Single-stranded replication intermediates of ribosomal DNA replicons of pea

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Replication of ribosomal DNA replicons in cells of *Pisum sativum* (cv. Alaska) occurs bidirectionally by displacement loops. Replication is initiated on opposite parental strands and nascent chains are elongated moving 5′ → 3′ along each parental template. Replicative intermediates were analyzed by 2-dimensional agarose gel electrophoresis under neutral–neutral and neutral–alkaline conditions. Southern blots of ribosomal DNA fragments separated in the second dimension under neutral conditions show slowly migrating replicative fragments that hybridize with specific probes in a manner consistent with bidirectional replication. The replicative fragments are present in root meristems with cells in S phase; they are absent or few in number in meristems with cells in G_2 phase. The following observations indicate that the replicative fragments are single stranded. The apparent length of the replicative fragments is not the same when separated under neutral and alkaline conditions. They contain rDNA without breaks and they do not exhibit the smaller nascent chains expected from replication bubbles and forks. They are not cleaved by restriction enzymes that require duplex DNA as substrate and they are digestible by S1 nuclease.

Key words: displacement loops/*Pisum sativum*/rDNA replicons/replication/single-stranded DNA

Introduction

The plasticity of eukaryotic chromosomal DNA replication is well documented. For example, over-replication or amplification occurs by unclear and, perhaps unusual mechanisms in cells subjected to stressful conditions (Schimke *et al.*, 1986; Stark *et al.*, 1989). Also, in rapidly dividing amphibian embryos, classical replication forks are rare but lengthy single-stranded segments are abundant. This finding suggests that single-stranded DNA, in some unknown way, replaces commonly accepted structures associated with bidirectional replication (Gaudette and Benbow, 1986). These observations and others on mammalian cells (Wanka *et al.*, 1977; Bjursell *et al.*, 1979; Carnevali and Filetici, 1981) indicate that the mechanisms responsible for eukaryotic DNA replication are still poorly understood. As a consequence, new methods and technology are continually being developed in an effort to grasp the details of this common and complex phenomenon.

The results presented in this paper are consistent with a model in which ribosomal DNA (rDNA) replication in pea occurs bidirectionally by a series of displacement loops. Nascent chains are elongated 5′ → 3′ on each parental template. Semi-conservative replication is achieved by converging forks from adjacent replicons that meet, are retarded temporarily and then resume replication of the remaining single-stranded template in a cooperative fashion.

Results

There are about 3900 copies of rDNA in pea var. Alaska (Cullis and Davies, 1975; Ingle and Sinclair, 1972) distributed into two major repeat size classes of 8.6 and 9 kb (Jorgensen *et al.*, 1987). The larger repeat, located on chromosome 7, is more abundant representing ~3000 tandem copies or a total of 27 000 kb (Polans *et al.*, 1986; Jorgensen *et al.*, 1987). There are ~900 tandem copies or ~8000 kb of the smaller repeats on chromosome 4 (Polans *et al.*, 1986; Jorgensen *et al.*, 1987). The repeats are located at or near the nucleolar organizers on their respective chromosomes (J.Van't Hof, unpublished data).

Restriction maps of the major repeats are in Figure 1. The repeats differ in size because of the number of 180 base subrepeat units located in the spacer region. The 9 kb repeat also has two *Eco*RI sites in the spacer region which are absent in the 8.6 kb repeats (Jorgensen *et al.*, 1987; Van't Hof *et al.*, 1987). The *Eco*RI fragments from the 9 kb repeats are 0.190, 1.3, 3.9 and 3.7 kb, those of the 8.6 kb repeats are 3.7 and 4.9 kb. The 3.7 kb fragments from each repeat are indistinguishable, containing most of the coding region. Both repeats have a *Hin*dIII site in the spacer region. Digestion with this enzyme cleaves the 3.9 kb *Eco*RI fragment of the 9 kb repeats into fragments of 1.5 and 2.4 kb. The same enzyme cuts the 4.9 kb *Eco*RI fragment of the 8.6 kb repeats into fragments of 2.5 and 2.4 kb. The 2.4 kb *Eco*RI–*Hin*dIII fragments from each repeat contain the same sequences, ~1 kb of spacer and 1.4 kb of the 18S gene.

Below the restriction maps, aligned with the sequences to which they hybridize, are the cloned probes used in this experiment. These probes detect sequences at increasing distances form the replication origin. Probe 790 hybridizes to the origin and the subrepeats, probe 1.3 to 1.3 kb located ~1 kb to the right of the origin and probe 1.6 hybridizes to coding sequences located ~3 kb to the right of the origin. Since the rDNA is tandemly repeated, probe 1.6 also hybridizes to sequences ~4 kb upstream and to the left of the origin (Figure 1).

Identification of replication intermediates of rDNA

Identification of replication intermediates of rDNA was achieved by two-dimensional gel electrophoresis as described by Brewer and Fangman (1987), by comparing results obtained with two samples of rDNA, one from tissue with cells in S phase and another with cells in G_2 phase. Because of their conformation, replication intermediates migrate more slowly than linear duplex molecules. Replication inter-

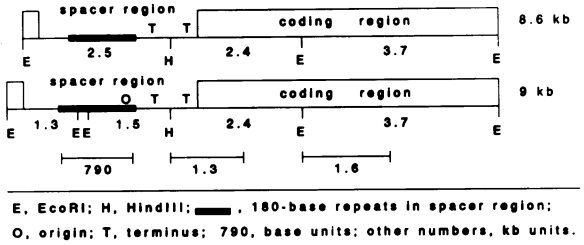

Fig. 1. Restriction maps of the 8.6 and 9 kb rDNA repeats of pea cv. Alaska. Below the maps, aligned below the sequences to which they hybridize, are the probes, 790, 1.3 and 1.6 used in the experiment. The origin, O, and termini, T, are positioned as determined by Hernández *et al.* (1988a,b).

mediates should be present in rDNA extracted from meristematic tissue with cells in S phase because the ribosomal genes are replicated throughout S phase (Van't Hof *et al.*, 1987). On the other hand, intermediates should be absent or few in meristems with cells in G_2 phase, since most of the genome is replicated by this time.

Autoradiograms of rDNA *Eco*RI fragments in Figure 2 support this expectation. There is a positive correlation between the presence of replication intermediates and cells in S phase. The autoradiograms in Figure 2 are Southern blots of *Eco*RI fragments of rDNA probed with 790 which hybridizes to the subrepeats and replication origin located in the spacer region (Figure 1). Virtually identical blots are obtained with rDNA fragments from asynchronously dividing meristems and from meristems with cells accumulated in S phase (Figures 2a and b, respectively). In each case, the probe hybridizes to three spots, labeled A, B and C, of rDNA fragments located on an arc above that formed by linear duplex fragments. These spots are not seen or are much reduced in rDNA from meristems with cells in G_2 (Figure 2c). Since the presence of the spots corresponds positively with cells in S phase and negatively with cells in G_2 phase, the spots are strong candidates for replication intermediates of rDNA.

The rDNA in the spots was further characterized by comparing their migration under neutral (Figure 3a) and alkaline conditions (Figure 3b). As in Figure 2, the blots in Figure 3 are of *Eco*RI fragments probed with 790. If the rDNA in the spots were double stranded, the length of the separated strands in alkali should correspond to that measured under neutral conditions. Further, if the rDNA in the spots has a bubble or fork conformation, the nascent strands would separate from parental chains in alkali and form a smear or bands below the spots.

The apparent size of the rDNA in spots A, B and C is 6.2, 3.6 and 3.2 kb, assuming that the spots in both gels migrated roughly according to mass in the first dimension (Bell and Byers, 1983; Brewer and Fangman 1987). In the second dimension under neutral conditions the migration of the rDNA in the spots was retarded forming an arc above the duplex *Eco*RI fragments (Figure 3a). The rDNA in the spots, therefore, has a different conformation than linear duplex DNA. In alkali (Figure 3b) neither spots A nor C have a smear or bands below them (this is substantiated later on in the text). Further, the length of the single-stranded molecules in spots A, B and C is 9, 5 and 3.6 kb, respectively. These sizes are larger than those determined from migration in the neutral first dimension where A, B and C migrated as 6.2, 3.6 and 3.2 kb fragments. The

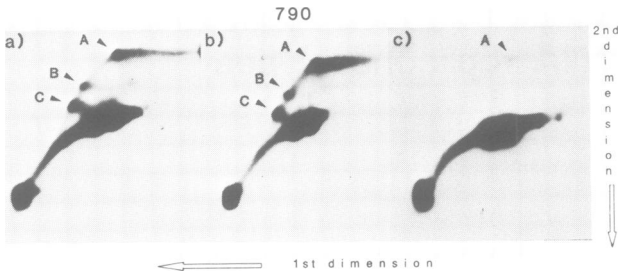

Fig. 2. Identification of replication intermediates of rDNA. Autoradiograms of Southern blots of rDNA *Eco*RI fragments of two-dimensional gels run under neutral conditions in both directions and probed with 790. (**a**) rDNA from asynchronously dividing meristematic cells; (**b**) rDNA from meristematic cells accumulated in S phase by treatment for 8 h with 5-aminouracil; (**c**) rDNA from meristematic cells in G_2 phase after synchronization with 5-aminouracil. A, B and C are spots of replicative intermediates on an arc located above a darker arc formed by linear duplex rDNA fragments.

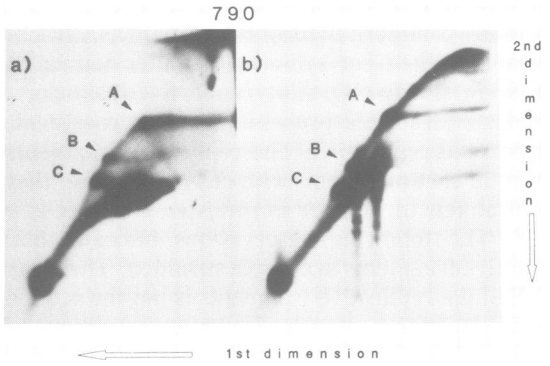

Fig.3. The migration of replication intermediates of rDNA under neutral and alkaline conditions. Autoradiograms of Southern blots of rDNA *Eco*RI fragments from asynchronously dividing pea root meristems separated by two-dimensional gel electrophoresis and probed with 790. (**a**) Separation under neutral conditions in both directions; (**b**) separation in the first dimension under neutral conditions and in the second dimension in alkali. Spots A, B and C as described in Figure 2.

inconsistency of length, the fact that the DNA in the spots is unbroken, and that *Eco*RI did not cleave the rDNA in spots A and B into smaller fragments as would be expected if they were double stranded, taken together indicate that the spots contain single-stranded rDNA.

Sequences in spots A, B and C

The rDNA sequences in spots A, B and C were determined by sequential hybridization of a single blot containing rDNA *Eco*RI fragments separated in both directions under neutral conditions. The blot was probed, stripped and probed again with 790, 1.3 and 1.6 probes (Figure 4). These probes detect sequences at different distances and orientation from the replication origin (Figure 1). In Figure 4, we see that the 790 probe hybridizes to all three spots. Probe 1.3 hybridizes to spots A and C and probe 1.6 hybridizes to spots A and B. Since they hybridize with different probes, the spots have different sequences. This conclusion is verified by experiments with rDNA double digested with *Eco*RI and *Hin*dIII discussed below.

A *Hin*dIII site is located in the spacer region of both the

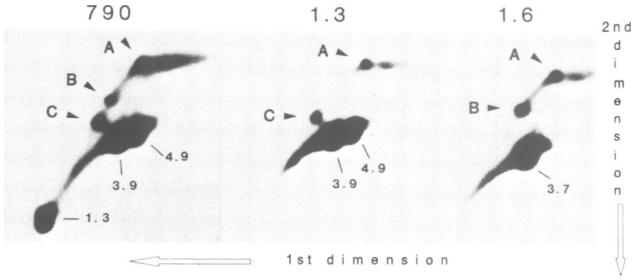

Fig. 4. Sequences in the spots of replication intermediates of rDNA. Autoradiograms of a Southern blot of rDNA *Eco*RI fragments from pea root meristems separated by two-dimensional gel electrophoresis under neutral conditions in both directions. The blot was probed, stripped and probed again sequentially with 790, 1.3 and 1.6. The probes are noted above the autoradiograms. A, B and C are spots of replicative intermediates; the numbers, in kb units, refer to duplex rDNA fragments.

8.6 and 9 kb repeat size classes (Figure 1). Cleavage with this enzyme should reduce the size of the rDNA in spots A and C, if the DNA is double stranded, since the *Hin*dIII site is located between the sequences detected by probes 790 and 1.3 and both probes hybridize with spots A and C. Double digestion of rDNA, however, did not change the size of the rDNA in the spots (Figure 5, top row of panels). They are identical in size, migration and hybridization characteristics to spots seen in rDNA digested with only *Eco*RI (Figure 4).

Spots A, B and C in double-digested rDNA fragments separated under neutral−alkaline conditions contain rDNA chains without breaks which migrate at sizes corresponding to 9, 5 and 3.6 kb, respectively (Figure 5, lower panels), indicating again that the rDNA in spots A and C was not cleaved by *Hin*dIII. Also in Figure 5 (lower panels) there is little evidence of smears or bands descending below the spots as would be expected of denatured replication bubbles and forks. We interpret these findings as further proof that the rDNA in the spots is single stranded.

Digestion of spots A, B and C by S1 nuclease

That the rDNA in spots A, B and C is single stranded is confirmed by digestion with S1 nuclease, an enzyme that preferentially cleaves single-stranded DNA (Figure 6). The autoradiograms in Figure 6 are of *Eco*RI rDNA fragments probed with 790. In each case, the fragments were separated in the first dimension under neutral conditions, the gel lane containing the fragments excised and equilibrated with S1 nuclease buffer. When equilibrated, the control lane received no enzyme (Figure 6a) while the treated lane was incubated with S1 nuclease (Figure 6b). Following digestion the fragments were further separated under neutral conditions in the second dimension, blotted and probed. As seen in Figure 6b the enzyme preferentially digested the rDNA in spots A, B and C as well as all other fragments that comprise the upper arc of replicative intermediates. The lower arc of duplex fragments, in contrast, is unchanged, being virtually identical to that of the untreated control. Thus, the rDNA in the spots and in the arc itself are single stranded.

Direction of the single-stranded rDNA in the spots

Spots A, B and C contain single-stranded rDNA chains of opposite direction i.e. some are 5′ → 3′ and others are 3′ → 5′. The rDNA in the spots hybridizes with single-

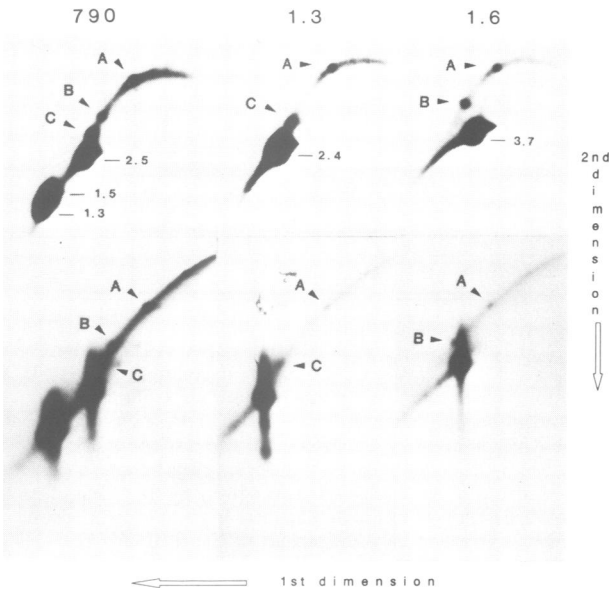

Fig. 5. *Eco*RI fragments of replication intermediates of rDNA are not cleaved by *Hin*dIII. Autoradiograms of Southern blots of double-digested (*Eco*RI and *Hin*dIII) rDNA fragments from pea root meristems. **Upper row**, a single blot of fragments separated by two-dimensional gel electrophoresis under neutral conditions in both directions. **Lower row**, a single blot of fragments separated in the first dimension under neutral conditions and in alkali in the second dimension. Each blot was probed, stripped and probed again sequentially with 790, 1.3 and 1.6. The probes are noted above the autoradiograms. A, B and C are spots of replicative intermediates; the numbers, in kb units, refer to duplex rDNA fragments.

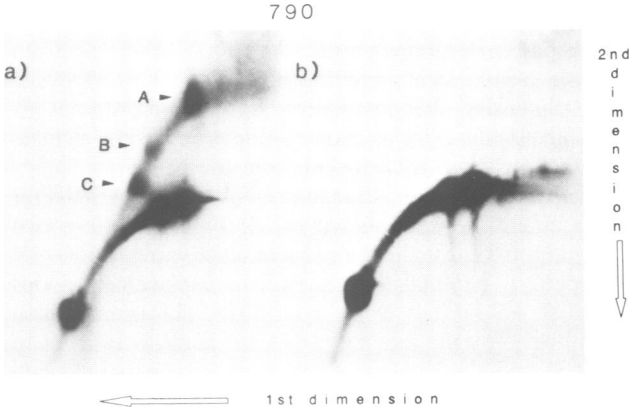

Fig. 6. The digestion of replicative *Eco*RI fragments of rDNA of pea by S1 nuclease. (**a**) An autoradiogram of a Southern blot of fragments treated without enzyme; (**b**) an autoradiogram of a blot of fragments treated with enzyme. Each blot was probed with 790. Gel lanes with fragments separated in the first dimension under neutral conditions were incubated with or without enzyme and then subjected to electrophoresis in the second dimension under neutral conditions before blotting. A, B and C are spots of replicative intermediates.

stranded 22 base oligonucleotide probes and with end-labeled ribosomal transcripts. The oligonucleotides (5′-GCC TAT GGT CTA TAG CCT ATG G-3′ and its 3′ → 5′ complement) represent a 22 base sequence located in or near the origin of the more abundant 9 kb rDNA repeats of pea (J.Van't Hof, unpublished data). This sequence is absent or degenerate in the 8.6 kb repeats. Consequently, the direction of DNA chains from this less abundant repeat are unknown.

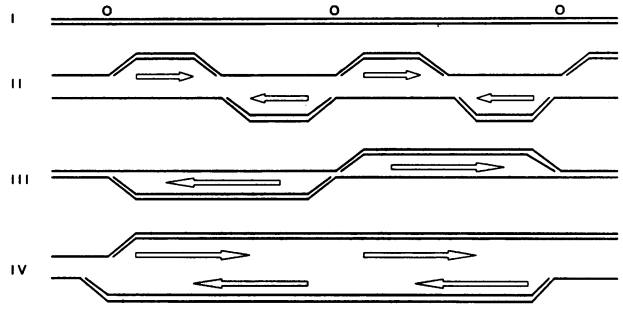

Fig. 7. Diagram representing a model of rDNA replication by displacement loops. I, duplex rDNA with three origins, the Os; II, a series of displacement loops initiated at each origin formed by replication forks travelling only 5′ → 3′ on opposite parental strands in the direction noted by the arrows; III, two displacement loops generated by diverging forks from a single isolated origin; IV, semi-conservative replication achieved cooperatively by the fusion of nascent chains replicated by forks in displacement loops moving in opposite directions. Formation IV is the product of II and III after converging forks from adjacent loops bypass each other and continue replication using the single-stranded parental chain as a template.

Further details about the direction of the chains in the spots are given in the Discussion.

Discussion

A model, shown in Figure 7, incorporates the findings reported in this paper. It shows a series of displacement loops in replicating rDNA (II, III and IV) below a duplex rDNA strand with three origins (I). The displacement loops in II are of three types. The one on the left is produced by a single fork moving left to right from the origin. In the middle are two displacement loops produced by forks diverging from a single origin each travelling only 5′ → 3′ in opposite directions using the complementary parental strands as template. On the right, is another loop produced by a single fork moving from right to left from the origin.

The source of single-stranded rDNA in spot A is larger displacement loops as shown in III, Figure 7. Here two diverging forks from a single origin, each moving 5′ → 3′ on opposite parental strands, form displacement loops extending ~9 kb from origin to terminus. This interpretation is confirmed by the fact the rDNA in spot A hybridizes to 5′ → 3′ and to 3′ → 5′ single-stranded oligonucleotide probes and to end-labeled RNA. Similar large loops would be produced in cases where only one fork is moving on opposite parental templates from adjacent origins, or from scattered origins located on the same duplex rDNA strand.

The rDNA in spot B is from the displacement loop formed by a fork moving left 5′ → 3′ from an origin as shown in II, Figure 7. The 790 bp and 1.6 kb probes hybridize to spot B which also hybridizes to end-labeled RNA indicating that it has chains that are 3′ → 5′.

The source of the rDNA in spot C is the loop formed by a fork moving right 5′ → 3′ from an origin (II, Figure 7). Spot C hybridizes to the 790 bp and 1.3 kb probes, strongly to a 3′ → 5′ single-stranded oligonucleotide probe and T-weakly to the RNA probe. The direction of most of the rDNA in spot C is, therefore, 5′ → 3′.

Semi-conservatively replicated rDNA is achieved in either case II or case III by cooperative converging forks from tandem origins that meet, resolve the supercoiled structure between them and resume replication moving 5′ → 3′ in opposite directions along the single-stranded parental templates (IV). Thus, cases II and III eventually resolve into case IV.

It is seen from the model that the source of the single-stranded rDNA in the spots is very likely the parental strand, assuming little to no extrusion of nascent chains. The double-stranded segment of the loop containing the nascent strand and its parental template are cleaved by *Eco*RI and *Hin*dIII but the parental chain is uncut where it is single stranded and is cut where it is double stranded.

On the other hand, the single-stranded rDNA in the spots may be the result of strand extrusion by branch migration, as suggested by Wanka *et al.* (1977), Zannis-Hadjopoulos *et al.* (1981) and others. If this were the case, the rDNA molecules in the spots are nascent chains.

A shortcoming of the analyses used, in this first of a series of experiments, is that parental and nascent rDNA chains are indistinguishable. Information on this important point requires further work involving experiments that differentiate between parental and daughter strands. These experiments are currently underway.

Nevertheless, the validity of the model in Figure 7 is not contigent on the source of the single-stranded rDNA in the spots. It stands regardless of their source, since the direction and sequence of the nascent strand is identical to that of the displaced single-stranded parental chain. All results observed are compatible with the proposal that tandem rDNA repeats in pea are replicated by a series of displacement loops. Further, the idea that some replicons of pea replicate by displacement loops is consistent with a previous finding pertaining to extrachromosomal DNA of pea. Electron microscopy of this DNA indicated that it replicates by strand displacement (Krimer and Van't Hof, 1983) and other experiments showed that rDNA is included in the extrachromosomal fraction (Kraszewska *et al.*, 1985).

Finally, the replication of the rDNA in pea by displacement loops suggest that highly repeated ribosomal genes in other plant species may be replicated in the same manner. It would be surprising if the mechanism is used only by pea cells, since other plants also have several thousand copies of ribosomal genes. Replication of rDNA by displacement loops, however, may be confined to higher plants, since yeast, a lower eukaryote, replicates most ribosomal genes unidirectionally and not by displacement loops (Brewer and Fangman, 1988; Linskens and Huberman, 1988). On the other hand, it is plausible that replication by displacement loops is characteristic of other tandem highly repeated sequences in both higher plant and animal genomes. Future work will tell us just how common this form of nuclear replication is.

Materials and methods

Seedling culture and cell synchronization

Pea seeds (*Pisum sativum*, cv. Alaska) were purchased from W. Atlee Burpee Co., Westminster, Pennsylvania, USA. Surface sterilized seed was germinated on moist filter paper in Petri dishes. After 4 days at 20°C, seedlings with 3–4 cm primary roots were transferred to Hoagland's nutrient solution (Conger, 1964) and grown for 8–16 h with continuous aeration before any additional treatment. Treatments with 0.2 mg/ml of 5-aminouracil (Sigma # A-4005) were also done in aerated nutrient solution.

To accumulate cells in S phase, seedling roots were treated with 5-aminouracil (0.2 mg/ml) for 8 h. In the presence of this inhibitor, cell progression into S phase continues (as determined by tritiated thymidine incorporation) (Van't Hof, 1966) but progession from G_2 to mitosis stops

within 4 h (Scheuermann and Klaffke-Lobsien, 1973; J.Van't Hof, unpublished data). Meristems with cells mostly in G_2 phase were obtained by treating primary roots of intact seedlings for 12 h with 5-aminouracil (0.2 mg/ml) and allowing them to recover for 3 h in the absence of the inhibitor. In roots treated in this manner, cells divide synchronously beginning at about the 6th hour of recovery (Prensky and Smith, 1965; J.Van't Hof, unpublished data).

DNA isolation for gel electrophoresis

Root tips, 1.5 cm in length, were cut from 400–800 primary roots over ice in Na-phosphate buffer (0.1 M, pH 6.8) containing 20 μg/ml of DNase inactivated pronase, transferred for 30 s to fresh buffer containing 2% formaldehyde and washed with three changes of ice-cold buffer. The 3 mm meristematic tips were cut and transferred to 1.5 ml Eppendorf tubes, 20 tips/tube, and stored on ice. 30 ml of buffer was added to each tube and the tissue crushed by a swift, downward movement of a plastic micropestle to release nuclei from the tissue. The pestle was rinsed with 50 μl of buffer and the solution added to the tube containing the nuclear suspension and cell walls. The cell wall debris was collected at the bottom of the tube by gentle tapping at 1 *g*. The supernatants of two tubes were combined and centrifuged at 2°C at 300 *g* for 10 min. The supernatant was removed leaving ~50 μl to cover the bottom of the tube, 100 μl of pronase (1 mg/ml, 100 mM NaCl, 10 mM Tris pH 7) was added to each tube to give a final concentration of buffer of 0.066 M and the mixture allowed to incubate at 21°C for 1 h. SDS and EDTA were added to give concentrations of 0.6% and 10 mM, pH 7.4, respectively. The solutions were mixed by tilting the tubes to a horizonal position once and allowed to stand at room temperature for 20 min. NaCl was added to give a concentration of 1 M, the solutions were mixed by tilting 10 times and stored at 4°C overnight. The precipitated DNA was pelleted in a microfuge at top speed for 30 min at 4°C, the supernatant discarded, and the pellet dissolved with 500 μl of TE, pH 8.0 at 37°C for 30–60 min. The DNA solutions were combined to a total of 16 ml in a 30 ml Corex tube, extracted with Tris-equilibrated (pH 8.0) phenol twice, with chloroform once and precipitated in 0.3 M Na-acetate, pH 7.0, 67% ethanol at −20°C. The pellet was washed with 80% ethanol cooled to −20°C, dissolved in TE, pH 8.0 and stored at 4°C.

The DNA was digested by restriction enzyme by the addition of enzyme buffer, enzyme and 0.1 mg/ml RNase A and incubation for at least 3 h at 37°C. The reaction was stopped by the addition of EDTA at 20 mM and the fragments were precipitated in 2.5 M NH_4-acetate, pH 7.0, 67% ethanol in a dry-ice ethanol bath. The precipitate was concentrated by lyophilization to *near* dryness and then suspended in TE, pH 8.0.

None of the DNA used in these experiments was centrifuged in CsCl. Two-dimensional gel electrophoresis of DNA banded by centrifugation in a CsCl gradient consistently failed to produce an upper arc of slowly migrating replicative rDNA intermediates.

Gel electrophoresis

Neutral–neutral two-dimensional gel electrophoresis was done according to Brewer and Fangman (1987) with minor modifications. In the first dimension, DNA fragments were separated in 6.5 × 10 cm, 30 ml 0.5% agarose gels dissolved in TBE buffer (Maniatis *et al.*, 1982) at 0.66 V/cm for 19.5 h. DNA samples were heated for 3 min at 65°C before loading and forced in the gel for 5 min at 6.7 V/cm before lowering the voltage gradient to 0.66 V/cm. The well size was 3 × 1 × 4 mm (l × w × h) and about 0.2–0.4 μg of DNA was loaded per well. In the second dimension, the excised lane was rotated 90° and 75 ml of 1.5% agarose dissolved in TBE buffer was poured around the isolated lane. The solidified gel was immersed in 4°C running buffer (TBE containing 0.3 μg/ml ethidium bromide) and allowed to stand for 15 min before separation at 3 V/cm for either 2.5 or 3 h at 4°C.

Neutral–alkaline two-dimensional gel electrophoresis followed the method of Hernàndez *et al.* (1988a) with minor modifications. The first dimension separation was identical to that described above for the neutral–neutral gels. In the second dimension, the fragments were separated at either 0.66 or 1.0 V/cm for 20 h. Under alkaline conditions, the gels were never exposed to sources of UV light, including standard fluorescent lights.

Photographs of the gels, in all cases, were taken just prior to Southern blotting and molecular standards, *Hin*dIII fragments of lambda DNA plus *Hae*III fragments of φX174 DNA, were used in both the first and second dimension gels.

Digestion by S1 nuclease

Gel lanes containing *Eco*RI fragments, separated in the first dimension, were equilibrated for 1 h with three changes of S1 buffer and placed in 50 ml of buffer at 37°C. Enzyme (13 units/ml) was added to the solution containing the treated lane and none to the control lane. Both lanes were incubated for 30 min at 37°C and washed twice for 15 min with TBE buffer at room temperature before casting the second dimension neutral gel as described above.

Southern blot hybridization

The procedure used for Southern blots was that of Hernández *et al.* (1988a).

Subcloning

The probes used in the present work were 0.790, 1.3 and 1.6 kb inserts, respectively, from plasmids pEE790, pHB1.3 and pEB1.6. pEE790 and pHB1.3 are subclones of the plasmid pEB2.8 obtained from the 9 kb rDNA of pea cv. Alaska. pEB2.8 contains the replication origin and two termination sites in the spacer region and ~0.5 kb of the 5′-end of the coding region (Figure 1). pEE790 has subrepeat sequences and the replication origin and was obtained by adding an *Eco*RI linker to the *Mlu*I site at the 3′-end of the origin (Hernández *et al.*, 1988b). pHB1.3 was obtained from the *Hin*dIII–*Bam*HI fragment of pEB2.8. pEB1.6 was obtained from pHA1 (Jorgensen *et al.*, 1987), an 8.8 kb rDNA clone, by subcloning. pEB1.6 contains a segment of the 18S and the 5.8S genes.

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