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RESTRICTION ANALYSIS OF RADISH NUCLEAR GENES CODING FOR rRNA : EVIDENCE FOR HETEROGENEITY

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INTRODUCTION

Our knowledge of the organisation of nuclear genes coding for ribosomal genes in higher plants is still very poor. They differ in several aspects from those of prokaryotes, lower eukaryotes and animals : they are usually repeated several thousand times and do not seem to be amplified during development (1). Although we have a good idea of the organisation of transcribed sequences (2, 3, 4) we do not know anything about the non-transcribed spacers. We lack information on the arrangement of the repeats and do not know whether or not they are all identical. In an attempt to answer these questions we have partially purified radish rDNA and digested it with several restriction endonucleases. Our results demonstrate that several distinct repeating units occur in this plant.

MATERIALS AND METHODS

DNA was prepared from seedlings germinated for 48-50 hours. A 6000 g pellet was treated according to MARMUR (5). Ribonucleic acids were then digested with RNase A and proteins were eliminated by proteinase K digestion and several chloroform extractions. High molecular weight DNA molecules were selected out by exclusion chromatography on a Sepharose 4 B column. They were further purified by equilibrium centrifugation on a CsCl gradient (6). DNA sequences hybridising to rRNA were located in such gradients (figure 1) and the corresponding fractions pooled, dialysed against 500 volumes of 10 mM Tris-HCl pH 8, 1 mM EDTA buffer and concentrated by ethanol precipitation.

The following restriction endonucleases were used : Pst I and Hae III were kind gifts of Dr G. ROIZES ; Eco RI and Bam HI were purchased from BOEHRINGER MANNHEIM GmbH. In most experiments DNA was digested using 1-5 units of enzyme per µg DNA in the following buffers. Bam H 1 and Hae III : 6 mM Tris-Hcl pH 7.6, 6 mM $MgCl_2$, 6 mM β mercaptoethanol ; Eco R 1 and Pst I : 100 mM Tris-Hcl pH 7.6, 50 mM NaCl, 10 mM β mercaptoethanol. Restriction fragments were separated by electrophoresis in 1 % agarose slab gels with ethidium bromide (0,5 µg/ml). Usually 5-8 µg digested rDNA were loaded in

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each slot. Molecular weight markers consisted of phage λ DNA (gifts of Drs G. ROIZES and B. JARRY) digested with Eco R I (7), phage PM 2 DNA (BOEHRINGER MANNHEIM Gm BH) digested with Hae III (8) and Cauliflower Mosaic Virus DNA (strain Cabb B-D) digested with Eco R I (9,10).

Denatured DNA was immobilized on Millipore filters either by filtration or by the SOUTHERN blot technique (11). Hybridisations were carried out in the presence of 40 % formamide, 4 x SSC (SSC is 0.15 M NaCl, 0.015 M Sodium citrate) at 40°C for 36 hours (4). Filters were then washed 6 times with 4 x SSC at 40°C followed by 0.2 x SSC at 25°C before drying and counting or autoradiography in the presence of a Cronex-4 "Parspeed" screen.Other hybridisation conditions, including more stringent ones (40-70 % formamide 50°C) and RNase treatments were also tested but with similar results. As a further control each batch of probe was hybridised under the above conditions with λ, PM 2 and CaMV DNA digests : non-specific hybridisation could not be detected.

RNA probes were prepared either from purified radish ribosomal subunits or from total RNA. Polysomes were prepared as previously described (12). They were dissociated in the presence of 20 mM EDTA and the subunits purified through a 5-25 % sucrose gradient centrifuged 3.5 hours in an SW 41 rotor (BECKMAN). Purified subunits were immediately extracted for rRNA. Whole cell RNA and ribosomal subunit RNA was extracted as already published (12) 25 S rRNA was separated from 18 S rRNA by sucrose gradient centrifugation (2-20 % Sucrose, 27 000 rpm 15 h in a SW 27 or a SW 41 rotor). Purified rRNA obtained in this way was then checked by polyacrylamide gel electrophoresis for cross-contamination (13). This was always found to be less than 10 %.

RNA was labelled in vivo by incubating seedlings for 20-36 hrs in ^{32}P Phosphate (100-200 µCi/ml) or in ^{3}H uridine (100 µCi/ml) for 8 hours. At the end of labelling contaminant bacteria were eliminated by surface sterilisation of the seedlings.^{32}P RNA preparations used in this work had specific activities ranging from 30 000-90 000 cpm/µg. The appropriate unlabelled rRNA was always added as a competitor in quantities which should have reduced the effects of cross-contamination by a factor of at least 10.

RESULTS

1) Partial purification of rDNA.

rDNA was partially purified by CsCl equilibrium ultracentrifugation since it is known that the density of rDNA in most plants is slightly higher than that of bulk DNA (14,15). This was confirmed for radish rDNA as shown in figure 1. The distribution of rDNA sequences is rather wide owing to overloading of the gradient. Fractions were pooled as indicated in figure 1 and the heavier fraction used as rDNA in subsequent experiments. Molecules at this step of purification were larger than 20.8 kbp. Saturation experiments were carried out in order to estimate the amount of rDNA and its degree of purification. Sequences complementary or identical to rRNA represent about 1.3 % of total DNA and 15 % of the partially purified fraction.

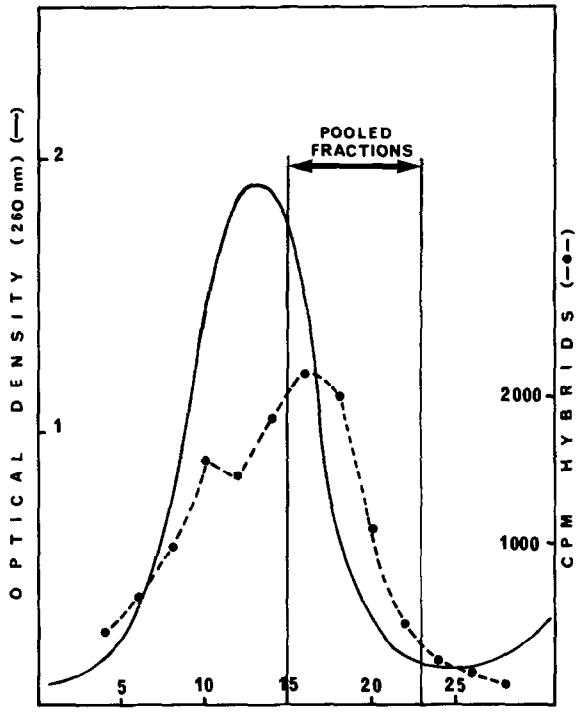

Figure 1. Partial purification of rDNA.

High molecular weight DNA was mixed with CsCl at a final density of 1.700 $g.cm^{-3}$. Gradients were formed during centrifugation (33 000 rpm in 50 Ti or 75 Ti rotor, 65-72 hrs at 20°C). After elution aliquots of even fractions were diluted 10 times with SSC, denatured and immobilised on Millipore filters. The filters were hybridised with an equimolecular mixture of ^{3}Huridine-labelled 25 and 18 S rRNA. Fractions corresponding to the peak of the hybridisation curve were pooled as indicated.

2) Restriction endonuclease digestion.

Figure 2 shows the electrophoretic pattern of an Eco R I digest of rDNA and its hybridisation pattern to 18 S and 25 S rRNA probes. The profiles obtained with either probe are quite similar and rather complex, since at least 6 fragments hybridise to the probes. The patterns are the same whether the probes derive from total RNA or from purified ribosomal subunits. However, two fragments ("a" and "c") hybridise more strongly, and differences in intensity can be detected between the 25 S hybridisation pattern and the 18 S one. It is noticeable that all the bands on the autoradiogram closely correspond to major bands in the ethidium bromide pattern and that "a" and "c", the more strongly hybridising fragments, correspond to the more stained bands.

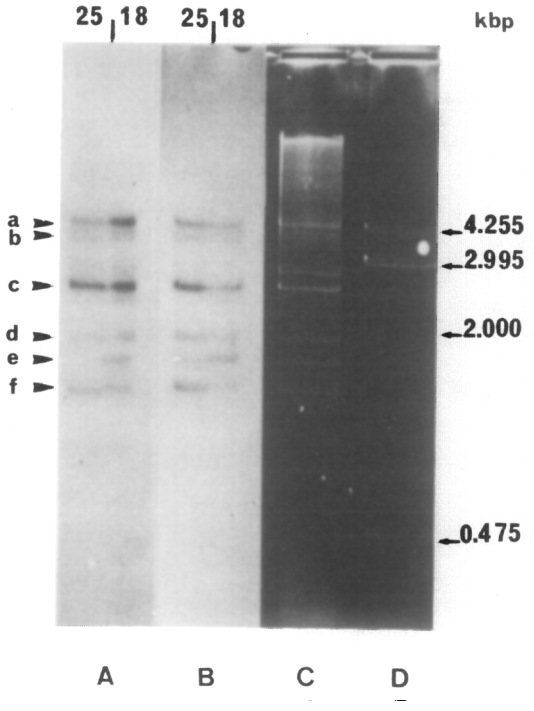

Figure 2. Eco R 1 pattern of radish rDNA.

The electrophoretic pattern of rDNA is shown in (C) together with a CaMV DNA Eco R 1 digest as marker (D). Millipore filter strips containing the denatured DNA fragments from Eco R 1 digests identical to (C) were cut in half down the middle. One half of each was hybridised to ^{32}P 25 S and the other half to ^{32}P 18 S rRNA. rRNA was derived from total RNA fractionated on a sucrose gradient (A) or from purified ribosomal subunits (B).

Figure 3 shows similar results obtained with a Bam H 1 digest. Six hybridising fragments are observed, of which two hybridise more strongly to both probes than the others, although with different intensities. Fragment "a" could be a partial digest product since when digestion is carried out for a long time or with an excess of enzyme its intensity decreases, correlated with an increase in the amount of fragments "c" and "e". However we never obtained a complete digestion of "a".

Figure 4 shows the results of a Pst 1 digest. Two major hybridising bands are visible. They hybridise to both probes and are rather broad. The larger fragment "a" is about twice as large as "b" and it disappears if the enzyme/DNA ratio is increased. This suggests that "a" is a partial digestion product.

3) Mapping information.

Several types of information can be used in attempting to map the above restriction sites : information relating to the size of hybridi-

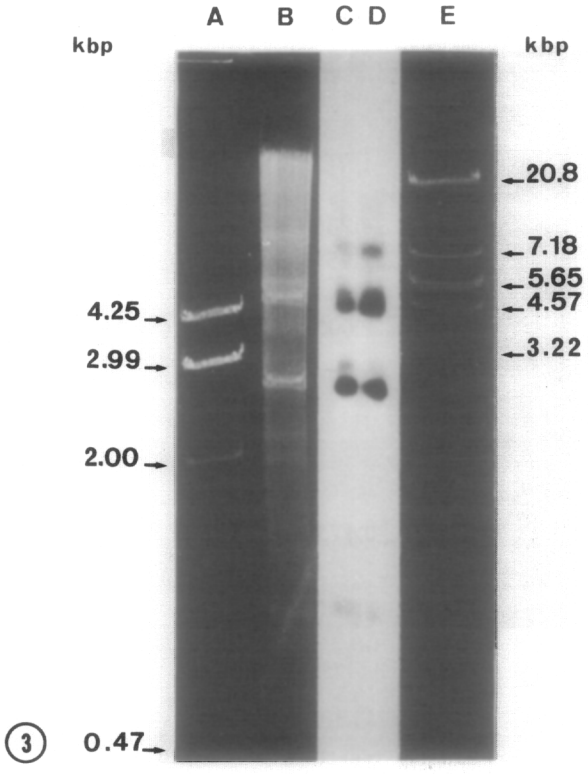

Figure 3. Bam H 1 pattern of radish rDNA.
(A) Eco R 1 digest of CaMV DNA (size marker). (B) Electrophoretic pattern of Bam H 1 digest of rDNA. (C) Hybridisation pattern to ^{32}P 18 S rRNA from 40 S subunit. (D) Hybridisation pattern to ^{32}P 25 S rRNA from 60 S subunit. (E) Eco R 1 digest of phage λ DNA.

Figure 4. Pst 1 pattern of radish rDNA.
(A) hybridisation pattern to ^{32}P 18 S rRNA from total RNA. (B) hybridisation pattern to ^{32}P 25 S rRNA. (C) Electrophoretic pattern of rDNA Pst I digest. (D) Eco R 1 digest of phage λ DNA.

sing fragments in single or double digests(table I) and information derived from studies on in vivo transcription of ribosomal genes in plants (2, 3, 4). The larger pre-rRNA clearly demonstrated in radish is 2.3 x 10^6 d (≃6750 nucleotides) (16, 17). The possibility of the occurence of a larger pre-rRNA in the range of 3 x 10^6 d (≃ 8 800 nucleotides) remains (3, 4). 25 S and 18 S rRNA have approximate sizes of 3800 and 2050 nucleotides respectively. The minimum size for transcribed non-conserved rDNA sequences is 900 bp, about two thirds of which are in the internal spacer.

From the Pst I digestion it appears that the minimum repeat size unit is 11 000 bp. This would indicate that two adjacent transcription units are separated by a 4 250 bp non-transcribed spacer if we assume a size of 6750 nucleotides for the pre-rRNA.

Table I

Size in kbp of the restriction fragments hybridising with 25 S and (or) 18 S rRNA.

Eco R I	Bam H I	Pst I	Eco R I + Bam H I	Eco R I + Pst I
a 4.5	a 8.0	a 22	a 2.3	a 4.5
b 4.1	b 5.1	b 11	b 1.72	b 4.1
c 2.43	c 4.5	33	c 1.48	c 2.4
d 1.86	d 2.9		d 1.07-1.04	d 1.86
e 1.55	e 2.5		e 0.95-0.80	e 1.55
f 1.35	f 0.7		7.52-7.34	f 1.35
15.79	23.7			15.76

We know, from studies on pre-rRNA that 18 S and 25 S rRNA genes are separated by an internal spacer. If we also assume that the genes are arranged in tandem repeats we should expect that a maximum of two restriction fragments hybridise to both 18 S and 25 S rRNA probes : one overlapping the transcribed spacer and a larger one overlapping the non-transcribed spacer. If we choose the alternative assumption that the genes are organised as inverted repeats, then only one fragment shoud be expected to hybridise to both probes. In fact with at least two enzymes (Eco R I and Bam H I) we observe that as many as 6 fragments hybridise to both probes. For both enzymes the sum of the sizes of these fragments is considerably larger than the repeat unit (Table I). The controls concerning stringency of hybridisation and cross-contamination of the probes rule out obvious possible artefacts. In addition double digestion by Eco R I and Bam H I yields two series of fragments ("d" and "e") which are clearly heterogeneous in size. Therefore, we conclude that some heterogeneity occurs among the highly repeated rDNA genes of radish. Since the double digestion by Bam H I and Eco R I yields a series of fragments (Table I) the sum of which is only slightly larger than the value expected for the minimum size of a transcription unit it seems likely that most of the heterogeneity lies in the non-transcribed spacer sequences. Each hybridising Eco R I fragment is reduced in size following further digestion with Bam H I. This means that at least one Bam H I site occurs in each Eco R I rDNA fragment. Examination of the Pst I + Eco R I double digestion shows that all hybridising Eco R I fragments are recovered. Thus the Pst 1 site, which has allowed us to define the repeat size unit, is located very near to one of the Eco R I sites. More precise mapping is hazardous due to heterogeneity.

DISCUSSION

Our results provide original information about the physical organisation of radish rRNA nuclear genes. Our data clearly demonstrate a heterogeneity in these genes. Preliminary reports of similar results in wheat, barley and cucurbits (18, 19) suggest that this phenomenon could be of wide occurence in higher plants. However in other cruciferae the ribosomal genes were found to be homogeneous (20). Recent experiments in our laboratory have demonstrated heterogeneity in rDNA prepared from a single plantet. More data are clearly required concerning the molecular basis of this heterogeneity. However our data suggests that, as in Xenopus (21-23), Drosophila (24-28) and several mammals (29-31), most of the heterogeneity seems to lie in the non-transcribed spacer sequences.Finally, the outstanding question which emerges is whether this heterogeneity could be correlated with some selective advantage which could be of interest for plant breeders.

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