the structures of these plasmids, incorporating molecular weights from gel analyses of restriction fragments and from EM methods show the actual data obtained and reflect the minor discrepancies between the two methods.

Restriction endonuclease digestion

Commercially obtained restriction endonucleases were used according to the instructions supplied.

Results and discussion

The sequences which code for mature 25S rDNA comprise 0.96% of the total genomic DNA of cucumber (Ingle et al. 1975). Allowing for the 18S sequence, the transcribed and untranscribed spacer and the complementary strand of the repeat unit indicates that about 7% of the cucumber genome is devoted to rDNA. This high proportion of rDNA is reflected in 5 major bands which appear when EcoR I digested total genomic DNA is electrophoresed on agarose gels (Fig. 1, track 2). These bands are clearly resolved in, and readily partially purified from, Actinomycin-D/CsCl gradients (Hemleben et al. 1977) (Fig. 1, track 4) and they all hybridize ^{32}P labelled 25S and 18S rRNA probes on Southern transfers (Fig. 1, track 5). The bands consist of 5 major fragments of 8.0, 6.7, 6.5, 3.7 and 3.3 kbp and a small band of 0.4 kbp, labelled E in Fig. 1, tracks 4 and 6. The three higher Mr EcoR I fragments were called L1, L2 and L3 in order of size, the next two S1 and S2 and the small fragment E. The latter small fragment hybridizes ^{32}P-rRNA as indicated

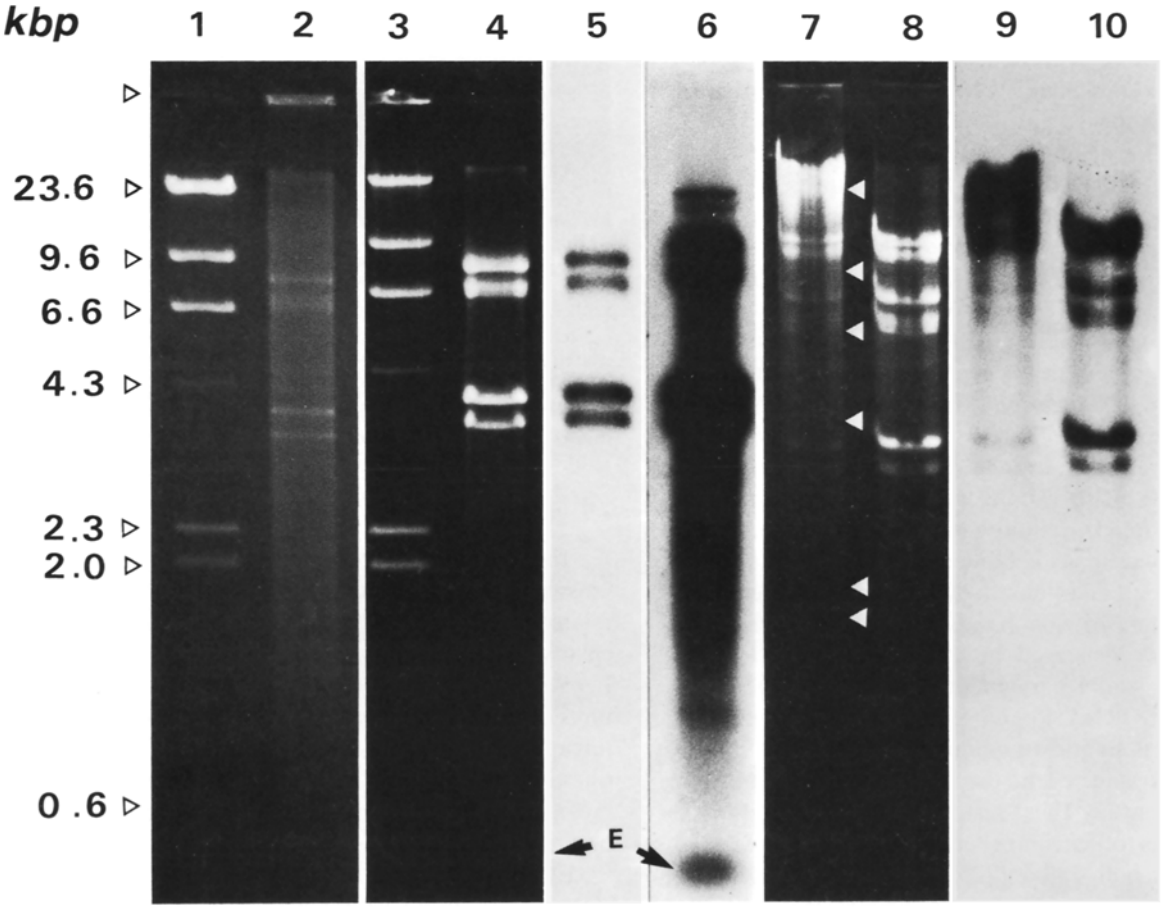


Fig. 1. Sizes of EcoR I fragments of the cucumber genome. *Tracks 1* and *3:* Hind III digested bacteriophage lambda DNA; *2:* total cucumber nuclear DNA; *4:* ribosomal DNA partially purified on actinomycin-D/CsCl gradients; *5:* Southern transfer of track 4 hybridized with ^{32}P-rDNA and autoradiographed; *6:* extended exposure of a similar Southern transfer and hybridization to track *5;* tracks *7* and *8:* partial digestion of actinomycin-D/CsCl purified rDNA with 1 unit of EcoR I/μg DNA for 5 and 15 min, respectively; *9* and *10:* Southern transfer of tracks *7* and *8* hybridized with ^{32}P-rRNA and autoradiographed. Fragments were resolved in 0.8% agarose. The *E* fragment refers to the 400 bp band not visible in the printed reproduction of the ethidium bromide stained gel

by prolonged exposure of the autoradiograph (Fig. 1, track 6).

The number, sizes and rRNA hybridization of EcoR I restriction fragments suggested that the rDNA repeat unit population in cucumber was heterogeneous in length and/or restriction sites, with each repeat unit containing one of the 3 larger fragments plus one of the 2 smaller fragments. A time course of EcoR I digestion confirmed that the 5 fragments were generated from at least 2 larger, partially digested fragments of 11.7 and 10.4 kbp, present in about equal numbers (Fig. 1, tracks 7–10). Prolonged digestion of native rDNA with excess EcoR I and hybridization with ^{32}P-rRNA (Fig. 1, track 6) indicated that some sites were apparently very resistant to cleavage, with a proportion of full sized repeat units often remaining.

In an attempt to simplify the analysis of these rRNA genes, an unsucessful search was made for restriction enzymes which cleaved the major repeat units once only. Cucumber rDNA was not digested by Bcl I, Hind III, Pst I or Pvu II and few sites existed for Sal I and Sma I, presumably because of modification of the sequence in genomic DNA. As with EcoR I, several fragments were generated by Hinc II, Kpn I and Xba I (results not shown).

To help clarify the physical arrangement of the EcoR I fragments within the probable different types of repeat unit and to determine which fragments coded for 18S and which for 25S rRNA, the native rDNA was analysed by crossed-contact hybridization (Rozek and Timberlake 1979). Actinomycin-D/CsCl purified rDNA was completely digested with EcoR I and fragments electrophoretically separated after loading into a wide well in 0.8% agarose (Fig. 2 a) and nucleic acids of artichoke explant cultures were labelled with ^{32}P in vivo and resolved from a wide well on denaturing agarose gels. The rDNA fragments were denatured, transferred to nitrocellulose membrane and the labelled artichoke nucleic acids blotted through the filter under hybridization conditions, at 90° to the cucumber rDNA bands. The restriction bands hybridizing each rRNA species were visualised by autoradiography (Fig. 2 b). The L1, L2 and L3 fragments almost exclusively contain the 18S rRNA coding sequence, while the S1 and S2 fragments hybridize mainly 25S rRNA. No hybridization was detected to the E fragment, probably for technical reasons. These results confirm that full length repeat units of cucumber rDNA, which contain sequences for both 18S and 25S rRNA, are comprised of at least one L and one S fragment. This was also indicated by the fact that purified L fragments contain sites for Xba I while S fragments alone contain BamH I sites (results not shown). The high degree of similarity within the L and within the S sequence suggests they are variants of a related molecular species.

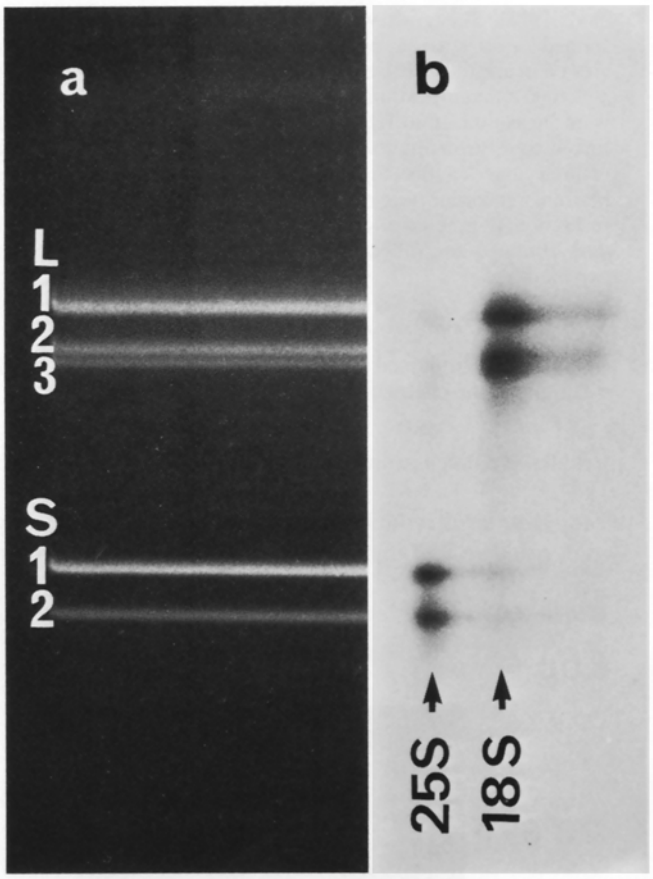


Fig. 2 a, b. Crossed-contact hybridization of native cucumber rDNA with artichoke rRNA. Native rDNA was fully digested with EcoR I and fragments resolved from a wide well in 0.8% agarose (**a**). Artichoke rRNAs, labelled in vivo with ^{32}P-orthophosphate, were separated on a denaturing agarose gel (not shown), turned through 90° and blotted through a Southern transfer of (**a**) under hybridization conditions. The gel fragments hybridizing the 25S and 18S rRNAs were detected by autoradiography (**b**)

The partial and complete EcoR I digestions, the crossed-contact hybridizations and the restriction site relatedness of the two size classes of EcoR I fragments clearly indicate that the 11.7 and 10.4 kbp partial EcoR I fragments represent full length rDNA repeat units. The involvement of the 0.4 kbp portion and the linkage arrangements of L and S fragments were questions which could be more easily approached after molecules representative of the native rDNA population had been cloned.

Extensive attempts to clone actinomycin-D purified rDNA in *E. coli* plasmids yielded no rDNA sequences and very few plasmids containing large inserts. The DNA was, however, readily cut by restriction endonucleases, efficiently ligated and yielded many recombinant colonies on transformation. The lack of rDNA clones from this donor DNA is therefore surprising,

especially as the method has been successfully used to clone barley and wheat rDNA (Gerlack and Bedbrook 1979). Numerous rDNA clones were, however, obtained following the enrichment procedure described in "Materials and methods". Partially digested nuclear DNA was first banded in neutral CsCl gradients and the dense side of the main band DNA peak was monitored for the presence of rDNA by agarose gel electrophoresis and hybridisation of ^{32}P-rRNA to nitrocellulose transfers. The fractions containing maximum rDNA content were pooled and full length (i.e. partially digested) repeat units enriched by size fractionation on sucrose gradients. Gradient fractions were analysed on agarose gels and the two fractions containing the purest rDNA full length repeat units used for cloning in pACYC184 (Chang and Cohen 1978).

Phosphatased plasmid was ligated with enriched rDNA repeat units and yielded 6.3×10^2 to 2.3×10^3 transformants per μg. These low transformation efficiencies reflect the large size of the donor DNA, most of which was between 8 and 15 kbp in size. However, a very high proportion (24%) of transformed colonies (Grunstein and Hogness 1975) hybridized ^{32}P-rRNA and 8 cucumber clones were found to contain full length rDNA repeat units on transfer and rRNA hybridization of EcoR I digested plasmid preparations. Five of these clones, representing all the major EcoR I restriction fragments of native rDNA (Fig. 3) were selected for further study. The composition of each clone in terms of L, S and E fragments is described in Table 1. It will be noted that the L fragment of pCU5 is slightly larger than the modal L1 fragments of native DNA suggesting further more subtle variations.

Table 1. EcoR I restriction fragment composition of cucumber rDNA clones. Nomenclature as in Fig. 3

Clone	Small fragment	Large fragment	E
pCU5	S1	L1[a]	–
pCU18	S1	L3	–
pCU26	S1	L1	–
pCU32	S2	L3	–
pCU34	S2	L2	+

[a] Slightly larger than mean L1 in native rDNA digests

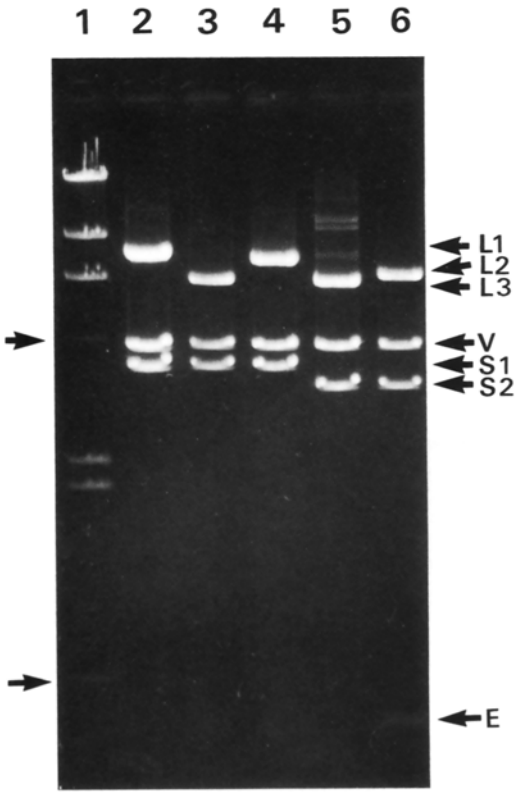


Fig. 3. Recombinant plasmids containing full length cucumber rDNA repeat unis. *Track 1:* Hind III digested bacteriophage lambda DNA with poorly stained bands indicated by *arrows; 2–6:* EcoR I digested pCU5, pCU18, pCU26, pCU32 and pCU34 respectively. *V:* plasmid vector pACYC184. Other labels refer to fragments described in Table 1

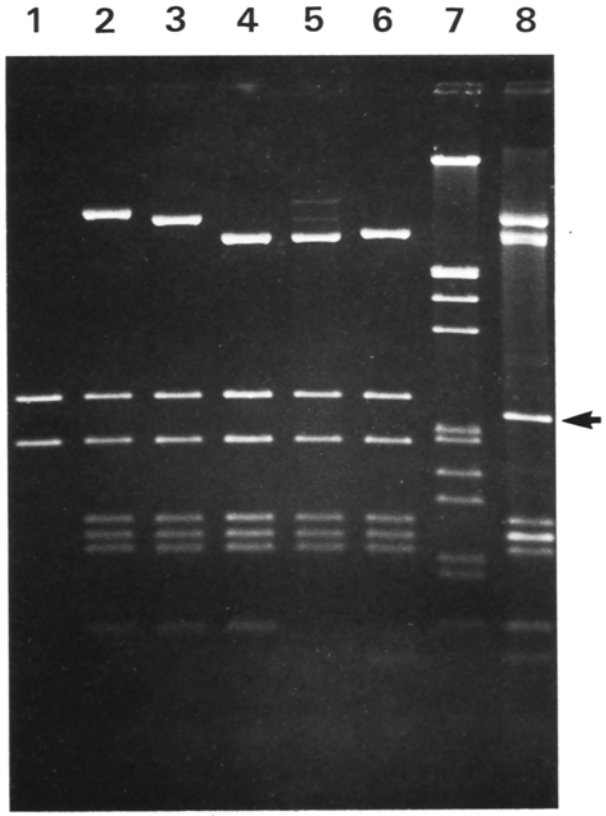


Fig. 4. A comparison of EcoR I+BamH I digested native cucumber rDNA with DNA from individual clones. *Tracks 1–6:* pACYC184 (vector), pCU5, pCU26, pCU18, pCU32 and pCU34; *7:* Hind III+EcoR I digested bacteriophage lambda DNA; *8:* actinomycin-D/CsCl native cucumber rDNA

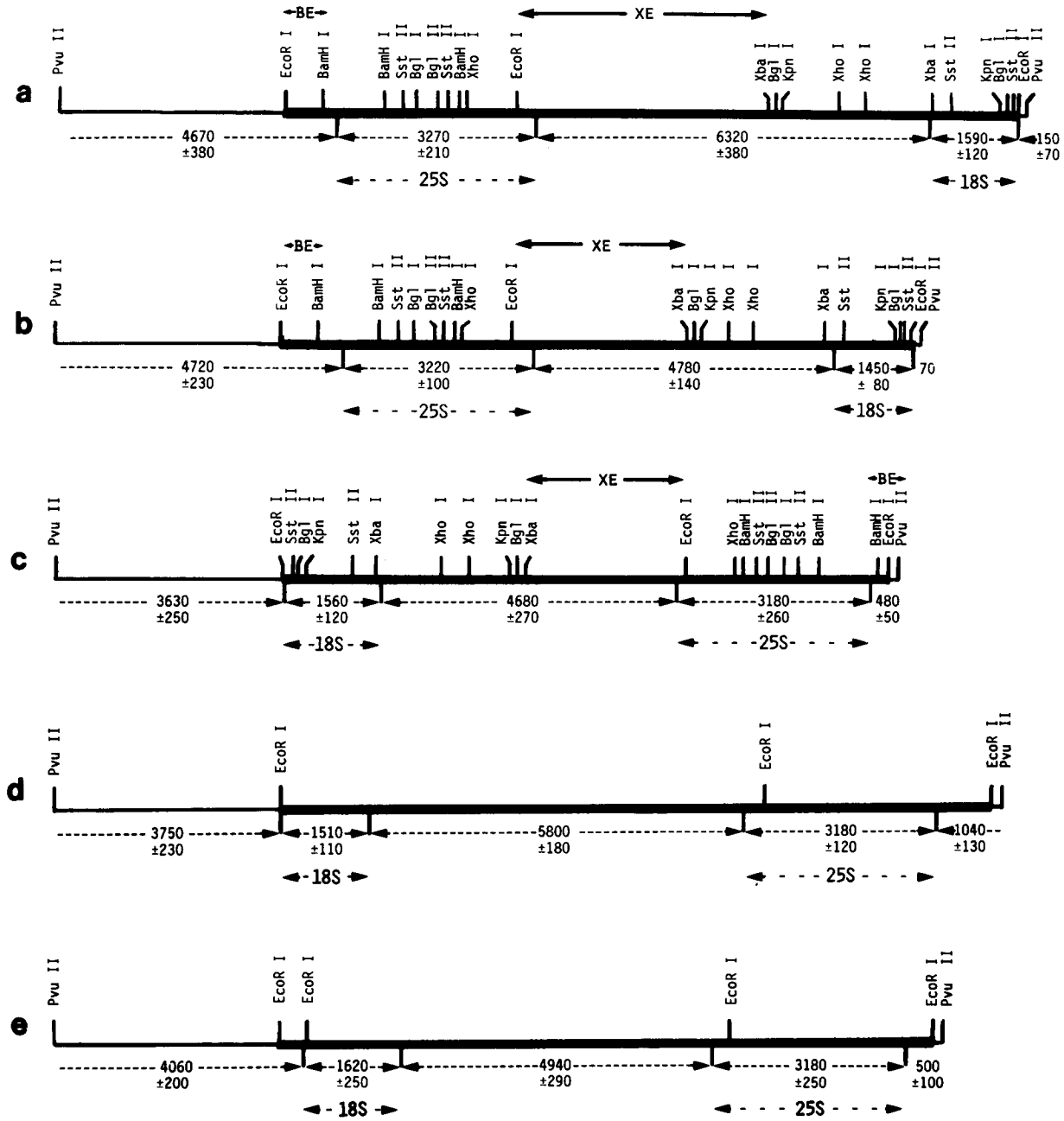
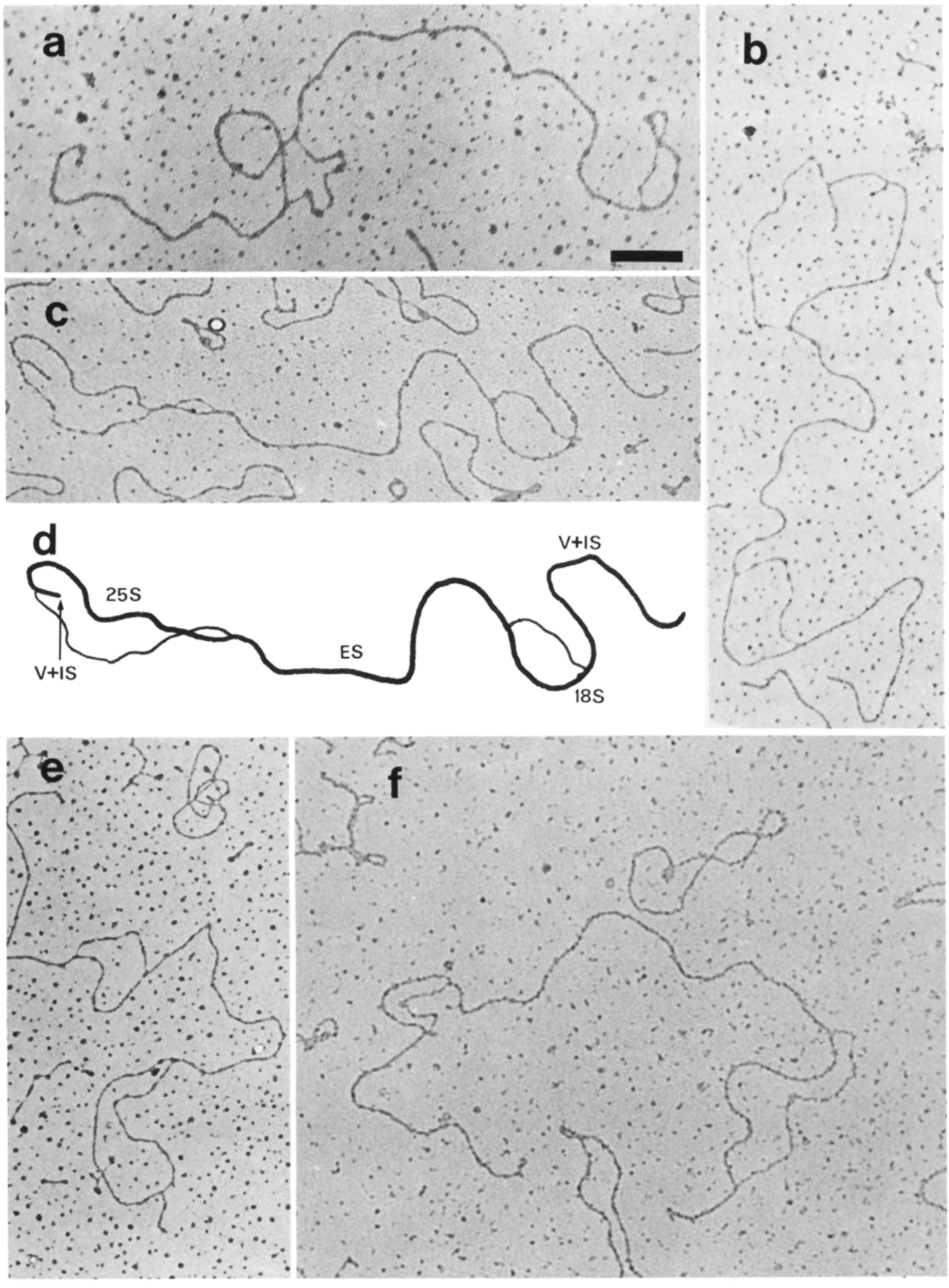


Fig. 5a–e. Aligned restriction and R-loop maps of recombinant plasmids containing cucumber rDNA repeat unit inserts. Positions of restriction sites for BamH I, Bgl I, EcoR I, Kpn I, Pvu II, Sst I, Xba I and Xho I are shown for (**a**) pCU5, (**b**) pCU18, (**c**) pCU32, (**d**) pCU26 and (**e**) pCU34. BE indicates the variable BamH I–EcoR I fragment, XE indicates the variable Xba I–EcoR I fragment. The number show mean molecular lengths (bp + standard deviations) of structural regions of R-looped molecules of each clone obtained from electron micrograph analysis represented in Fig. 6. The 18S and 25S labels indicate the regions which give rise to these mature rRNAs

Fig. 6a–f. R-loop molecules of recombinant plasmids containing cucumber rDNA repeat unit inserts. Plasmids were linearized with Pvu II and hybridized under R-loop conditions with pure cucumber 18S and 25S rRNAs. (**a**) pCU5, (**b**) pCU34, (**c**) pCU32, (**d**) line drawing explanation of (**c**), (**e**) pCU34 and (**f**) pCU26. Small circular molecules (eg. in (**f**)) are bacteriophage ΦX174 double stranded DNA standards. *V:* vector, *IS:* internal spacer, *ES:* external spacer, *18S* and *25S:* the structural regions giving rise to these two mature rRNAs. All micrographs are at the same magnification in bar in (**a**) represents lkbp


a
b
c
d
25S
V+IS
ES
V+IS
18S
e
f

The association of cucumber rDNA EcoR I fragments is not random, as not all possible combinations are found in the clones. However all the Xba I + EcoR I digestion products of native DNA are accounted for in the 5 clones (result not shown). In contrast, BamH I + EcoR I digestion of native rDNA, while yielding 8 fragments also found in the clones, shows an additional band not present in the clones (Fig. 4). This band of 2.1 kbp, strongly hybridizes rRNA and its likely origin is an uncleaved BamH I site between the 0.9 kbp BamH I – EcoR I and the 1.2 kbp BamH I – BamH I fragment in some native repeat units. In a high proportion of native rDNA this site therefore appears to be protected from BamH I digestion, presumably by methylation of the internal adenosine of the recognition sequence (McClelland 1983).

Restriction maps were determined for the major size variants pCU5, pCU18 and pCU32 (Fig. 5 a–c). To resolve some mapping ambiguities it was necessary to use fragment purification and ^{32}P-end labelling techniques as well as classical single and double digestions of the cucumber clones. The restriction maps are essentially similar to each other with the exception of a variable length Xba I – EcoR I fragment (XE), which lacks sites for any of the other enzymes used. This fragment is 4.1 kbp in pCU5, 2.8 kbp in pCU18 and 2.6 kbp in pCU32; accounting for the differences in size between L1, L2 and L3, respectively. A variable BamH I –EcoR I fragment (BE), 0.55 kbp in pCU5 and pCU18 and 0.15 kbp in pCU32, accounts for the S fragment variation. The difference between these fragments is 400 bp, the precise size of the E fragment found in native rDNA and in pCU34 (Fig. 3). This suggests that the S fragment variation is caused by a restriction site polymorphism with all rDNA units containing a 0.55 kbp BE fragment while a proportion of units contain an additional EcoR I site which on cleavage generates the S1 and S2 fragments. This explanation is confirmed by results described later.

The XE and BE regions were located relative to the 18S and 25S coding regions by R-loop mapping (Fig. 6). The five clones were each linearised with Pvu II (which cuts pACYC184 but not the insert) and hybridized under R-loop conditions to pure cucumber rRNA. Each molecule contains 2 different sized, uninterrupted R-loops locating the larger 25S gene and the smaller 18S sequence. The two R-loops are separated by a variable length of unhybridized DNA, indicating that the EcoR I site within the transcribed spacer was the insertion site in all the clones. This observation indicates a strong preference for cleavage of this site in partial digestions, suggesting some protection of the alternative EcoR I site which was later shown to be within the 25S gene. This result is consistant with the difficulty of complete digestion of rDNA referred to earlier.

The arrangement of coding, spacer and vector regions in the various clones was determined by alignment of R-loop and restriction maps (Fig. 5). In pCU5 and pCU18, the 25S R-loop and most of the internal spacer (IS) are adjacent to the long (3.6 kbp) Pvu II – EcoR I vector arm (Fig. 5 a and b, respectively) and are separated from the 18S R-loop by non-transcribed or external spacer (ES). The 18S R-loop is therefore attached to the very short (150 bp) Pvu II – EcoR I arm of the vector. In the other three clones the rDNA lies in the alternative orientation to pCU5 and pCU18 (Fig. 5 c–e). From these physical maps it is clear that the variable BE fragment lies within the internal spacer, adjacent to the vector, supporting the previously described origin of the S fragment variation as EcoR I restriction site polymorphism. With this interpretation, pCU32 represents an incomplete rDNA repeat unit which would be flanked at one end or the other by the 400 bp E fragment. The major length variation in cucumber rDNA repeats is therefore confined to the XE fragment which starts near one end of the 25S coding sequence and extends, by a variable amount, into the external spacer. This finding is consistent with those of Yakura et al. (1984), who found that length variation in *Vicia faba* repeat units was located within the external spacer in a precisely similar location. They were, however, able to identify and sequence a Mlu I and Acc I subrepeat present in variable numbers in different gene lengths. So far in a search using 10 tetranucleotide recognising enzymes (Langridge and Timmis, unpublished observations) we have been unable to simplify the origin of the cucumber rDNA heterogeneity. The indications are that the XE fragment is a heterogeneous sequence containing little or no internal repetition.

These observations show that there is both length and sequence heterogeneity within cucumber similar to that described for several other species (e.g. Gerlach and Bedbrook 1979; Siegel and Kolacz 1983; Ellis et al. 1984; Yakura et al. 1984). Indeed, where sufficient clones have been examined, the presence of length and sequence variation seems to be the rule rather than the exception; making cases of apparent homogeneity, such as in flax (Goldsbrough and Cullis 1981) and melon (Kavanagh and Timmis, in preparation), of increased interest. The present work indicates that there is likely to be a minority of rDNA repeat units which are rarer in the native population: e.g. the L fragment of pCU5 is slightly larger than the majority of L1 fragments possibly indicating that sequence rearrangements are quite common and that any possible fixation to homogeneity is in a very dynamic state (Dover and Flavell 1984). We favour the explanation that the variation has a biological explanation (Reeder 1984), particularly as the rDNA population described is very similar in all

cucumbers examined and is also found in several other members of the Cucurbitaceae (Kavanagh and Timmis, in preparation). The different responses of the different gene varients to the implied stringent selection, and the varied biological roles of different classes are, however, not at present clear.

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