Extrachromosomal DNA from a dicot plant *Vigna radiata*

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Received 18 September 1986

Extrachromosomal DNA was isolated from germinated mung bean as well as from its callus culture. Methods followed for the isolation of cccDNA gave a heterogeneous mixture of DNA from 4.2 kb to 140 bp in size. Hybridization with a repetitive DNA probe from mung bean chromosomal DNA showed extensive dispersion, similar in chromosomal and extrachromosomal locations. An electron micrograph of purified extrachromosomal DNA revealed some circular DNA molecules with hanging tails.

extrachromosomal DNA *Polydisperse size* *Repetitive sequence*

1. INTRODUCTION

There are several reports in the recent literature about the occurrence of EC DNA in higher eukaryotes [1,2]. Like prokaryotes, higher organisms can also have both plasmid and viral DNA in the extrachromosomal form. Organelles have their own genomes and in addition may harbour extra DNA, in either linear or circular forms [3]. In particular, plant mitochondria are known to contain many copies of circular and linear EC DNA [4], a fraction of which contributes to cytoplasmic male sterility [5].

In the course of our studies on a repetitive DNA family in mung bean (*Vigna radiata*), we observed extensive dispersion of the family members, throughout the chromosomal high-M_r DNA [6]. We therefore wanted to determine whether these family members also occur extrachromosomally. An analogous situation occurs in human and African green monkey kidney cells, where the repetitive *Alu*I and *Kpn*I sequences were shown to occur both chromosomally and extrachromosomally [7–9]. It has been postulated that these are transposable elements, generated by the reverse transcription of RNA products containing the interspersed repeat elements from the chromosome and are called retroposons [10]. This paper describes the isolation and characterization of mung bean EC DNA and the distribution of a family of repetitive sequences in it. Using both the cloned family member as well as the heterogeneous repetitive DNA probes, it was found that the EC DNA fraction from the whole cell contains a highly dispersed arrangement of the repetitive sequences. Electron micrographs of the EC DNA showed a heterogeneous population with some circular molecules.

2. MATERIALS AND METHODS

Mung bean (variety B1) seeds were obtained from the Pulse and Seed Research Station, Baharampur. Callus culture from leaf explant was grown in MS medium with synthetic auxin and Kinetin. The enzyme, S_1 nuclease and exonuclease III were purchased from BRL (USA) and nitrocellulose filter paper (BA 85) was obtained from Schleicher and Schuell. Biogel HTP-DNA grade was obtained from Bio-Rad Laboratories.

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Abbreviations: EC DNA, extrachromosomal DNA; ccc, covalently closed circular; HAP, hydroxyapatite; bp, base pair; EtBr, ethidium bromide; MS medium, Murashige and Skoog's medium

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2.1. *Isolation of EC DNA*

Mung bean germinated at 30°C in darkness under sterile conditions for 3 days or 2 weeks growth of callus culture was processed for isolation of EC DNA according to the plasmid isolation method of Birnboim and Doly [11], as adapted by Junakovic and Ballario [12] for *Drosophila* cell line.

2.2. *Purification of EC DNA*

For CsCl-EtBr equilibrium density gradient centrifugation, the mixture was spun at 45000 rpm for 18 h in a VTi 80 rotor at 25°C. After fractionation, an aljquot from each fraction was loaded onto a 0.8% agarose gel, electrophoresed and Southern hybridized to look for the presence of the repetitive DNA.

EC DNA isolated from the leaf callus culture was further purified by HAP column chromatography according to the method of purification of cccDNA [13]. The isolated EC DNA was purified by passing through a nitrocellulose (BA 85) filter. This DNA was heat-denatured and immediately chilled in ice and adjusted to 8 M urea, 0.24 M sodium phosphate buffer (pH 6.5) and 0.9% SDS. This mixture was loaded on an HAP column previously equilibrated with the same buffer and washed extensively with 10 mM sodium phosphate buffer (pH 6.5) and the EC DNA eluted with 0.3 M phosphate buffer (pH 6.5). This DNA was dialysed extensively against TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and finally alcohol precipitated, after making 0.3 M with NaOAc (pH 5.2).

This EC DNA mixture was treated with single-strand specific nuclease S_1 using S_1 digestion buffer (30 mM NaOAc buffer, pH 4.6; 1 mM $ZnCl_2$, 25 mM β-mercaptoethanol). The HAP-purified and nitrocellulose-filtered EC DNA was further treated with exonuclease III for limit digestion, to eliminate single-stranded and double-stranded linear DNA contaminants. For isolation of inverted repeat, the method of Ohtsubo and Ohtsubo [14] was followed.

2.3. *Other procedures*

Mung bean repetitive DNA was isolated by heat denaturation, then renaturation and S_1 digestion from total mung bean DNA [6]. The total repetitive DNA, ~300 bp in length, was nick-translated by the method of Rigby et al. [15] to a specific activity of 10^8 cpm/μg DNA, using DNA polymerase I, [α-^{32}P]dCTP (Amersham, England) but no DNase I. Southern blotting, hybridization and washings were done according to standard procedures [16]. Repetitive DNA insert was obtained by restricting the recombinant plasmid SG 78 which was selected from a shotgun cloning of mung bean genomic DNA in pBR322 with *Eco*RI and *Bam*HI. After separation using 1% agarose gel electrophoresis, the 400 bp insert was eluted from the gel slice by diffusion and labelled by nick-translation. Grid preparation for electron microscopy of HAP-purified EC DNA was according to Dubochet et al. [17].

3. RESULTS AND DISCUSSION

Total EC DNA was isolated from aseptically germinated mung bean. To rule out the possibility of any bacterial contamination in the seedlings, callus culture from leaf of mung bean was also used for the isolation of EC DNA. It was found that they are the same (fig.1). The size range of the polydisperse EC DNA obtained was determined from the known M_r of λ DNA fragments cut with *Hin*dIII and pBR322 fragments produced by *Alu*I. This turns out to be 4.2 kb to 140 bp in length. The small size range of this DNA fraction indicates the absence of organelle DNA as a whole, e.g. chloroplast and mitochondrial DNAs. However, wheat mitochondria are known to contain polydisperse small circular DNA ranging from 0.1 to 2 μm, i.e. 300 bp to 6 kb in length [4]. Therefore, our preparation may contain such intra-organelle circular DNAs. To remove single-stranded DNA produced from alkaline-SDS treatment of the cell extract the following steps were performed: (i) phenol, saturated with 0.5 M NaCl, was used for extraction [18]; (ii) the EC DNA was filtered through nitrocellulose (BA 85) in the presence of 0.5 M NaCl and (iii) this was purified through an HAP column. That all the single-stranded DNA was removed was proved by S_1 nuclease treatment of purified EC DNA. There was no change evident from the gel electrophoresis pattern. To show the occurrence of cccDNA in our EC DNA preparations, we digested these extensively with exonuclease III. A control digestion of a mixture of form I, II and III of puR250, a

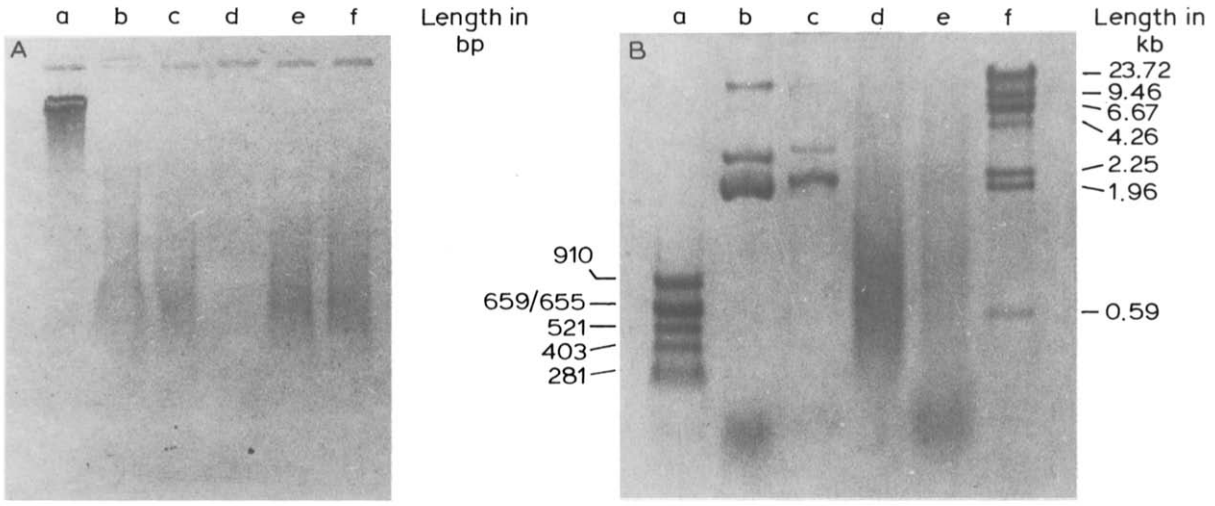

Fig.1. EtBr-stained 1% agarose gel electrophoresis of (A) lane a, CsCl-purified mung bean leaf DNA, 4 μg; b, EC DNA isolated from 3 day germinated mung bean, 1.5 μg; c, HAP-purified callus EC DNA, 1 μg; d, flow through of HAP column, 0.2 μg; e, S_1 nuclease-treated (2 U/μg at 37°C for 30 min) HAP-purified callus EC DNA, 2.5 μg; f, same as lane c. (B) Lane a, pBR322 DNA, 1.5 μg restricted with *Alu*I; b, puR250 DNA, 2 μg; c, the same digested with Exo III (70 U at 37°C for 18 h); d, HAP-purified callus EC DNA, 2 μg; e, the same, digested with Exo III under the conditions of lane c; f, λ DNA, 2 μg cut with *Hin*dIII.

pBR322-derived plasmid DNA with exonuclease III showed that most of the reaction product was in form I or supercoiled circular DNA. So, it is expected that the exonuclease III-protected EC DNA is circular double-stranded DNA, though the diminution in band intensity of form I plasmid DNA indicated that some nonspecific DNase action or digestion from the single nicks of cccDNA took place. From electron microscopy of purified EC DNA, a heterogeneous population including a few circular DNA molecules with a tail (sigmoid) was observed. This is shown in fig.3.

To show the occurrence of the 300 bp family of repetitive DNA sequences [6] in the EC DNA preparation of mung bean and compare it with that of chromosomal origin, EC DNA was fractionated by CsCl-EtBr equilibrium density gradient centrifugation. After fractionating the

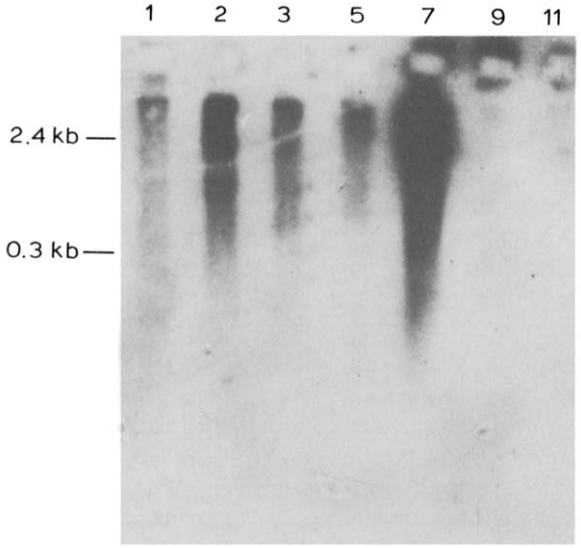

Fig.2. CsCl-EtBr density gradient centrifugation of total EC DNA. After fractionation of the gradient from the bottom, a 20 μl aliquot of each was run on 0.8% agarose gel electrophoresis, denatured, renatured and Southern blotted on nitrocellulose. On hybridization with ^{32}P-labelled total repetitive DNA probe from mung bean, in 50% formamide, 5 × SSPE (saline sodium phosphate EDTA), 0.2% SDS and 2 × Denhardt's reagent [17] at 42°C for 17 h, the filter was washed extensively with 2 × SSPE, 0.2% SDS, followed by 0.1 × SSPE, 0.2% SDS at 50°C and autoradiographed. The marker lane contained puR250 DNA digested with *Pvu*II. The lane numbers are the corresponding fraction number.

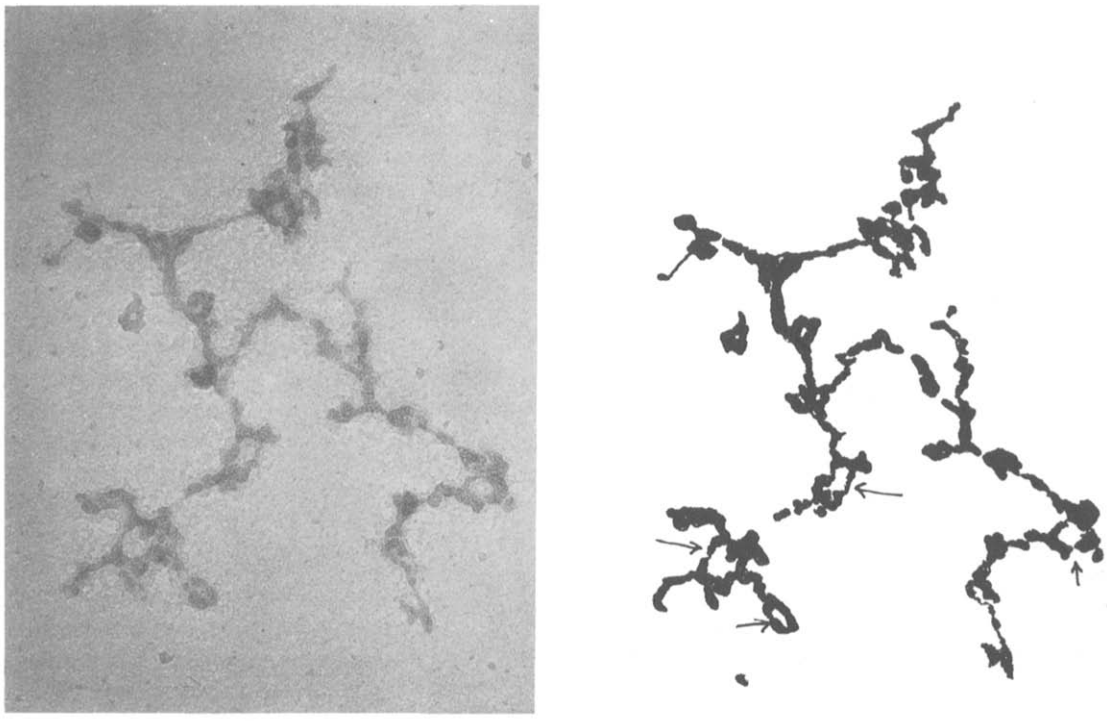

Fig.3. Electron micrograph along with its schematic diagram of a selected field of callus EC DNA (magnification × 150000).

gradient, an aliquot from each fraction was electrophoresed on agarose gel and then blotted onto nitrocellulose. Using ^{32}P-labelled, nick-translated repetitive DNA as a probe, it was found that the repetitive sequences were also polydispersed in EC DNA (fig.2). Using the cloned repetitive DNA probe from SG 78, similar polydisperse hybridization was obtained with EC DNA, without any distinctive band (not shown).

Fig.4. Autoradiograph of the Southern hybridization of denatured, renatured and S_1 nuclease-treated callus EC DNA [14] with nick-translated total mung bean DNA. 10 μg HAP-purified callus EC DNA was denatured with 0.15 M NaOH at room temperature for 15 min, neutralized with HCl, then the solution was chilled in ice, and the Na^+ concentration was adjusted to 0.3 M with NaCl, renaturation of the inverted repeat was done by incubating the mixture at 68°C for 30 s, S_1 nuclease buffer was added, then S_1 nuclease (6.6 BRL U/μg DNA) treatment was performed at 37°C for 30 min. Hybridization and washings were done as described in the legend to fig.2.

Alkali-denatured, briefly renatured, EC DNA was treated with S_1 nuclease to determine the presence of inverted repeat, if any [14]. On Southern hybridization with labelled total mung bean DNA, among many fragments, one prominent hybridizable band of ~1 kb in length was observed on the autoradiogram (fig.4). The EM picture of sigmoid DNA molecules might originate from the inverted repeat sequences which on isolation appear circular with a hanging tail [19].

On calculation of the percentage of this repetitive DNA in total EC DNA from dot-blot hybridization, it was found to be 4.6% (w/w), assuming the haploid genome size of mung bean to be 4.7×10^8 bp [20]. The function of the repeat family is still unknown in the EC DNA. Two hypotheses have been proposed in the literature: (i) they are the by-products of recombination and/or reverse transcription of transcripts of the mobile genetic elements [1] and (ii) they are produced as a result of 'replicon misfiring', which may occur at the chromosomal origins of replication [21].

ACKNOWLEDGEMENTS

We gratefully acknowledge Professor B.B. Biswas for encouragement and support, Dr Amita Pal for tissue culture, Dr Sribir Sen for various help and Dr Anima Sen for grid preparation for electron microscopy; also the Department of Science and Technology, Govt. of India, for financial support to the Genetic Engineering Unit in the Department of Biochemistry, Bose Institute.

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