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Conserved features in the mode of replication of eukaryotic ribosomal RNA genes

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It was previously shown that a 1.5 kb fragment located in the non-transcribed spacer (NTS) is the earliest replicating region of pea (*Pisum sativum*) rDNA in synchronized root cells. In the present report the structure of this region was characterized. It contains a cluster of four 11 bp near matches to the *Saccharomyces cerevisiae* ARS consensus sequence (ACS). These near matches are embedded in an A+T rich domain located upstream from the transcription initiation site. We identified and mapped an intrinsic DNA bending locus 5′ to the cluster of near matches. Several eukaryotic origins including the ARS from the budding yeast show very similar structural features. This observation strengthens the notion that pea rDNA replication initiates at or near this region. Replication of the entire pea rDNA repeat was analysed by two-dimensional (2D) agarose gel electrophoresis. The results obtained indicate that only a small fraction of the potential origins is used in each replication round. Forks moving in the direction opposite to rRNA transcription are stalled at a polar replication fork barrier (RFB), which mapped near the 3′ end of the transcription unit. Consequently, most of pea rDNA appears to replicate in a unidirectional manner. These results show that the strategy used to replicate pea and yeast rRNA genes is very similar, suggesting that it has been conserved and might be common to most eukaryotes.

Key words: Pisum sativum/rDNA/replication fork barrier/replication origin/two-dimensional agarose gel electrophoresis

Introduction

Initiation of DNA replication in eukaryotic chromosomes occurs at numerous discrete sites along parental DNA (Huberman and Riggs, 1968). In yeast, there is evidence that these sites, called replication origins, are specific DNA sequences. Some but not all genomic DNA fragments from yeast confer autonomous extrachromosomal replication to the plasmids containing them. Autonomously replicating sequences (ARSs) isolated by this plasmid assay share several structural features (Campbell and Newlon, 1991). They are 100–200 bp long sequences containing a highly conserved 11 bp core consensus sequence (ACS) (A/TTTTATA/GTTTA/T). This ACS is part of a 15 bp sequence called domain A (Marahrens and Stillman, 1992). ACS is indispensable for ARS function and it is known to be a binding site for protein factors (Bell and Stillman, 1992; Diffley and Cocker, 1992 and references therein). Domain A is flanked 3′ to the T-rich strand of the ACS by a less conserved sequence (domain B), which is also required for an efficient ARS activity. Domain B is an A+T rich sequence that contains several near matches to the ACS (Palzkill and Newlon, 1988). Two-dimensional (2D) agarose gel electrophoresis led to the demonstration that ARS elements serve as true plasmid replication origins. Some but not all of them also do so in their original chromosomal context (reviewed by Fangman and Brewer, 1991; Deshpande and Newlon, 1992). Some genomic DNA fragments from other organisms, including plants, also function as ARSs in yeast (Stinchcomb *et al.*, 1980). There is no evidence, however, that those sequences that behave as ARSs in yeast also function as origins of replication in their native context.

Contrary to the situation in yeast, the nature and organization of chromosomal replication origins in higher eukaryotes is still poorly understood (Fangman and Brewer, 1992). Some recently developed techniques for origin mapping suggest that initiation of DNA replication takes place also at a short specific DNA region within the dihydrofolate reductase (dhfr) domain in CHO cells (Burhans *et al.*, 1990; Vassilev *et al.*, 1990). On the other hand, 2D gels showed bubble-containing replicative intermediates (RIs) along a broad zone within the dhfr domain (Dijkwel and Hamlin, 1992 and references therein). This latter observation suggests that initiation can actually occur at multiple locations in higher eukaryotes. The nature of this apparent contradiction is still unknown. Supporting the hypothesis for random initiation, substitution of the dyad region of an Epstein–Barr virus (EBV)-derived plasmid by almost any large enough (12 kb or more) human DNA fragment, promotes autonomous replication of the recombinant plasmid in human cells (Heinzel *et al.*, 1991). Furthermore, replication initiates at multiple sites in at least one of these plasmids (Krysan and Calos, 1991).

Replication of the ribosomal RNA genes (rDNA) has been studied using different approaches in a variety of species. Electron microscopy revealed that in *Tetrahymena* (Cech and Brehm, 1981), *Physarum* (Vogt and Braun, 1977), yeast (Saffer and Miller, 1986), *Xenopus* larvae (Bozzoni *et al.*, 1981), sea urchin embryos (Botchan and Dayton, 1982) and *Drosophila* embryos (McKnight *et al.*, 1978) initiation of rDNA replication takes place at the non-transcribed spacer (NTS). *In vivo* preferential labelling also indicates that replication of *Xenopus* rDNA initiates at the NTS in synchronized cultured cells (Bozzoni *et al.*, 1981). On the contrary, replication of plasmids containing one rDNA repeat from *Xenopus* appears to initiate at random locations both in microinjected eggs and *in vitro* (Hyrien and Méchali, 1992; Mahbubani *et al.*, 1992). Moreover, initiation events are not restricted to the rDNA sequences but occur also throughout vector DNA sequences. These results support

the notion that almost any DNA molecule injected in *Xenopus* eggs replicates with an efficiency that depends only on the size of the molecule (Harland and Laskey, 1980). Consistently, replication of histone gene repeats also seems to initiate at multiple locations in *Drosophila* embryos (Shinomiya and Ina, 1991). In all the latter cases, random initiation might be due to a hypothetical abundance of the factors required to initiate DNA replication in rapidly dividing embryonic cells. As indicated above, however, specific initiation within the NTS has been reported in the rDNA of *Drosophila* and sea urchin embryos (McKnight *et al.*, 1978; Botchan and Dayton, 1982).

In yeast, the rDNA origin of replication was recently mapped at or near an ARS present in the NTS2 (Linskens and Huberman, 1988; Fangman and Brewer, 1991). These studies also revealed the presence of a replication fork barrier (RFB) near the 3′ end of the yeast RNA ribosomal genes. This barrier stops only forks moving in the direction opposite to rRNA transcription (Brewer and Fangman, 1988; Linskens and Huberman, 1988).

In pea, a 1.5 kb *Eco*RI−*Hin*dIII fragment located within the NTS is the earliest replicating rDNA fragment in synchronized root cells (Hernández *et al.*, 1988a). This observation suggests that the replication origin of pea rDNA also resides within the NTS. The principal aims of the present work were to characterize this putative replication origin region and to analyse replication of the complete pea rDNA repeat using 2D agarose gel electrophoresis.

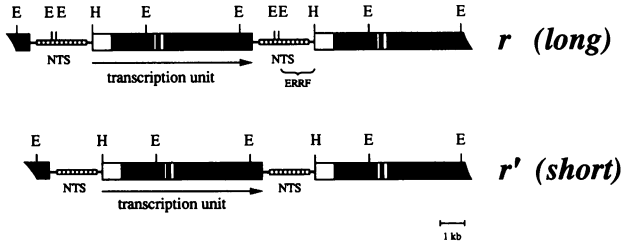

Fig. 1. Organization of the two size-class repeats of pea rDNA. Each tandem repeat comprises a transcription unit (arrow) and a non-transcribed spacer (NTS). The NTS contains an array of 11 (*r* size class) or nine (*r′* size-class) imperfect sub-repeats of ~180 bp each (open small boxes). This variability accounts for the size heterogeneity. Black boxes within the transcription units represent the regions coding for the 18S, 5.8S and 25S mature rRNAs. There is a single *Hin*dIII (H) site per repeat, close to the transcription initiation site. The *r* repeats (9.0 kb long) contain four *Eco*RI cleavage sites (E). Among them two located in the NTS are missing in the *r′* repeats (8.6 kb long). The earliest replicated restriction fragment (ERRF), which was proposed to contain the putative replication origin (Hernández *et al.*, 1988a), is also indicated.

Results

Ribosomal RNA genes (rDNA) of *Pisum sativum* cv. Alaska consist of ~3900 repeat units arranged in tandem (Ingle *et al.*, 1975). Each repeat contains a transcription unit for the rRNA precursor and a non-transcribed spacer (NTS). There are two major size-classes of rDNA repeats in pea: a large size-class of 9.0 kb (*r*) and an 8.6 kb small size-class (*r′*) (Figure 1). The NTS of the *r* repeats contains an array of 11 imperfect sub-repeats of ~180 bp each, whereas

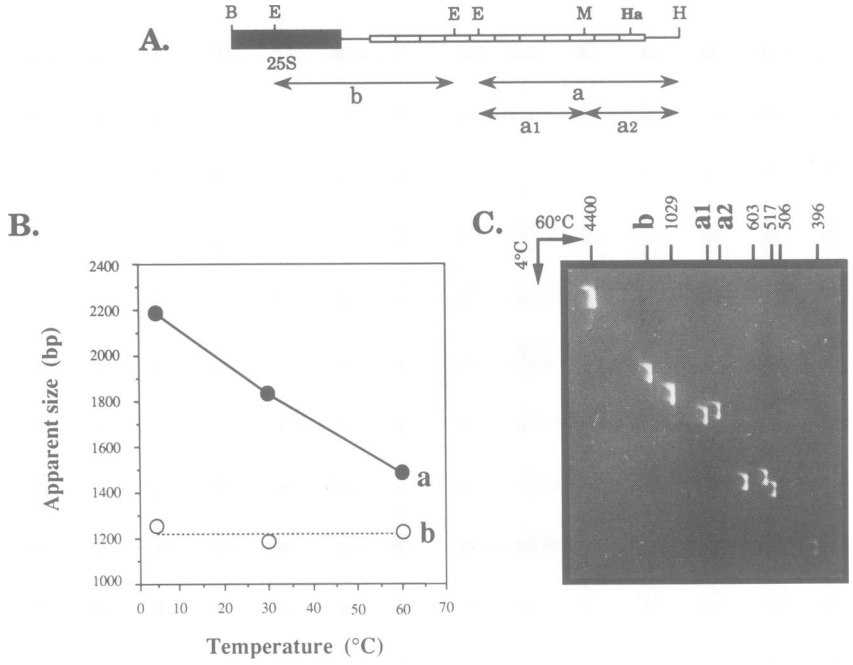

Fig. 2. Analysis of DNA bending in a restriction fragment containing the complete NTS of pea rDNA. (**A**) Restriction map of the *Bam*HI−*Hin*dIII fragment containing the complete NTS corresponding to the *r* repeats. The fragments analysed in this study are indicated below the map (double-headed arrows). B, *Bam*HI; E, *Eco*RI; M, *Mlu*I; Ha, *Hae*III. Only the *Hae*III site nearest to the 3′ end is indicated. (**B**) A plasmid containing the complete *Bam*HI−*Hin*dIII fragment (pHB3.3) was digested with *Eco*RI+*Hin*dIII and electrophoresed at 4, 30 and 60°C on 5% polyacrylamide gels. The apparent size of restriction fragments a and b from the insert was determined and plotted against the temperature of electrophoresis. (**C**) The plasmid pHB3.3 was further digested with *Mlu*I and analysed by 2D PAGE. The first dimension (left to right) was run at 60°C and the second dimension at 4°C (top to bottom). Before loading, the sample was mixed with restriction fragments generated by digestion of pBR322 with *Hin*fI+*Hin*dIII, which were used as internal markers. The size of these markers in bp and their position at the end of the first dimension are indicated along with the position of fragments a1, a2 and b derived form the insert. The largest ~4400 bp fragment consists of vector plus a small *Bam*HI−*Eco*RI fragment located at the 5′ end of the insert.

the NTS corresponding to the *r′* repeats has only nine of these sub-repeats. This variability in the number of sub-repeats accounts for the size heterogeneity. In addition, the *r* repeats have two *Eco*RI cleavage sites, one in each of two contiguous sub-repeats. These *Eco*RI sites are absent in the *r′* repeats (Figure 1). The *r* size-class is assigned to chromosome 4 and the *r′* class to chromosome 7. These locations correspond to those of pea nucleolar organizer regions (Piller *et al.*, 1990).

The DNA fragment containing the putative replication origin of pea rDNA is intrinsically bent and contains a cluster of near matches to the yeast ARS consensus sequence

Intrinsic DNA bending is closely associated with several replication origins (Caddle *et al.*, 1990 and references therein). To find out whether this is also the case in pea rDNA, we looked for DNA bending in a recombinant plasmid (pHB3.3) containing the complete NTS and the 3′ end of the coding region (Figure 2A). This plasmid was digested with *Eco*RI and *Hin*dIII and the apparent size of the two larger restriction fragments corresponding to the insert (Figure 2A, a and b) was determined in polyacrylamide gels run at different temperatures. A hallmark of bent DNA fragments is that they show low electrophoretic mobility in polyacrylamide gels run at low temperatures (Koo *et al.*, 1986). Only fragment a, which contains the putative replication origin, showed an increasing apparent size as the electrophoresis temperature decreased (Figure 2B). To map the bent locus more precisely, fragment a was further digested with *Mlu*I resulting in two restriction fragments: a1 and a2 (Figure 2A). Fragment a1 contains only sequences corresponding to the sub-repeats. Fragment a2, on the other hand, consists of the 3′ end of the sub-repeat array and the NTS sequence that separates the sub-repeats from the transcription initiation site. Initiation of transcription actually takes place 4 bp downstream from the *Hin*dIII restriction site (Piller *et al.*, 1990). These two fragments were analysed by 2D PAGE (Figure 2C). Before loading, *Hin*fI+*Hin*dIII digested pBR322 was added to the sample as an internal standard. The first dimension took place at a high temperature (60°C) to minimize the anomalous mobility of bent DNA fragments. The second dimension was run at a lower temperature (4°C). Under these conditions bent DNA restriction fragments move more slowly during the second as compared with the first dimension, while non-bent DNA fragments show a similar behaviour in both dimensions. Consequently, at the end of the 2D electrophoresis, bent DNA fragments are detected above a diagonal formed by non-bent fragments (Mizuno, 1987). As shown in Figure 2C, fragment a2 clearly exhibited a lower mobility during the second as compared with the first dimension, indicating that it is bent. Fragment a1, on the other hand, showed almost the same mobility in both dimensions. Although at 60°C (first dimension) fragment a2 run faster than fragment a1, due to its smaller size, at 4°C (second dimension) their relative mobilities were reversed. The 517 and 506 bp pBR322-*Hin*fI restriction fragments also showed low

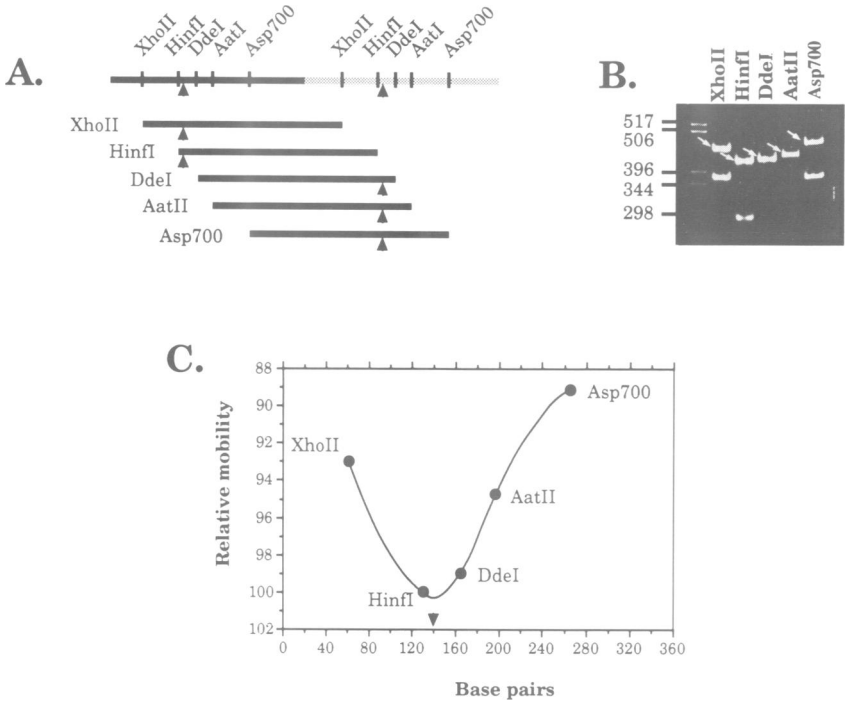

Fig. 3. Mapping of the centre of bending within the 356 bp *Hae*III−*Hin*dIII restriction fragment by circular permutation analysis. (**A**) The bent *Hae*III−*Hin*dIII fragment showed in Figure 2A was cloned as a tandem dimer in pUC18 (see Materials and methods for details). The cleavage sites for restriction enzymes that cut once in each element of the dimer are indicated. The dimer was isolated and separately digested with these enzymes. The permuted fragments generated are represented by black lines below the map of the dimer. (**B**) The relative mobility of each permuted fragment, pointed by white arrows, was determined by electrophoresis in a 5% polyacrylamide gel run at 4°C. The first lane of the gel corresponds to size markers (*Hin*fI digest of pBR322). (**C**) The relative mobility of each permuted fragment was plotted against the restriction site in one element of the dimer. The relative mobility was estimated as a percentage of the distance migrated by the *Hin*fI fragment that showed the highest mobility. The inflexion on the curve points to the position (arrow head) where the bending is centred (Wu and Crothers, 1984). This position is also indicated in (A) by arrowheads.

electrophoretic mobility during the second dimension. They served as an internal control since it is well known that these fragments are bent (Stellwagen, 1983).

The pea rDNA bending was further mapped to the *Hae*III−*Hin*dIII fragment using the same analytical procedure. This fragment covers the 3′-half of the a2 fragment (Figure 2A). The bending centre within this fragment was determined by the circular permutation method of Wu and Crothers (1984). This method is based on the observation that electrophoretic mobility decreases as the centre of bending approaches the centre of the fragment. A plasmid containing a tandem dimer of the *Hae*III−*Hin*dIII segment was constructed (see Materials and methods). This dimer was isolated and cleaved with restriction enzymes that cut only once per monomer, to produce a family of circularly permuted molecules of identical size (Figure 3A). These molecules, that differ only in the position of the bending centre, were analysed in a polyacrylamide gel run at 4°C. The relative mobility of each of these permuted molecules (pointed by arrows in Figure 3B) was plotted against the position of the restriction site within the fragment (Figure 3C). The lowest point on the curve corresponds to the bending centre (Wu and Crothers, 1984). It mapped around position 140 of the *Hae*III−*Hin*dIII fragment (Figure 3C), which is 20 bp downstream from the 3′-end of the sub-repeat array and 216 bp upstream from the *Hin*dIII site.

A radioactively labelled synthetic 11 bp oligomer corresponding to the yeast ACS specifically hybridizes to the 1.5 kb *Eco*RI−*Hin*dIII fragment of pea rDNA (Hernández *et al.*, 1988a). We determined the nucleotide sequence of this fragment by the dideoxy method (Figure 4). It consists of seven imperfect elements of the sub-repeat array covering the 5′ 1221 bp of the fragment (Figure 4, large box) and a unique 236 bp sequence that separates the sub-repeats from the 5′-end of the coding region. Sequence homology searching revealed the presence, in the latter unique sequence, of a cluster of four near matches (two 10/11 and two 9/11) to the 11 bp yeast ACS (Figure 4, small boxes). The centre of bending maps ~30 bp upstream from the first near match (Figure 4, thick line). These near matches are embedded in a block of 135 bp sequence (nucleotides 1272−1406) that is 73% A+T rich.

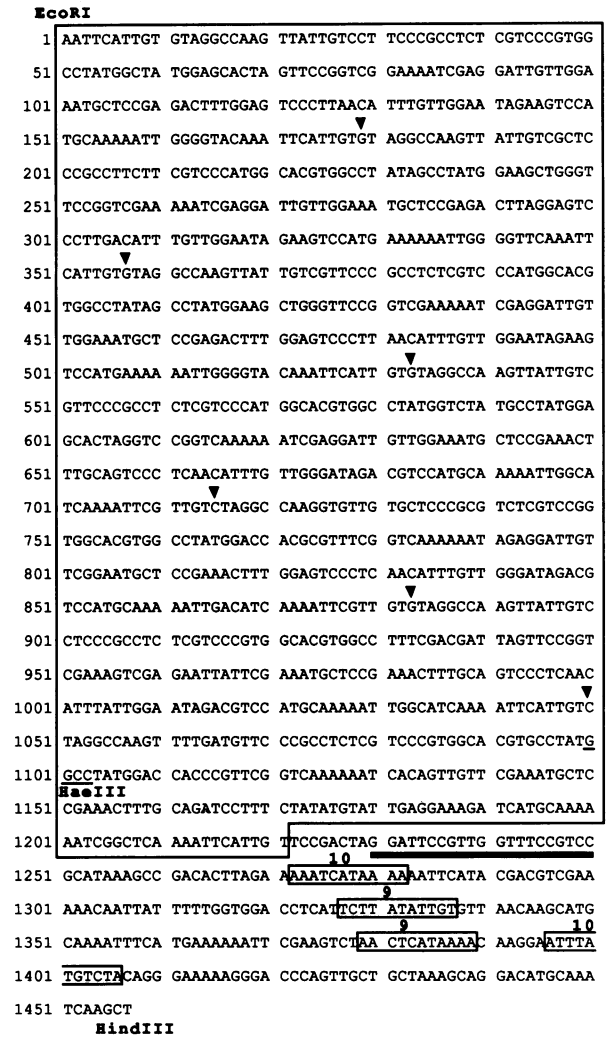

Fig. 4. Nucleotide sequence of the *Eco*RI−*Hin*dIII fragment containing the putative replication origin of pea rDNA. The large box contains the NTS sub-repeats separated by arrowheads. The remaining corresponds to a unique sequence where four near matches to the yeast 11 bp ACS were found (small boxes). Numerals above each small box indicate the number of matches. The position of the centre of bending is indicated by a thick horizontal black line. Only the *Hae*III restriction site nearest to the *Hin*dIII end is indicated.

Initiation of DNA replication is an infrequent event in each repeat of pea rDNA

Replication of pea rDNA was analysed using the 2D neutral/neutral agarose gel electrophoresis procedure developed by Brewer and Fangman (1987). This technique allows identification of the shape of RIs corresponding to any specific restriction fragment. In short, DNA is digested with the selected restriction enzyme(s) and the resulting fragments are separated predominantly according to their mass in the first dimension, which is run in a low percentage agarose gel at low voltage. The second dimension maximizes the effect of retardation caused by the molecular topology of RIs. This second dimension is run in a high percentage agarose gel at high voltage. Once electrophoresis is completed, the gel is blotted and then hybridized with a probe specific for the fragment to be analysed. After the first dimension, the RIs corresponding to any given DNA fragment are distributed between the positions where non-replicating linear forms (1×) and molecules of double its mass (2×) have moved. After the second dimension, due to the low mobility caused by their shape, RIs move above the diagonal corresponding to non-replicating linear fragments. Unique hybridization patterns are obtained depending on how the fragment is replicated: by a single fork moving from one end to the other (simple Y pattern), by a bidirectionally growing bubble initiated at the centre of the fragment (bubble pattern) or by two forks moving in opposite directions that meet at the centre of the fragment (double Y pattern). Composite patterns also can be found if the fragment replicates from an origin asymmetrically located within the fragment (bubble-to-simple Y composite pattern) or by two forks entering the fragment asynchronously (simple Y-to-double Y composite pattern).

If initiation of DNA replication occurs at a specific site in every rDNA repeat, a bubble pattern would be the only one generated by a restriction fragment where the origin is symmetrically centred. If not all the potential origins are used, on the other hand, some molecules would lack an active origin and would be replicated passively by a single fork

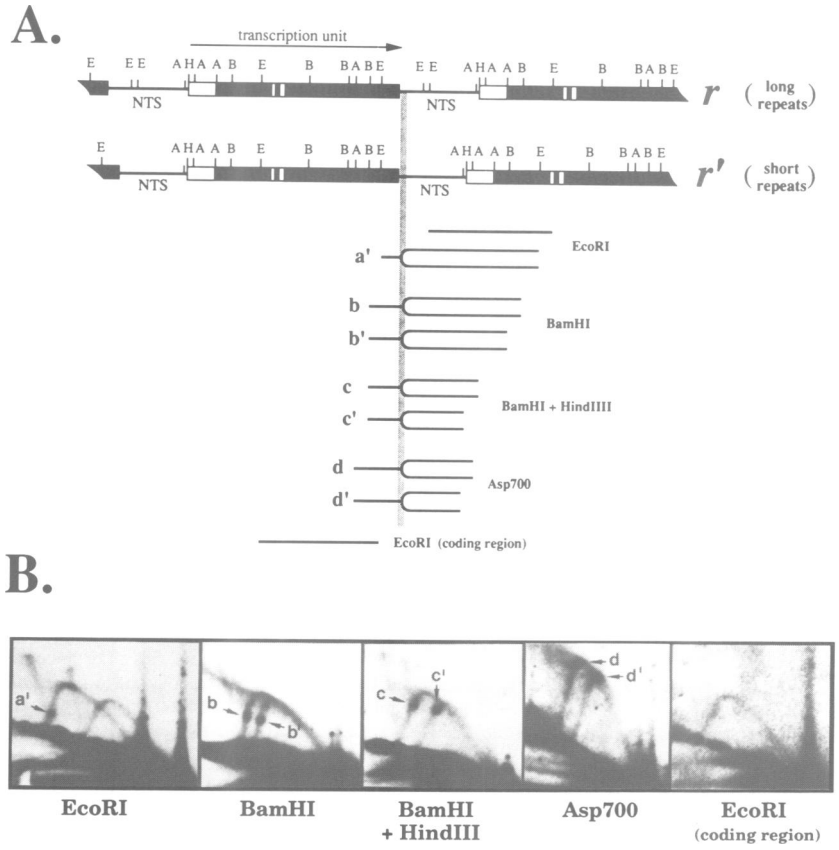

Fig. 5. Analysis of pea rDNA RIs by 2D agarose gel electrophoresis. (**A**) Restriction maps corresponding to the two size-class repeats (*r* and *r'*) of pea rDNA are shown at the top. The sites for the restriction enzymes used in the study are indicated (E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; A, *Asp*700). The 3' end of the transcription unit of both size-classes have been arbitrarily aligned. The restriction fragments analysed in each 2D gel are represented below the maps with the name of the enzyme(s) used to generate them on the right. Those fragments containing the RFB (vertical grey bar) are drawn as Y structures and named by a letter on the left. Due to the size heterogeneity of the NTS, restriction fragments spanning this region show two different sizes. The long ones (b, c and d) derive from the *r* repeats and the small ones (b', c' and d') from the *r'* repeats. An exception occurs when *Eco*RI is used. The two *Eco*RI sites present in the NTS of the *r* repeats are missing in the *r'* repeats. Therefore, among the two *Eco*RI fragments analysed from the NTS the longest one (a') derives from the *r'* repeats. (**B**) 2D gel autoradiograms corresponding to the pea rDNA regions diagrammed above. Pea DNA from asynchronously dividing root-cells was digested with the enzyme(s) indicated below each autoradiogram. For the probes used see Materials and methods. Arrows indicate the accumulated simple Y RIs with the fork stalled at the RFB.

initiated elsewhere. As a consequence, both a simple Y and a bubble pattern would be obtained in the same autoradiogram (Schvartzman *et al.*, 1990; Martín-Parras *et al.*, 1991). The relative intensity of these two signals will depend on the frequency of origin usage (Martín-Parras *et al.*, 1992). The bubble pattern might not even be detected if activation of the potential origins is a rare event.

Intact pea chromosomal DNA from asynchronously growing root-cells was obtained from isolated nuclei embedded into agarose plugs. The embedded DNA was digested with the indicated restriction enzyme(s) and the restriction fragments were separated in 2D gels. After transfer to nitrocellulose, specific probes were used to investigate the RIs of a series of overlapping restriction fragments covering the entire rDNA repeat. Figure 5A (upper part) shows a restriction map of the two major size-classes of pea rDNA units. The NTS of the *r* repeats is ~350 bp longer than the NTS corresponding to the *r'* repeats. Therefore, those restriction fragments containing the NTS are doublets. In each doublet, the larger fragment arises from the *r* repeats while the shorter one derives from the *r'* repeats. An exception occurs when *Eco*RI is used. The NTS of the *r* repeats contains two *Eco*RI cleavage sites located very close to each other. These sites are absent in the NTS of the *r'* repeats. Therefore, here the largest *Eco*RI fragment derives from the *r'* repeats and contains the complete NTS. On the other hand, the coding region generates single-sized restriction fragments since no heterogeneity exists within this region.

In the 2D gel autoradiogram shown at the bottom left of Figure 5B, RIs of the *Eco*RI fragments containing the NTS were analysed. The probe used detected two fragments of 4.9 and 3.8 kb, respectively. The 4.9 kb fragment derived from the *r'* repeats and contained the complete NTS including both ends of the transcription unit. The 3.8 kb fragment arose from the *r* repeats and lacked the 3' end of the coding region as well as part of the NTS next to it (Figure 5A). Two arcs of RIs were visible above the diagonal corresponding to linear molecules. These arcs merged from the linear forms of each *Eco*RI fragment and returned to the diagonal of linear molecules with duplicated masses. These arcs correspond to simple Y patterns indicating that these fragments were primarily replicated by a single fork that proceeded from one end to the other. Two more signals were also visible. They appeared as spikes rising from the point where the simple Y arcs returned to the diagonal of linear molecules (Figure 5B, *Eco*RI). These signals correspond to X-shaped recombination intermediates.

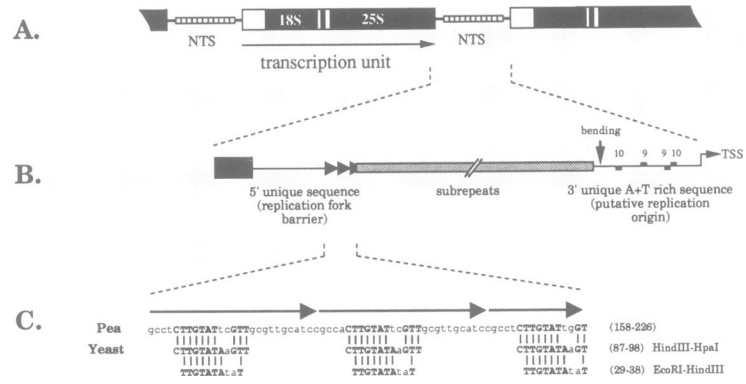

Fig. 6. Structural features of pea rDNA involved in replication. (**A**) Organization of pea rDNA repeats. (**B**) Structure of the NTS. The 5′ unique sequence separates the end of the transcription unit from the sub-repeat array. This region contains the RFB. Adjacent to the sub-repeats, there are two complete and one truncated 26 bp direct repeats (arrowheads) (Piller *et al.*, 1990). The 3′ unique sequence separates the sub-repeat array from the transcription start site (TSS). This region is suggested to contain a weak DNA replication origin. It is a bent A+T rich sequence (the centre of bending is indicated by the arrow) containing four near matches to the yeast ACS represented by black boxes. The position of the box above or below the line indicates the position of the T rich strand in the upper or the lower strand of the sequence, respectively. The number above each box indicates the number of bp that match the 11 bp ACS. (**C**) Sequence homologies found between yeast and pea RFBs. The upper sequence corresponds to the direct repeats in the 5′ unique sequence of pea rDNA. The position of the first and last nucleotides counted from the end of the 25S coding region are indicated in parenthesis (according to Piller *et al.*, 1990). The middle sequence below each repeat corresponds to a unique 12 nucleotides from the *Hin*dIII−*Hpa*I yeast rDNA fragment where replicating forks are stalled (Brewer *et al.*, 1992; Kobayashi *et al.*, 1992). The positions of the first and last nucletoides of this sequence from the *Hin*dIII site are indicated in parenthesis. The bottom sequence corresponds to another unique 11 nucleotides from the *Eco*RI−*Hin*dIII yeast rDNA fragment located upstream and adjacent to the *Hin*dIII−*Hpa*I fragment (from Elion and Warner, 1984). It has been proposed that this *Eco*RI−*Hin*dIII fragment plays a role in fork arrest in yeast (Brewer *et al.*, 1992). The positions of the first and last nucleotides of this sequence from the *Eco*RI site are indicated in parenthesis. Matches are denoted in upper case letters.

All the other restriction fragments analysed also generated signals corresponding to simple Y patterns (Figure 5B). As expected, the *Eco*RI fragment from the transcription unit gave a single simple Y arc due to the size homogeneity within that region (Figure 5B, *Eco*RI, coding region). Longer exposure times failed to detect any complete or even partial 2D hybridization signal corresponding to RIs containing an internal bubble (data not shown). Therefore, initiation of DNA replication seems to be a very rare event in each repeat of pea rDNA.

Replication forks moving in the direction opposite to transcription are stalled at a barrier near the 3′ end of the transcription unit

A conspicuous spot was detected over the simple Y arc generated by the largest *Eco*RI fragment (***a′*** in Figure 5B, *Eco*RI). This *Eco*RI fragment derives from the ***r′*** repeats and contains the complete NTS as well as both ends of the transcription unit (Figure 5A). The spot appeared close to the point where the simple Y arc ends at the diagonal formed by linear molecules. It was produced by an accumulated RI containing a single fork positioned at a specific site. This site, therefore, functions as a replication barrier where forks are stalled. Based on its mobility in the first dimension, the estimated mass for this accumulated intermediate was 9.1 kb (1.86× the mass of the linear form). Since the linear fragment is 4.9 kb, this means the fork stopped once it replicated 4.2 kb of the fragment. Therefore, the barrier should be located 0.7 kb apart from either end of the fragment. If it were located at the right end of the fragment it would block forks moving rightward, whereas if it were at the left end it would be active on leftward moving forks. The smaller 3.8 kb *Eco*RI fragment that derived from the ***r*** repeats does not contain a barrier, since no spot of RIs appeared over the simple Y arc generated by this fragment (Figure 5B, *Eco*RI). This 3.8 kb fragment is similar to that one coming from the ***r′*** repeats but lacks ~1.5 kb on the left end that includes the 3′ end of the transcription unit and part of the NTS (Figure 5A). This observation strongly suggests that the barrier maps 0.7 kb apart from the left end of the 4.9 kb *Eco*RI fragment. Therefore, the accumulated RI would be due to blockage of the leftward moving fork. The position of the barrier would then map in the NTS ~200 bp apart from the 3′ end of the transcription unit in both size-classes (Figure 5A, grey vertical bar). If this hypothesis is correct, the position of the spot would vary along the simple Y arc depending on the relative position of the barrier along the restriction fragment. In addition, the spot of accumulated RIs should be visible on the simple Y arcs generated by both size-class fragments whenever they span the barrier. The series of overlapping fragments covering the complete NTS analysed in Figure 5 varied in their endpoints relative to the position of the presumptive RFB. The results obtained satisfied all the expectations. Both simple Y arcs generated by each of the two *Bam*HI fragments containing the NTS showed a spot corresponding to accumulated intermediates (b and b′ in Figure 5B, *Bam*HI). As expected, the relative position of each of these spots is slightly closer to the inflection point of the simple Y arc than in the *Eco*RI digest. The relative masses estimated for these accumulated intermediates fit well with the expected ones: 1.78× and 1.76×, respectively. Moreover, both *Bam*HI−*Hin*dIII fragments produced spots even closer to the inflection point (c and c′ in Figure 5B, *Bam*HI+*Hin*dIII). Replication of each *Asp*700 fragment generated a spot located almost at the inflexion point of the simple Y arc (d and d′ in Figure 5B, *Asp*700). Finally, the single *Eco*RI fragment corresponding to the coding region produced no spot of accumulated intermediates (Figure 5B, *Eco*RI, coding region).

These results proved that both size-classes of pea rDNA repeats contain an RFB in the NTS near the transcripton termination site. This barrier blocks only leftward moving forks, since the putative spots corresponding to accumulated

intermediates for rightward moving forks were absent. Therefore, the barrier is specific for forks moving in the direction opposite to transcription, impeding them from entering the transcription unit. Consequently, most of pea rDNA appears to replicate unidirectionally.

Discussion

We determined the DNA structure of the region containing the putative replication origin of pea rDNA (Hernández *et al.*, 1988a). We also studied replication of the entire rDNA repeat by 2D agarose gel electrophoresis. Within the putative replication region, we found an A+T rich 135 bp sequence containing a cluster of four near matches to the yeast ACS. Yeast ARSs are also A+T rich sequences containing several matches to the ACS. Deletion analyses indicate that these multiple matches are important for ARS funetion (Palzkill and Newlon, 1988). However, other experimental results showed that only the match contained in domain A is critical for ARS function and the other near matches stimulate ARS activity simply becuase they are easily unwound (Natale *et al.*, 1992 and references therein). Unwinding of this region would permit the entry of replication enzymes into the DNA helix. The coincidence of sequence features with yeast ARSs supports the idea that pea rDNA replication might initiate by a similar mechanism within the region of the NTS mentioned above.

We found intrinsic DNA bending in the NTS of pea rDNA centred ~30 bp usptream from the cluster of near matches. Yeast ARS1 and other DNA fragments with ARS activity also contain a bending locus (Snyder *et al.*, 1986; Eckdahl and Anderson, 1987; Valenzuela, 1990). Deletion of the natural bending impairs ARS1 activity, which is restored to a near wild type level with synthetic bent DNA (Williams *et al.*, 1988). The role of DNA bending in ARS1 function was recently questioned, however, by Marahrens and Stillman (1992) who were able to separate origin function from DNA bending.

Bent DNA is also present near other putative eukaryotic replication origins (Caddle *et al.*, 1990; Heck and Spradling, 1990). Moreover, several replication initiation binding factors are known to recognize (Zahn and Blattner, 1987), induce (Mukherjee *et al.*, 1985) or enhance (Koepsel and Khan, 1986; Stenzel *et al.*, 1987) DNA bending at several prokaryotic origins. The presence of a DNA bending locus at the putative replication origin of pea rDNA supports the idea that this structural feature may be a *cis*-acting element in at least some eukaryotic replication origins.

In pea rDNA the bent locus, the transcription initiation site and the putative DNA replication origin are all clustered in a 250 bp region (Figure 6B). The proximity of these three elements led us to suggest that they might be functionally related. There is evidence that transcriptional elements can enhance the activation of replication origins (reviewed in DePamphilis, 1988; Heintz *et al.*, 1992). It is also known that DNA bending near transcription initiation sites regulates promoter function in bacteria (Bracco *et al.*, 1989; McAllister and Achberger, 1989; Claveri-Martin and Magasanik, 1992) and probably in eukaryotes as well (Shuey and Parker, 1986; Inokuchi *et al.*, 1988; Schroth *et al.*, 1992). Therefore, the DNA bent locus mapped near the transcription initiation site in pea rDNA might indirectly influence initiation of DNA replication by affecting promoter function. This hypothesis implies that the frequency of origin usage in pea rDNA, which seems to be low (see Results and discussion below), would be related to the level of transcriptional activity of the rRNA genes. The observation that in higher plants only a small fraction (~20%) of the ribosomal genes shows an active open chromatin configuration is consistent with this hypothesis (Conconi *et al.*, 1992).

None of the pea rDNA restriction fragments analysed generated a complete or even a partial hybridization signal corresponding to RIs containing an internal bubble (Figure 5B). The cluster of near matches to the yeast ACS is close to the centre of the 4.9 kb *Eco*RI fragment and within the central third of the 3.8 kb one. Therefore, if bidirectional replication initiates at this region, RIs of these fragments should generate a bubble signal when analysed by 2D agarose gel electrophoresis. The only visible signal generated by RIs from these and all the other fragments that were analysed, however, corresponded to a simple Y pattern (Figure 5B). Since lysis of the nuclei, DNA purification and digestion with restriction enzymes were all carried out in agarose plugs, we believe that the possible breakage of bubble-containing molecules at the forks during sample manipulation (Martín-Parras *et al.*, 1992) was significantly reduced and cannot account for their total absence. Pea rDNA from samples isolated from S-phase synchronized cells and enriched for RIs by column chromatography on benzoylated naphthoylated DEAE-cellulose produced similar results (data not shown). We explain the lack of a bubble signal in the autoradiograms by the low frequency of origin usage in pea rDNA. If only a fraction of the potential origins is activated in each particular S phase, those that are used would replicate a stretch of rDNA that contains several repeats. Consequently, many molecules of a restriction fragment containing the potential origin would be replicated by a single fork initiated several repeats apart. If the frequency of origin usage is low enough, fragments where the origin is active would be so scarce that it would be unlikely to detect bubble-containing RIs in 2D gels. A low frequency of origin usage was also found for yeast rDNA replication (Walmsley *et al.*, 1984; Linskens and Huberman, 1988; Fangman and Brewer, 1991), where a weak ARS maps within the NTS (Skryabin *et al.*, 1984). Analysis of yeast rDNA fragments containing the ARS by 2D gel electrophoresis only detected single fork-containing RIs (Brewer and Fangman, 1988) or a strong signal generated by simple Y RIs together with a very faint bubble signal (Linskens and Huberman, 1988; Fangman and Brewer, 1991). In the case of pea, it should also be considered that in *in vivo* growing root tips, proliferating cells are only a small fraction of the total cell population. Therefore, the percentage of replicating DNA in a given sample is significantly lower than in yeast or cultured mammalian cells.

Based on the intensity of the signal in long exposed autoradiograms we deduced that a bubble pattern at least 10-fold weaker than the simple Y pattern would still be visible. Since bubble-containing fragments are replicated by two moving forks, they are expected to replicate twice as fast as single fork-replicated fragments. Thus, our results suggest that at most one out of five potential replication origins would be functional in each individual replication round of pea rDNA. The specific replicon size of pea rDNA is unknown. If it does not differ much from the average replicon size in pea, which is ~80 kb (Schvartzman *et al.*, 1984), each replicon would accommodate about nine rDNA repeats. Therefore, only one out of nine potential origins

would be active. This is consistent with the frequency of origin usage estimated above. Moreover, initiation of DNA replication in higher plants may occur in a delocalized manner, as already suggested for mammalian cells (Dijkwel and Hamlin, 1992 and references therein). This would make the detection of a discrete bubble pattern even more difficult.

A displacement loop model has been recently proposed for replication of rDNA in pea meristematic root cells (Van't Hof and Lamm, 1991). According to this model, pea rDNA would replicate through single-stranded RIs. In the present study we found no evidence suggesting such a mode of replication. Two-dimensional gels of all fragments analysed showed simple Y arcs arising from the linear form of each fragment. These signals had an inflection at a position corresponding to 1.5× the mass of the linear fragments and they ended at the diagonal corresponding to linear fragments with a duplicated mass. Therefore, in contrast to what would be expected according to the displacement loop model (Van't Hof and Lamm, 1991), both parental strands appeared to be simultaneously copied. The model proposed by Van't Hof and Lamm (1991) is based on the detection of three spots corresponding to rDNA molecules showing low mobility during the second dimension of neutral/neutral 2D agarose gel electrophoresis. We did not find these hybridization signals in our 2D gel analysis. One possible explanation for the discrepancy between our results and those by Van't Hof and Lamm (1991) is that during the DNA isolation procedure followed by these authors, samples were somehow enriched for rare rDNA molecules that generated the spots mentioned above. Extrachromosomal DNA might be the source of these molecules since rDNA is included in this DNA fraction (Kraszewska *et al.*, 1985) and replicates by strand displacement (Krimer and Van't Hof, 1983). In addition, we have noticed that standard isolation of DNA by phenol extraction of lysed nuclei leads to significant degradation of RIs. This problem is avoided when nuclei embedded into agarose plugs are used (see Materials and methods).

A relevant observation of this work is the presence of a polar RFB in the NTS close to the transcription termination site of pea rDNA. An RFB was also found within the rDNA repeat of *Saccharomyces cerevisiae* (Brewer and Fangman, 1988; Linskens and Huberman, 1988). In both species the barrier maps near the 3′ end of the transcription unit and blocks only leftward moving forks, preventing them from entering into the coding region in the direction opposite to transcription. These striking coincidences suggest that (i) the mechanism of fork blockage may be similar in these two distant organisms, (ii) such a barrier could be a common feature for rDNA replication in all eukaryotes and, therefore, (iii) it probably plays an important physiological role.

Two specific single-strand gaps or nicks were detected in the rDNA of synchronized pea root cells (Hernández *et al.*, 1988b). These discontinuities, whose locations do not coincide with the RFB mapped in the present work, were suggested to be related to termination of DNA replication. This interpretation was based on the observation that they were preferentially detected in the rDNA of cells positioned at the S/G_2 boundary and early G_2 phase (Hernández *et al.*, 1988b). Joining of replicon-size nascent DNA to give mature DNA is known to occur precisely at this stage (Schvartzman *et al.*, 1981, 1984). However, the fact that these nicked restriction fragments showed the mobility expected for linear molecules in agarose gels (Hernández *et al.*, 1988b) suggests that they were not due to replication-fork-containing structures. Their relationship to termination of DNA replication is still an open question.

The nature of the RFB in the rDNA of pea and yeast is still unknown. Fork arrest might be intrinsically induced by the DNA structure at the barrier. Then, a DNA fragment containing the barrier would also be active when replicated in other organisms. We cloned the 3.3 kb *Bam*HI−*Hin*dIII fragment from the *r* size-class of pea rDNA (see Figure 5A) in both orientations in unidirectionally replicated pUC vectors. Analysis by 2D gel electrophoresis showed no accumulation of Y-shaped RIs in either of the two recombinant plasmids replicating in *Escherichia coli* (data not shown). This observation strongly suggests that the RFB is not caused by the DNA structure itself.

It has been shown recently that the arrest of replication forks in yeast rDNA occurs independent of rRNA transcription (Brewer *et al.*, 1992; Kobayashi *et al.*, 1992). This observation proves that fork arrest does not occur as a consequence of collision between the replication machinery and RNA polymerase I (Pol I) travelling in opposite directions. In *E.coli* and *Bacillus subtilis*, head-on collision between RNA polymerase and DNA replication is specifically avoided by the appropriate orientation of actively transcribed genes so that the promoters are proximal to the bidirectional origin of replication (Brewer, 1988; Ziegler and Dean, 1990). In addition, collision is also prevented by polar fork barriers (*Ter* sites) at the *E.coli* replication termination region (Kuempel *et al.*, 1989). The physiological role of the RFB in pea and yeast rDNA might also be to prevent such a collision between replication and transcription, which would have deleterious consequences.

As already suggested for yeast (Brewer *et al.*, 1992; Kobayashi *et al.*, 1992), fork arrest in pea rDNA might be also mediated by one or several *trans*-acting factors that would bind to one or several specific DNA binding sites. Bound sequences would inhibit some activity of the replisome in an orientation-dependent manner. Fork blockage mediated by DNA binding proteins also occurs at yeast centromeres and at the EBV family of repeats. Centromere binding proteins and the EBNA-1 protein, respectively, are responsible for fork pausing in these systems (Gahn and Schildkraut, 1989; Dhar and Schildkraut, 1991; Greenfeder and Newlon, 1992). The best known mechanism for replication fork blockage operates at the *Ter* sites in *E.coli* (reviewed by Kuempel *et al.*, 1989). *Ter* sites are 22 bp binding sequences recognized by the *ter* binding protein (TBP), which is encoded by the *tau*(*ter*) gene. The *Ter*−TBP complex seems to arrest replication forks by obstructing helicase action in an orientation-specific manner. All helicases tested so far from *E.coli*, virus and mammalian cells are inhibited by this complex in a polar manner (Hidaka *et al.*, 1992 and references therein; Lee and Kornberg, 1992).

The fact that the site where forks stall in pea and yeast rDNAs coincides near the 3′ end of the transcription unit, suggests that factor(s) responsible for transcription termination also might be involved in fork arrest. Transcription termination of mouse rRNA genes is mediated by a specific nuclear factor (TTF1) that binds to a repetitive motif (Sal box) at the 3′ end of the intergenic spacer (Grummt *et al.*, 1986). Interestingly, the termination signal is orientation dependent and specific for Pol I. The mechanism of

transcription termination at the human rDNA is similar to the one operating in mouse (Pfleiderer *et al.*, 1990). Similar transcription termination factors operating at pea and yeast rDNA also might be involved in replication fork arrest. It would be interesting to find out whether Sal boxes of mouse and human rDNA function as replication barriers with the opposite polarity as they direct transcription termination.

In yeast, rDNA forks are stalled within the 129 bp *Hin*dIII−*Hpa*I restriction fragment of the NTS1 (Brewer *et al.*, 1992; Kobayashi *et al.*, 1992). We contemplated the possibility that the sequence signal(s) for fork arrest in yeast rDNA could be similar to that in pea rDNA. To test this hypothesis we looked for nucleotide sequence homology between the yeast 129 bp fragment and the 5′ region of the NTS of pea rDNA containing the fork barrier. The DNA sequence of this region has been recently determined (Piller *et al.*, 1990). A sequence of 12 bp from the yeast fragment showed significant homology with a sequence that is tandemly repeated three times in the pea NTS (Figure 6C). These repeats are adjacent to the 5′ end of the subrepeat array (Piller *et al.*, 1990). Brewer and coworkers (Brewer *et al.*, 1992) reported that in their plasmid constructs, this 129 bp *Hin*dIII−*Hpa*I fragment is necessary but not sufficient to achieve an efficient fork arrest. They suggested the possibility for an essential function present in the adjacent 188 bp *Eco*RI−*Hin*dIII fragment. This fragment contains the terminator sequence for transcription by yeast Pol I (van der Sande *et al.*, 1989). A sequence of 10 bp from this fragment (Figure 6C) also shows homology with the sequences mentioned above. Although these sequence homologies may simply be fortuitous, more investigation is clearly needed to determine their possible meaning.

The main conclusion of the present report is that the organization of pea and yeast rDNA for replication shares several important features: (i) a similar structure of the putative replication origin, (ii) a low frequency of initiation events, and (iii) the presence of a polar replication barrier close to the 3′ end of the transcription unit. These similarities between a higher eukaryote and a eukaryotic microorganism raise the possibility that these features were conserved and might be common to the rDNA of most eukaryotes.

Materials and methods

Plant material

Pea seeds (*P.sativum*, cv. Alaska) were supplied by W.Altee Burpee Co., Warminster, PA. Seeds, surface-sterilized with 10% (v/v) sodium hypochloride, were aseptically germinated in Petri dishes on three layers of Whatman no. 1 filter paper moistened with distilled water. After 4 days at 18°C the apical 3 mm from primary roots were excised and subsequently used for nuclei isolation.

Two-dimensional polyacrylamide gel electrophoresis

First dimension was run on a 5% polyacrylamide gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA) at 7 V/cm at 60°C. The gel was then reoriented 90° relative to the first dimension and the second dimension was run under the same conditions except that temperature was maintained at 4°C. DNA fragments were detected by staining the gel with 0.5 μg/ml ethidium bromide dissolved in TBE buffer.

DNA sequencing

Sequencing was performed by the dideoxy method (Sanger *et al.*, 1977) using a Sequenase Version 2.0 sequencing kit (US Biochemical Corp.). Plamid pHB3.3 contains a 3.3 kb *Bam*HI−*Hin*dIII fragment from the long pea rDNA size-class cloned in pBR322 (see Figure 2A) (Hernández *et al.*, 1988a). This fragment consists of the 3′ end of the coding region and the NTS. The *Eco*RI−*Hin*dIII subfragment from the insert in pHB3.3 was isolated, made their ends blunt with the Klenow fragment of DNA polymerase I and subcloned in both orientations in the *Sma*I restriction site of the phagemid pEMBL18(+) (Dente *et al.*, 1985), yielding plasmids pEHE1.5(+) and pEHE1.5(−). A nested set of external deletions of the insert in each of these plasmids was generated by the exonuclease III/nuclease S1 procedure (Promega). The resultant deleted plasmids were propagated in the *E.coli* strain DH5αF′. Single stranded DNA was obtained by superinfection with f1 as helper phage. The universal 17-mer (−40) oligonucleotide was used as primer in the sequencing reactions and [α-^{35}S]dATP as the labelled precursor. Sequencing gels (0.35 mm thick, 6% polyacrylamide, w/v) were run in a Hoefer apparatus following the suppliers' instructions.

Plasmid construction for circular permutation analysis

The bent *Hae*III−*Hin*dIII fragment was isolated from pHB3.3 and cloned in the *Sma*I site of pUC18 after filling the *Hin*dIII end using the Klenow enzyme. The resultant plasmid (pHH356−) was double digested with either *Sca*I+*Eco*RI or *Sca*I+*Bam*HI. *Sca*I cuts within the ampicillin resistance gene. *Eco*RI and *Bam*HI cleave in the pUC18 polylinker at either side of the insert. Therefore, one of the two restriction fragments generated by each double digest contains the insert at one end and the 5′ (*Sca*I+*Eco*RI digest) or the 3′ (*Sca*I+*Bam*HI digest) region of the ampicillin resistance gene at the other end. The region of the ampicillin resistance gene that is missing in one of these two fragments is present in the other. These two fragments were isolated and ligated to each other after their ends were made blunt with the Klenow enzyme. DH5αF′ *E.coli* cells were transformed with the ligation products and plated in the presence of ampicillin. Ligation can occur in two possible orientations, but only that in which the ampicillin resistance gene is reconstituted will contain a head-to-tail dimer of the insert. One clone was selected and confirmed by restriction enzyme analysis to contain a plasmid (pDimHH356) bearing a tandem dimer of the *Hae*III−*Hin*dIII fragment. This dimer was isolated from pDimHH356 by *Eco*RI+*Bam*HI digestion.

DNA sample preparation for 2D agarose gel electrophoresis

Nuclei were extracted from pea meristematic root tips according to the procedure described by Chiatante *et al.* (1990) using McLeish buffer (66 mM Na_2HPO_4, 66 mM NaH_2PO_4, pH 6.8) as extraction buffer. Nuclei suspension was filtered through a 20 μm nylon mesh and centrifuged at 1500 *g* for 30 min at 4°C. Nuclei pellet was resuspended in McLeish buffer at a concentration of 5×10^7 nuclei/ml and the suspension warmed to 42°C. An equal volume of 1% low-melting-temperature agarose prepared in McLeish buffer and cooled at 42°C was added to the nuclei suspension. After mixing by swirling, the molten mixture was transferred to a hand-made Plexiglas mould and allowed to set at 4°C for 30 min. The hardened agarose was extruded from the mould and sliced in small blocks ($6 \times 3 \times 2$ mm). Blocks were then incubated with gentle shaking in 5 vol of KS solution (10 mM Tris−HCl, pH 7.6, 100 mM EDTA, 1 mg/ml proteinase K, 1% *N*-lauryl-sarcosine) at 42°C for 18 h. KS solution was then replaced by 10 vol of PMSF solution (10 mM Tris−HCl, pH 7.6, 1 mM EDTA, 1 mM phenyl-methylsulphonyl fluoride) and agarose blocks were incubated for 1 h at room temperature with gentle shaking. This incubation was repeated with fresh PMSF solution. Blocks were finally washed twice for 15 min with 10 vol of Tris−HCl (pH 7.6), 1 mM EDTA at room temperature and stored for a few days in this solution at 4°C. For longer term storage (a few months) blocks were washed with and stored in 2 mM Tris−HCl (pH 8.0), 5 mM EDTA at 4°C. For restriction endonuclease digestion of agarose-embedded DNA, blocks were individually transferred to microfuge tubes and incubated twice for 15 min with 300 μl of the appropriate restriction enzyme buffer at 4°C. Blocks were then incubated overnight at 37°C with 100 μl of fresh restriction buffer containing 30−50 U of restriction enzyme (Boehringer-Mannheim) and 200 μg/ml RNase A. Before loading on to the agarose gel, blocks were equilibrated in 10 mM Tris−HCl (pH 8.0), 1 mM EDTA. Each block contained ~10 μg of total DNA.

2D agarose gel electrophoresis

The first dimension was electrophoresed at room temperature for 40 h in a 0.4% (w/v) agarose gel in TBE buffer at 0.6 V/cm. The lane containing the phage λ DNA/*Hin*dIII marker sizes was excised, stained with ethidium bromide and photographed. The second dimension was in a 1% agarose gel in TBE buffer containing 0.5 μg/ml ethidium bromide at a 90° angle with respect to the first dimension. The dissolved agarose was poured around the excised lane from the first dimension and electrophoresis was carried out at 5 V/cm for 7 h in a 4°C cold-room.

Southern transfer and hybridization

Gels were washed twice for 15 min in 50 mM HCl, then twice for another 15 min in 0.5 M NaOH containing 1 M NaCl followed by another 60 min

wash in 1 M Tris–HCl (pH 8.0) with 1.5 M NaCl. The DNA was transferred to nitrocellulose supported membrane (BAS-85, Schleicher and Schuell, Inc.) in 10 × SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 16–18 h and the membranes were baked at 80°C for 2 h. Prehybridization was carried out in 50% formamide, 5× SSC, 5× Denhardt's solution (100× Denhardt's contains 2% bovine serum albumin, 2% Ficoll and 2% polyvinylpyrrolidone), 0.1% SDS, and 250 μg/ml sonicated salmon testes DNA at 42°C for 16–18 h. Membranes were hybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 250 μg/ml sonicated salmon testes DNA and 10% dextran sulfate with 10^6 c.p.m./ml probe DNA labeled with [^{32}P]dCTP by random priming (~10^9 c.p.m./μg), at 42°C for 24–48 h. After hybridization, the membranes were washed twice for 15 min in 2× SSC and 0.1% SDS at room temperature followed by two to three washes in 0.1× SSC and 0.1% SDS at 60°C for 30 min each time. Exposure of XAR-5 films (Kodak) was carried out at −80°C with two intensifying screens for 1–7 days. Two cloned pea rDNA fragments were used as probes. The 1.5 kb *Eco*RI–*Hin*dIII fragment from the NTS of *r* repeats (named ERRF in Figure 1) was used to analyse RIs of restriction fragments containing part or the complete NTS. The 3.7 kb *Eco*RI fragment from the coding region was used as probe to detect RIs of the transcription unit.

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