ORGANIZATION OF 5 S RNA GENES IN *VICIA FABA*

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1. Introduction

Data concerning the genetic organization of 5 S genes in eukaryotes are scarce. In *Drosophila melanogaster* there are ~160 5 S genes/haploid genome [1] with an average size of 375 bp (base pairs)/repetition unit [2]. In *Xenopus laevis* three 5 S RNA species [3,4] have been found. The DNA repeat length of two of them is 700 bp and 350 bp [4]. Although there is obviously a remarkable variation in size of the 5 S repeat units (5 S DNA), all contain an A+T-rich spacer region. If A+T-rich spacers are a general feature of 5 S repeats, despite their size variation, one could speculate that the spacer fulfils an important function in regulation of transcription and/or evolution of the 5 S RNA.

No data are available to date concerning the organization of 5 S genes in plants. We report here evidence that in the broad bean, *Vicia faba*, 5 S genes are organized in repeats ~600 bp in length containing the gene and a 480 bp long spacer rich in A+T.

2. Materials and methods

DNA extracted from root tips by a modification of the method in [5] were digested by restriction enzymes using the following buffers: *Eco*RI – 100 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$; *Hin*dII – 10 mM Tris–HCl (pH 7.9), 60 mM NaCl, 7 mM $MgCl_2$, 6 mM 2-mercaptoethanol; *Bam*I – 6 mM Tris–HCl (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol; *Bsp*RI – 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol; *Eco*RI$^+$ – 2 mM $MgCl_2$, 25 mM Tris–HCl (pH 8.5). Terminal digestion was indicated when, after prolonged incubation or the addition of more enzyme, the digestion pattern remained unaltered. Electrophoretic separation of restriction fragments was accomplished in 1.2% agarose gels in 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8 (for analytical purposes 4 μg DNA/slot). DNA was visualized by ethidium bromide staining. Hybridization of restriction fragments immobilized on nitrocellulose filter with ^{125}I-labelled 5 S RNA occurred as in [6] in the presence of unlabelled 18 S and 25 S RNA. The isolation and iodination of RNA was as in [7]. After hybridization filters were rinsed with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate), treated with RNase (10 μg/ml) in 3 × SSC for 30 min and rinsed again with 2 × SSC, 0.1 M KI. After drying at 60°C the filters were prepared for fluorography [8] and exposed to flashed X-ray film (ORWO) at −70°C for 12 days.

Isolation of restriction fragments from 1% agarose gels was accomplished by repeated freezing and thawing of the gel slices in 50 mM Tris, 1 mM EDTA (pH 7.8). Gel particles were removed by centrifugation.

For isopycnic sedimentation DNA was mixed with CsCl (final density, 1.700 g/cm) in 5 ml and centrifuged for 68 h at 40 000 rev./min in a Beckman type 50 rotor at 20°C. DNA of the pooled fractions from the gradient were denatured by alkali, neutralized by the addition of 20 vol. 2 M NaCl and immobilized on nitrocellulose filters [9]. After hybridization the filters were treated with RNase (see above), dried, immersed in Toluol and counted in a Packard scintillation counter.

3. Results and discussion

After digestion of *V. faba* DNA with *Eco*RI, *Eco*RI$^+$,

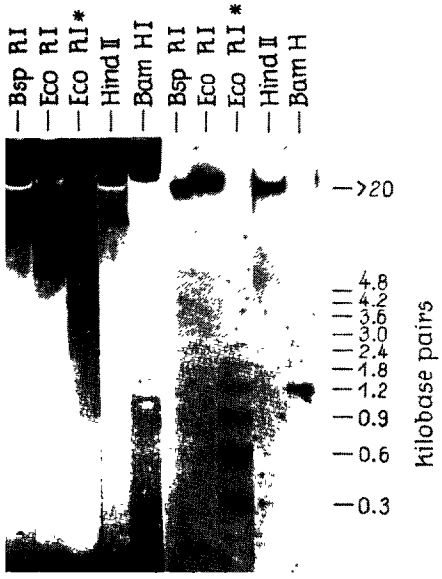

Fig.1. Hybridization of 5 S RNA with restriction fragments. DNA (5 μg) was completely digested with either *Eco*RI, *Hin*dII, *Bam*I, *Bsp*RI, or *Eco*RI$^+$ at 37°C for 5 h. Electrophoretic separation of restriction fragments was in 1.2% agarose slab gels at 25 V for 8 h. Hybridization with ^{125}I-labelled 5 S RNA (5.2×10^6 dpm/μg) occurred in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 21 h using a 5 ml reaction volume with 6 μg labelled 5 S RNA and the 20-fold amount of unlabelled 18 S and 25 S RNA. Size determination of the restriction fragments was carried out using λ-*Eco*RI [14], λ-*Hin*dIII [15] and PM2-Hin [16] restriction fragments for size calibration. An exact determination of the molecular weight of the F1 fragments by electrophoresis was impossible due to their large size which is between the largest λ-*Eco*RI fragment and the uncleaved λ molecule (i.e., between 20.7 kbp and 46.9 kbp) [14]. Left. Ethidium bromide-stained gels; right: fluorography.

*Hin*dII, *Bam*I, and *Bsp*RI, the resulting fragments were separated by electrophoresis, transferred to a cellulose nitrate filter [6] and hybridized with ^{125}I-labelled 5 S RNA. As is evident from fig.1, the 5 S RNA hybridizes to a single size class of *Eco*RI, *Hin*dII, and *Bsp*RI restriction fragments (called F1) ~30 kbp in length (for a more detailed characterization of this fragment class see [10]). However, in the *Eco*RI$^+$ and *Bam*I digests hybridization resulted in a multimeric series of bands arranged in an arithmetic order based on a monomer size of 0.6 kbp. This series proceeds at least up to the dodecamer. Two additional bands on the fluorographs are due to hybridization of 5 S RNA with restriction fragments 0.3 kbp and 0.9 kbp in length. It is assumed that these bands belong to a second arithmetic series based on a monomer size of 0.3 kbp.

In order to ascertain the buoyant density of the 5 S DNA, F1 restriction fragments from *Eco*RI digests were eluted from 1% agarose gels and fractionated in CsCl gradients. As revealed by analytical centrifugation [10] F1(*Eco*RI) fragments consist of mainly two subpopulations of molecules characterized by buoyant densities of 1.7215 g/cm and 1.6955 g/cm. Hybridization with ^{125}I-labelled 5 S RNA across a preparative CsCl gradient showed (fig.2) that the 5 S DNA repre-

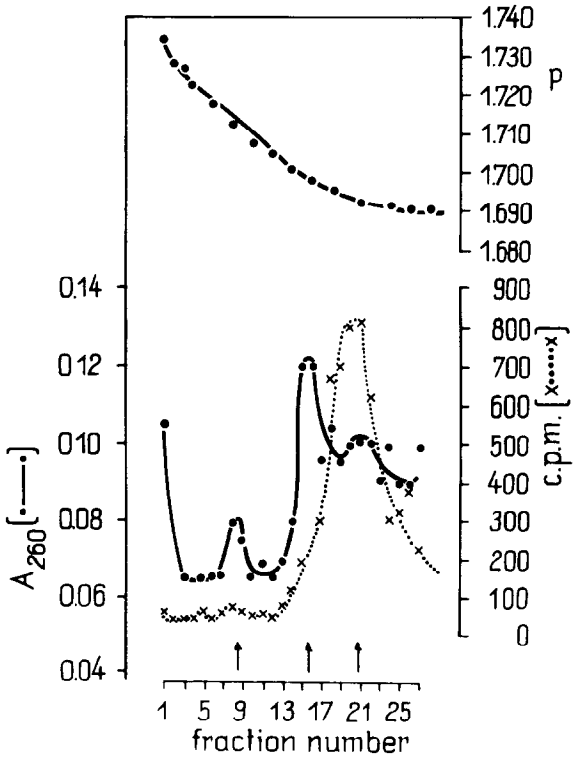

Fig.2. Hybridization of F1(*Eco*RI) restriction fragments with 5 S RNA. DNA, 2 mg, were digested by *Eco*RI to completion and F1 restriction fragments were isolated from the digest. F1 DNA, 80 μg, was fractionated by centrifugation in CsCl and hybridized with 5 S RNA for 16 h at 64°C in 2 ml 2 × SSC containing 2.5×10^5 dpm of ^{125}I-labelled 5 S RNA (5.2×10^6 dpm/μg) and the 20-fold amount of 18 S and 25 S RNA (unlabelled). Arrows indicate the positions of the three F1(*Eco*RI) restriction fragment populations. p = buoyant density (g/cm).

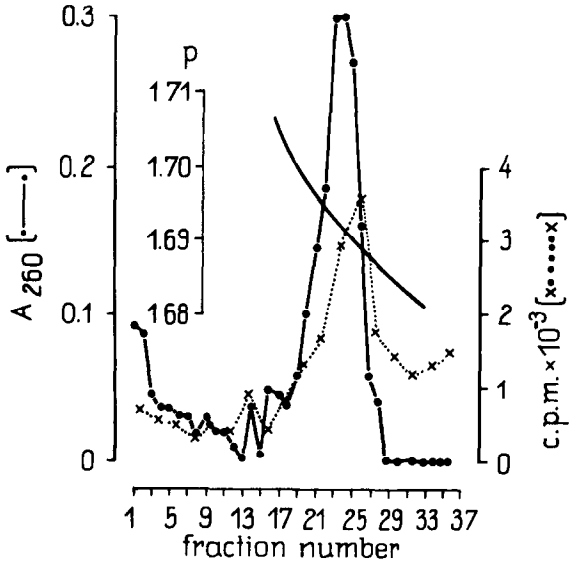

Fig.3. Buoyant density of 5 S DNA. Undigested DNA, 0.1 mg, was centrifuged in CsCl for 67 h at 40 000 rev./min. The experimental conditions for collection of the fractions, hybridization with iodinated 5 S RNA and scintillation counting were the same as described in fig.2.

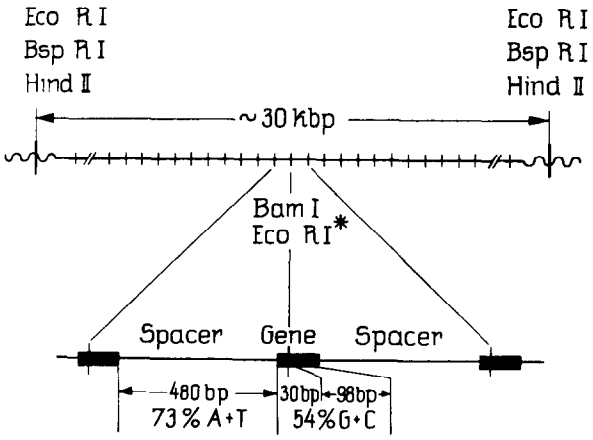

Fig.4. Diagrammatic representation of the physical map of the 5 S genes in *V. faba*. The size of the F1 restriction fragments generated by *Eco*RI, *Hin*dII, and *Bsp*RI was estimated to be ~30 kbp according to their electrophoretic mobility in horizontal 0.4% agarose slab gels. As a size standard uncleaved λ and λ-*Eco*RI fragments [14] were used. The number of repetition units per F1 fragment is unknown.

sents a minor fraction within the F1(*Eco*RI) fragment class characterized by a buoyant density of 1.6920 g/cm, corresponding to a calculated G+C content of 32%. The same result was obtained by using uncleaved *V. faba* DNA in gradient hybridization experiments with 5 S RNA (fig.3). This strongly indicates the majority, if not all, of the 5 S DNA to be rich in A+T.

The sequence of 5 S RNA of *V. faba* is known to have a length of 118 bp and a G+C content of 54% [11]. If the repetition unit within the 5 S DNA is 600 bp in length, the 5 S genes are separated by a spacer of ~480 bp and an A+T content of 73%. A summary of data leading to the physical map of 5 S genes (5 S DNA) in *V. faba* is given in fig.4.

Utilizing the 5 S RNA sequence data [11] the cleavage sites of *Eco*RI$^+$ and *Bam*I are located within the gene at identical position. The gene does not contain cleavage sites for either *Eco*RI nor *Hin*dII, but there is one site for *Bsp*RI 54 bp from the end of the gene. Obviously this site is not a target for the enzyme. The reasons are unknown but modification of this site due to methylation seems to be a reasonable assumption.

We do neither know how many repetition units are present per F1 fragment nor do we know the nature of the DNA flanking these fragments and containing sites for *Eco*RI, *Hin*dII and *Bsp*RI. With a size of 30 kbp/F1 fragment, a maximum of 50 repetition units/F1 fragment, 0.6 kbp in length, are expected.

Two interesting observations demand further explanation.

1. The arithmetic series of bands which might be explained:
   (i) By partial digestion of the sequence;
   (ii) By the presence of mutations affecting the *Eco*RI$^+$/*Bam*I site;
   (iii) By methylation of this site.

The following arguments favour the third interpretation. Experiments were carried out under terminal digestion conditions as indicated by unaltered microdensitograms when increasing enzyme concentrations were used. Mutations randomly involving the *Eco*RI$^+$/*Bam*I sites in the 5 S genes seem to be unlikely because the 5 S sequence has, with respect to these sites, been reported to be remarkably conservative in different plant species [11]. On the other hand partial hydrolysis, either due to partial methylation of a given sequence or to an incomplete inhibition of the enzyme's

ability for cutting, has been reported in other species, e.g., in calf [12]. If this is true in our case, differences between *Eco*RI$^+$ and *Bam*I as to their cleavage ability of methylated sites would account for the observed quantitative differences in the hybridization pattern shown in fig.1.

2. The 0.3 kbp and 0.9 kbp long fragments containing 5 S genes are indicative of the existence of at least two 5 S DNA families in *V. faba* differing in their monomer size.

In this respect it should be noted that the 5 S genes in this species have been located on the satellite of the satellite chromosome at two separate loci [7,13]. It would be of interest to find out whether or not the organization of the 5 S genes within these chromosome regions is different.

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