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# Replication termini in the rDNA of synchronized pea root cells (*Pisum sativum*)

**P.Hernández, S.S.Lamm, C.A.Bjerknes and J.Van't Hof**

Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA

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**In synchronized root cells of *Pisum sativum* (cv. Alaska) the joining of nascent replicons is delayed until cells reach the S−$G_2$ boundary or early $G_2$ phase. To determine if the delayed ligation of nascent chains occurs at specific termination sites, we mapped the location of arrested forks in the ribosomal DNA (rDNA) repeats from cells in late S and $G_2$ phases. Two-dimensional (neutral-alkaline) agarose electrophoresis and Southern blot hybridization with specific rDNA sequences show that only cells located at the S−$G_2$ boundary and early $G_2$ phase produce alkali-released rDNA fragments of discrete size. The released fragments are from a particular restriction fragment, demonstrating that the replication forks stop non-randomly within the rDNA repeats. Indirect end-labeling with probes homologous to one or the other end of the fork-containing restriction fragment shows that there are two termination regions, $T_1$ and $T_2$, where forks stop. $T_1$ is located in the non-transcribed spacer and $T_2$ is at the junction between the non-transcribed spacer and the 18S gene. The two termini are separated by ~1.3 kb. Replication forks stop at identical sites in both the 8.6- and 9.0-kb rDNA repeat size classes indicating that these sites are sequence determined.**

*Key words:* replication termini/rDNA/*Pisum sativum*

## Introduction

In eukaryotes replication of chromosomal DNA occurs at numerous sites, called replicons, that are tandemly arranged along the parental DNA. Each replicon has an origin where replication begins and two replication forks that diverge from the origin while replicating nascent DNA chains bidirectionally. The replication origins are active in groups or clusters, and each cluster is replicated at a particular time during S phase.

Several studies show that the increase in size of chromosomal nascent DNA occurs in a step-wise manner as cells progress through S phase (Kowalski and Cheevers, 1976; Walters *et al.*, 1976; Funderud *et al.*, 1978; Schvartzman *et al.*, 1981, 1984). In synchronized meristematic cells of *Pisum sativum*, replicon-size DNA fragments remain unjoined until cells achieve a 4C DNA content. This delayed maturation of nascent DNA to chromosomal size suggests that replication termini occur in chromosomal eukaryotic DNA, but evidence for termini in eukaryotic genomic DNA is lacking. Replication termini are found, however, in *Escherichia coli* (de Massy *et al.*, 1987; Hill *et al.*, 1987), *Bacillus subtilis* (Weiss and Wake, 1984), animal viruses (Tapper and DePamphilis, 1980) and plasmids (Germino and Bastia, 1981). In these cases, movement of replication forks is impeded as they approach the terminus region and the forks are temporarily stalled before the terminus sequence is finally replicated. The observation of temporarily stalled bacterial replication forks at a known terminus region is analogous to the delayed ligation of nascent replicons seen in pea and other eukaryotic organisms.

In this paper we describe experiments aimed at determining whether or not termination of replication occurs at specific sites in the ribosomal DNA (rDNA) in synchronized pea root cells. The results show that replication forks involved in the replication of rDNA stop in two different regions of the rDNA repeats. One of these regions is located within the non-transcribed spacer and the other is at the junction between the 18S gene and the non-transcribed spacer. These two replication termini give rise to the accumulation of site-specific gapped rDNA repeats when cells reach the S−$G_2$ boundary of the cell cycle. The recent finding that replication origins in pea rDNA map at or near one of these regions (P.Hernández *et al.*, in preparation) suggests that the regulation of eukaryotic DNA replication may involve a functional relationship between sequences that act as origins and those that act as termini.

## Results

The rDNA of pea, as in other higher plants, is organized in tandem repeats. The tandem arrangement of the rDNA repeats produces replicons that contain only the rRNA genes and their spacers. There are ~3900 copies of rDNA in the pea genome (Cullis and Davies, 1975; Ingle and Sinclair, 1979), representing ~35 000 kb of the chromosomal DNA. This amount of rDNA is replicated by replicons of 54−72 kb (Van't Hof and Bjerknes, 1977; Schvartzman *et al.*, 1984), a size range large enough to accommodate 6−8 of the 8.6- and 9.0-kb rDNA repeats and one potential replication terminus.

### *Design and rationale of the experiments*

The diagrams in Figure 1 show how we detected the replication termini in rDNA. In Figure 1a are six clustered rDNA repeats with one repeat having a functional terminus (*ter*). In (b) the rDNA array is replicated during S phase by two replication forks traveling in opposite directions. In (c) the converging forks are retarded at the *ter* sequence, causing accumulation of unjoined rDNA replicons in cells located at the S−$G_2$ boundary. To determine if the forks stop at specific sites within the rDNA, the DNA is digested with a selected restriction enzyme (d) and separated by two-dimensional (2-D) gel electrophoresis, the first carried out under neutral conditions and the second under denaturing conditions (e−g). The neutral first dimension separates the restriction fragments, and the alkaline second dimension

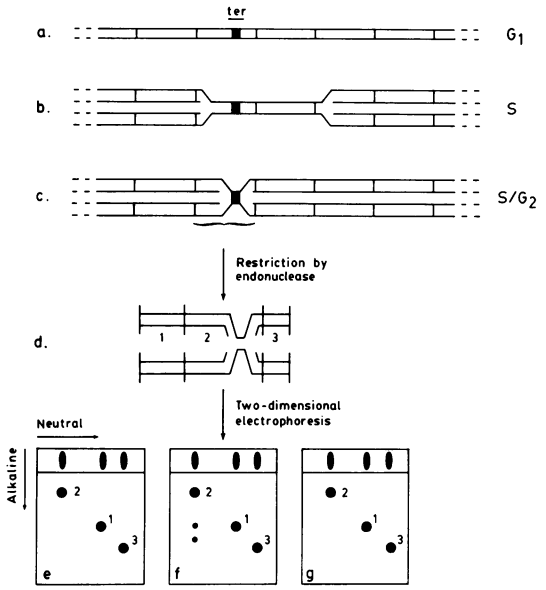


**Fig. 1.** Diagrammatic representation of the replication of six rDNA repeats. See text for details.

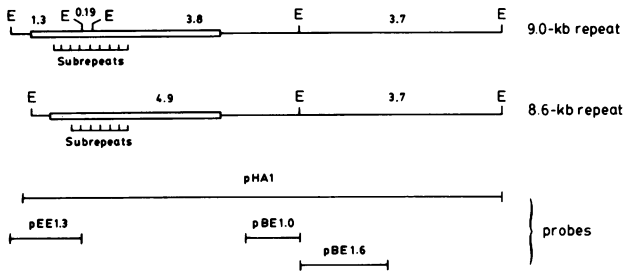


**Fig. 2.** *Eco*RI (E) restriction map of the two major size classes of rDNA repeats in pea (**upper section**), and location of the plasmid clones of fragments used as probes in the present work (**lower section**). The open box indicates the non-transcribed spacer in each size class, and below it, the location of the subrepeat region (from Jorgensen *et al.*, 1987). Size of the restriction fragments is in kilobases.

separates the smaller DNA molecules from those fragments with gaps at the replication forks. Southern blots of these gels, probed with cloned rDNA, reveal where replication forks stop or are retarded. From such blots there are three possible results. First, if there are no termini in the rDNA, none of the restriction fragments separated in the first dimension will give smaller DNA molecules in the second (Figure 1e). Second, if the forks stop at specific loci, discrete spots corresponding to uniformly sized fragments will be seen in blots of the second dimension (Figure 1f). Finally, if the forks stop at random positions within the rDNA, a smear descending from the fragments is expected (Figure 1g).

### *Accumulation of arrested replication forks in the rDNA of cells at S−$G_2$ boundary and early $G_2$ phase*

Each pea rDNA repeat has a coding region for the 18S, 5.8S and 25S rRNAs and an intergenic non-transcribed spacer separating the 18S and 25S coding regions (Jorgensen *et al.*, 1987). Pea variety Alaska has two major size classes of repeats of 9.0 and 8.6 kb (Figure 2), with the latter being the more abundant by a factor of 3 or 4 (Ellis *et al.*, 1984; Jorgensen *et al.*, 1987; Watson *et al.*, 1987). The variation

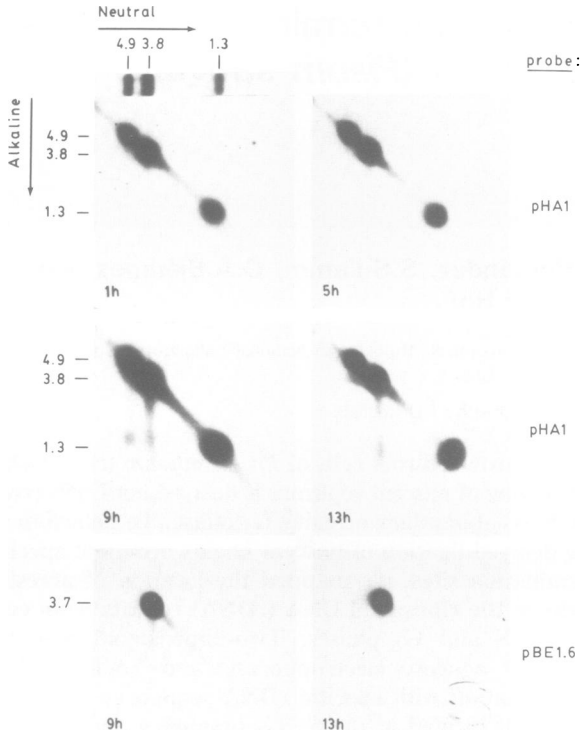


**Fig. 3.** Autoradiograms of blotted rDNA after 2-D electrophoresis. The DNA was isolated from cells at 1, 5, 9 and 13 h after release from the $G_1$−S boundary. The first dimension electrophoresis was carried out under neutral conditions (horizonal lane over the upper left autoradiogram) and the second dimension under denaturing conditions (see Materials and methods). The plasmid used as probe in each pair of autoradiograms is indicated at the right.

in size of the two major rDNA repeats reflects a different number of 180-base subrepeat units located in the spacer (Figure 2) (Jorgensen *et al.*, 1897; Watson *et al.*, 1987).

In Figure 2 we show the *Eco*RI restriction map of both size-classes and the plasmids we used as probes. The spacer of the 9.0-kb repeat has two *Eco*RI sites separated by ~190 bases that are absent in the 8.6-kb repeat. This difference is useful in distinguishing between fragments from the two major size classes of rDNA repeats.

To determine if converging forks of contiguous replicons stop temporarily at separate locations within the rDNA we synchronized root cells at the $G_1$−S boundary and allowed them to progress through S phase for increasing lengths of time before extracting their DNA for *Eco*RI digestion and electrophoretic analysis. The results obtained with this protocol are shown in Figure 3. The uppermost left-hand panel is an autoradiogram showing hybridization of labeled pHA1 to rDNA *Eco*RI fragments of cells 1 h into S phase. Beside this panel, to the right, is an autoradiogram of rDNA from cells that progressed 5 h into S phase. Both autoradiograms show that the pHA1 probe, which has an insert homologous to the complete 8.6- and 9.0-kb rDNA repeats, detects no spots or descending smears from the rDNA *Eco*RI fragments. On the other hand, blots of rDNA from cells 9 (middle, left-hand panel) and 13 (middle, right-hand panel) hours after their release from the $G_1$−S boundary do show spots corresponding to fragments released by the alkali. Of the released fragments one is clearly from the 4.9 *Eco*RI fragment of the 8.6-kb repeat but the others have two pos-

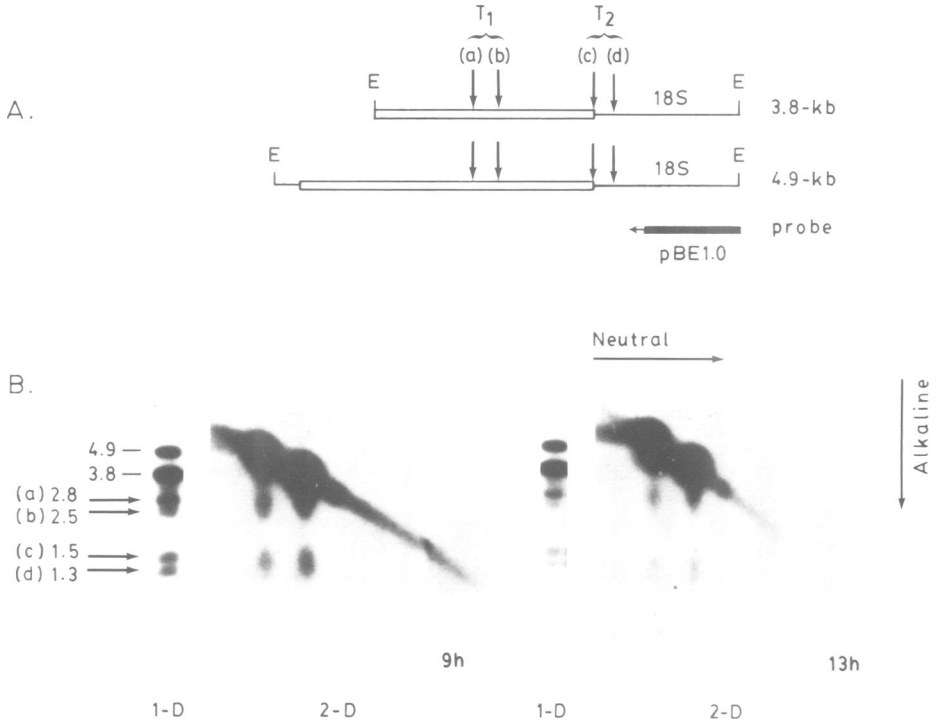


**Fig. 4.** Mapping of replication termini in the rDNA of cells at S−$G_2$ boundary and $G_2$ phase by indirect 3′-end labeling. (**A**) Fork-containing *Eco*RI (E) restriction fragments from each of the two size classes of rDNA repeats (3.8-kb fragment from the 9.0-kb repeat and 4.9-kb fragment from the 8.6-kb repeat). The open box represents the non-transcribed spacer and the line represents coding sequences. Vertical arrows point to the location of the termination sites ($T_1$ and $T_2$) determined by the sizes of the alkaline released rDNA sequences observed in the autoradiograms presented in (**B**). The closed darkened box indicates the homologous region of the insert in pBE1.0 used as probe. (**B**) Autoradiograms of DNA from synchronized cells at 9 and 13 h after release from the $G_1$−S boundary. The DNA was separated by alkaline 1-D and 2-D, (neutral alkaline) agarose electrophoresis, transferred to nylon membranes and hybridized with the labeled probe indicated in (**A**). The sizes of the two native *Eco*RI fragments (4.9 and 3.8 kb) are indicated by horizontal lines and the arrows point to the four alkaline-released rDNA fragments (2.8, 2.5, 1.5 and 1.3 kb) named **a**−**d**, respectively.

sible sources: the 3.8-kb *Eco*RI fragment of the 9.0-kb repeat or the 3.7-kb fragment containing only coding sequences from both repeats (Figure 2). These two fragments are too similar in size to be distinguishable by pHA1 hybridization. To discern the actual source, we used a probe, pBE1.6, which contains a 1.6-kb *Eco*RI−*Bam*HI fragment homologous only to the 3.7-kb *Eco*RI fragment (Figure 2). As seen in the two lowest panels of Figure 3, pBE1.6 fails to hybridize with the released fragments. Thus, the released fragments seen in the 2-D blots are from gapped sequences in the 3.8- and 4.9-kb *Eco*RI fragments containing the non-transcribed spacers.

The results shown in Figure 3 fit those predicted in Figure 1f. They indicate that only certain restriction fragments have gaps that generate smaller DNA molecules under alkaline conditions, that the molecules are released from fragments containing most of the non-transcribed spacer of the 8.6- and 9.0-kb rDNA repeats and that these released molecules have a discrete size producing a spot on the 2-D autoradiograms of rDNA isolated at 9 and 13 h. The two middle panels further show that the hybridization signals of the released fragments are less intense in rDNA isolated from cells at 13 h than in rDNA isolated at 9 h, indicating that some gaps are sealed during the 4-h interval.

### *Two separated replication termini in the pea rDNA*

The gaps in the 3.8-kb and 4.9-kb *Eco*RI fragments were mapped using two cloned fragments as indirect end-labeling probes homologous to one or the other end of these restriction fragments. One clone, pBE1.0, contains a 1.0-kb *Bam*HI−*Eco*RI fragment which is homologous to the right-hand end of both 3.8-kb and 4.9-kb *Eco*RI fragments (Figures 2 and 4A). The other, pEE1.3, is the 1.3-kb *Eco*RI fragment from the 9.0-kb repeat (Figures 2 and 5A). This clone is homologous with the left-hand end of the 4.9-kb fragment from the 8.6-kb repeat and contains some of the subrepeats located in the non-transcribed spacer (Figure 2). Thus, pEE1.3 is also homologous to the left-hand end of the 3.8-kb *Eco*RI fragment of the 9.0-kb repeat.

Figure 4B shows the hybridization of [$^{32}$P]pBE1.0 to alkaline one-dimensional (1-D) and to 2-D blots of rDNA extracted from cells 9 and 13 h after release from the $G_1$−S boundary. The 1-D blot of rDNA from cells at 9 h shows six bands (Figure 4B, left lane). Of these, the 4.9- and 3.8-kb bands correspond to the two native ungapped *Eco*RI restriction fragments from the 8.6- and 9.0-kb rDNA repeats, respectively. The others of 2.8, 2.5, 1.5 and 1.3 kb named (a), (b), (c) and (d), respectively, are released from gapped sequences by the alkali. The 2-D blot shows that these alkaline released rDNA molecules are from both 4.9- and 3.8-kb *Eco*RI restriction fragments and that the hybridization signals of the released fragments are more intense in rDNA at 9 h than at 13 h (Figure 4B, right 1-D and 2-D autoradiograms).

Above the autoradiograms, in Figure 4A, we diagram the position of gaps in the 8.6- and 9.0-kb rDNA repeats. The positions are determined from the size of alkaline-released rDNA fragments shown in Figure 4B and they pertain to

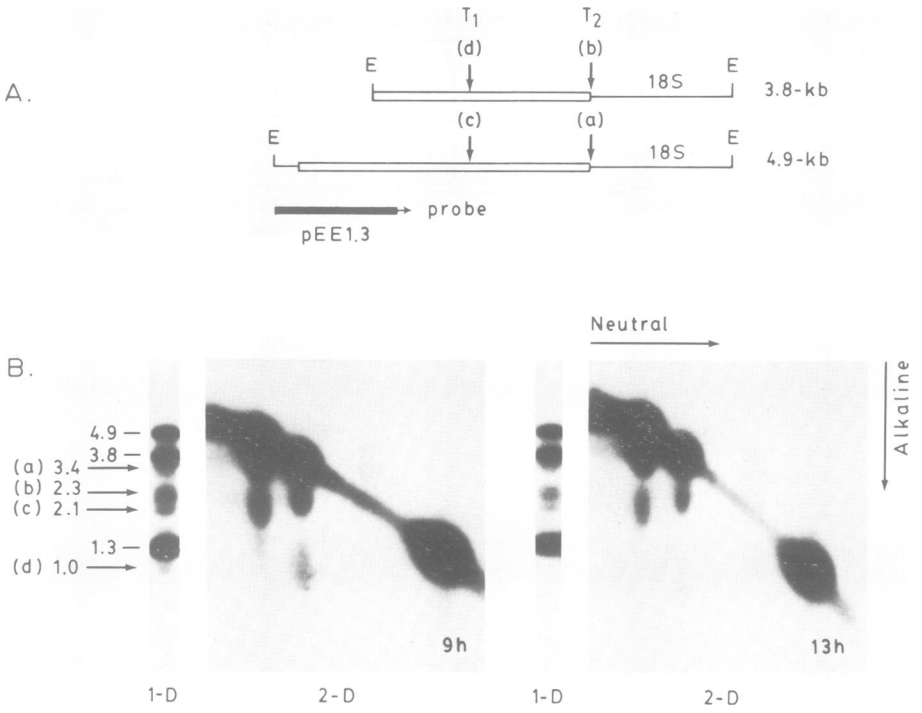


**Fig. 5.** Mapping of replication termini in the rDNA of cells at $S-G_2$ boundary and $G_2$ phase by indirect 5′-end labeling. (**A**) Fork-containing *Eco*RI (E) restriction fragments from each of the two size classes of rDNA repeats (3.8-kb fragment from the 9.0-kb repeat and 4.9-kb fragment from the 8.6-kb repeat). The open box represents the non-transcribed spacer and the line represents coding sequences. Vertical arrows point to the location of the termination sites ($T_1$ and $T_2$) determined by the sizes of the alkaline released rDNA sequences observed in the autoradiograms presented in (**B**). The closed darkened box indicates the homologous region of the insert in pEE1.3 used as probe. (**B**) Autoradiograms of DNA from synchronized cells at 9 and 13 h after release from the $G_1-S$ boundary. The DNA was separated by alkaline 1-D and 2-D, (neutral-alkaline) agarose electrophoresis, transferred to nylon membranes and hybridized with the labeled probe indicated in (**A**). The sizes of the three native *Eco*RI fragments homologous to the probe used (4.9, 3.8 and 1.3 kb) are indicated by horizontal lines and the arrows point to the four alkaline-released rDNA fragments (3.4, 2.3, 2.1 and 1.0 kb) named **a**–**d**, respectively.

the location of replication termini ($T_1$ and $T_2$) for forks traveling in the direction from right to left. The arrowheads below the letters point to the location of the termini and the size of the released fragments corresponds to the distance between the *Eco*RI site on the right and the arrowheads.

The location of termini for forks moving from left to right are shown in Figure 5A. These locations are based on the results of the autoradiograms seen in Figure 5B. To map the termini of forks moving left to right, we stripped the blots seen in Figure 4B and probed them with pEE1.3. This probe detected four released fragments in the 1-D alkaline gel in rDNA from cells in late S phase (9 h; Figure 5B, left lane). The sizes of these released fragments are about 3.4, 2.3, 2.1 and 1.0 kb, and they are named (a), (b), (c) and (d), respectively. The 2-D blot shows that fragments (a) and (c) are released only from the 4.9-kb *Eco*RI fragment of the 8.6-kb rDNA repeat size class and that (b) and (d) are generated from the 3.8-kb *Eco*RI fragment of the 9.0-kb repeat size class.

## Discussion

The experiments described provide evidence for specific replication termini in the rDNA of synchronized pea root cells. These termini are detectable because: (i) in pea, as in other eukaryotes, nascent replicon chains remain unjoined and accumulate as cells reach late S or $G_2$ phase of the cell cycle (Kowalski and Cheevers, 1976; Funderud *et al.*, 1978; Schvartzman *et al.*, 1981, 1984); (ii) the tandem rDNA repeats contain only rRNA coding regions and non-transcribed spacers, thus the sequences in which the replication forks stop and accumulate are easily detected, and (iii) the presence of two repeat size classes that have different sized spacers and *Eco*RI restriction sites provides the needed asymmetry to differentiate between the handedness of converging forks stopped at specific termini.

By using specific probes we demonstrate that gaps are confined to the 3.8-kb *Eco*RI fragment belonging to the 9.0-kb size-class repeat and to the 4.9-kb *Eco*RI fragment from the 8.6-kb size-class. These two fragments contain most of the non-transcribed spacer that separates adjacent coding regions.

Hybridization with rDNA probes homologous to one or the other end of these *Eco*RI fragments shows that there are two regions ($T_1$ and $T_2$) where forks preferentially stop. $T_1$ is located within the non-transcribed spacer outside the subrepeat region ~1.3 kb from the 5′-end of the 18S gene; $T_2$ maps to the junction between the non-transcribed spacer and the 18S gene (Figures 4A and 5A). These two termini are separated by ~1.3 kb.

Recently, it was shown that the terminus region of the chromosome of *E.coli* contains two separated sites, $T_1$ and $T_2$ (Hill *et al.*, 1987; de Massy *et al.*, 1987). These sites act in a polar manner, where the counterclockwise-moving fork passes through $T_2$ but is inhibited at $T_1$, and, conversely, the clockwise fork is not inhibited at $T_1$ but stopped by $T_2$. Unlike *E.coli*, *B.subtilis* has only one terminus which inhibits the movement of the clockwise-moving fork (Weiss and Wake, 1984; Weiss *et al.*, 1986; Handley *et al.*, 1987). The plasmid R6K also has only one termination site which inhibits both replication forks (Kolter and Helinski, 1978;

Bastia *et al.*, 1981). The results reported herein show that pea, like *E.coli*, has two separate regions where fork movement is impeded but whether or not the two sites in pea also have a polar effect is unknown.

The use of the pBE1.0 probe for indirect end-labeling of sequences of forks that approach the termini from the right-hand side shows that the alkaline-released rDNA molecules, produced by gaps at either $T_1$ or $T_2$, have two different sizes giving two double bands with alkaline one-dimensional electrophoresis (Figure 4). For forks that approach the terminus from the left-hand side, using pEE1.3 as a probe, the result is less clear (Figure 5). This probe, because of the asymmetry of the two fragments containing the non-transcribed spacer, should resolve eight different sized alkali-released fragments. The resolution of these expected fragments is difficult because they come in pairs of similar size. Nevertheless, the length of the dots generated in the two-dimensional electrophoresis suggests that each dot represents two molecules of slightly different size (Figure 5B). The presence of these similarly sized double fragments may be explained by assuming that the leading and lagging strands of the replication fork have trailing nascent chains of different lengths and this difference is detected by the indirect end-labeling of the nascent chains.

Tapper and DePamphilis (1980) report that during replication of simian virus 40 (SV40) DNA more forks are arrested when bidirectional DNA replication is 91% completed, and the two converging forks are separated by ~470 bp of unreplicated DNA centered at the expected terminus site. However, when the size of the SV40 genome or the position of the origin of replication is changed, termination occurs at sites other than that of the wild-type SV40. This led Weaver *et al.* (1985) to conclude that the arrest of replication forks at specific DNA sites is not the cause, but the consequence of termination. In contrast, in *E.coli, B.subtilis* and R6K the terminus region acts as if specific DNA sequences inhibit fork movement thus ensuring that forks meet in a certain region (Kuempel *et al.*, 1977; Louarn *et al.*, 1977; Kolter and Helinski, 1978; Kuempel and Duerr, 1979; Germino and Bastia, 1981; Weiss and Wake, 1984).

Our findings show that replication forks stop at equivalent sites in the 8.6- and 9.0-kb rDNA repeats of pea even though they reside on different chromosomes (Polans *et al.*, 1986). This is strong evidence that the arrest of forks is determined at the sequence level in the termini regions.

The mechanism of inhibition of fork movement is unknown, even for bacteria, but the fact that the origin of replication of the 9.0-kb rDNA repeats of pea maps at or near one of the termination regions ($T_1$) (Van't Hof *et al.*, 1987; P.Hernández *et al.*, in preparation) suggests a possible relationship between origins and termini in the regulation of eukaryotic DNA replication. What this relationship may be requires more investigation.

## Materials and methods

### *Root culture and cell synchronization*

Pea seeds (*Pisum sativum*, cv. Alaska) from the 1985 harvest were purchased from W.Altee Burpee Co., Westminster, Pennsylvania, USA. Seeds, surface sterilized with Clorox and washed with distilled water, were aseptically germinated in Petri dishes on three layers of water-moistened Whatman no.1 filter paper. After four days at 22°C, 1–1.5 cm tips were excised from 2.5–4 cm primary roots of selected seedlings and the meristem cells synchronized as previously described (Schvartzman *et al.*, 1981).

### *DNA isolation*

DNA from synchronized pea-root cells was purified according to Burr and Burr (1981). After banding in CsCl and dialysis against 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, the DNA was stored at 4°C. Large scale purification and minipreps of plasmids were carried out using alkaline lysis standard procedures (Maniatis *et al.*, 1982).

### *Gel electrophoresis*

After digestion with *Eco*RI, the DNA was loaded in two wells of a 0.6% agarose gel and separated by electrophoresis at 1 V/cm for 13 h. The buffer used was 0.04 M Tris-acetate, 0.002 M EDTA, pH 8.0. Next, one lane was excised, placed at the end of a tray of a submarine electrophoresis cell and 1.0% agarose, dissolved in 50 mM NaCl, 1 mM EDTA, was poured around it. (The DNA fragments separated in neutral buffer are neither stained nor photographed before separation under alkaline conditions in the second dimension.) The solidified gel was then soaked for 30 min in alkali (30 mM NaOH, 1 mM EDTA) and electrophoresis carried out in the same alkaline buffer, perpendicular to the first dimension, at 1 V/cm for 17.5 h with constant circulation. After electrophoresis, the gel was washed in water, neutralized with 1.0 M Tris–HCl, pH 7.4, 1.5 M NaCl, stained with ethidium bromide and photographed. The remainder of the first dimension gel, that also had marker DNA, was stained with ethidium bromide and photographed. The DNA from both the neutral first dimension and alkaline second dimension was transferred to nylon membrane and hybridized with labeled cloned rDNA fragments.

For one-dimensional alkaline electrophoresis DNA samples dissolved in alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5% w/v, Ficoll 400, 0.025%, w/v, bromocresol green) were separated in 1.0% agarose gels prepared in 50 mM NaCl, 1 mM EDTA at 1 V/cm for 17.5 h.

### *Southern blot hybridization*

Gels were soaked in 0.25 N HCl for 7 min, rinsed with distilled water and the DNA transferred to cationized nylon membranes (Zeta-Probe, Bio-Rad) in 0.4 M NaOH. The blotted membranes were neutralized with 0.5 M Tris–HCl (pH 7.5), 1.0 M NaCl for 10 min and washed with 2 × SSC (pH 7.5). After baking at 80°C in a vacuum oven, membranes were treated with 50% deionized formamide, 6 × SSC (pH 7.4), 2.5 mM EDTA and 5% (w/v) non-fat powdered milk, for 6 h at 42°C. Hybridization was carried out under the same conditions by adding about $10^7$ c.p.m. of heat denatured probe (0.02 μg). After hybridization, blots were washed at room temperature for 30 min each in 2 × SSC (pH 7.4), 0.1% SDS then in 0.1 × SSC, 0.1% SDS and, finally, for 1 h in 0.1 × SSC, 0.1% SDS at 60°C. The blots were exposed to Kodak XAR-5 X-ray film at −70°C with intensifying screens.

For repeated hybridization, blots previously hybridized with radioactive probe were stripped of radioactivity by washing for 30 min in 0.4 N NaOH at 42°C and neutralized at the same temperature with 1 × SSC, 0.5% SDS, 0.2 M Tris–HCl (pH 7.5). The efficiency of the stripping procedure was checked by exposing the blots at −70°C for 2 days with intensifying screens.

### *Subcloning*

The probes used in the present work were pHA1 (gift of Dr R.E.Cuellar) or subcloned pHA1 fragments, except pEE1.3 which was subcloned from pBH3.3, described elsewhere (Hernàndez *et al.*, in preparation). Restriction fragments from the pHA1 or pBH3.3 insert were extracted from agarose gels using DEAE membranes NA-45 (Schleicher and Schuell) following the supplier's protocol and cloned into appropriately cleaved pBR322 or pBR325.

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