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# Location of the replication origin in the 9-kb repeat size class of rDNA in pea (*Pisum sativum*)

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**Abstract**

The replication origin of the 9-kb rDNA repeat size class of pea (*Pisum sativum* cv. Alaska) was identified by benzoylated naphthoylated DEAE-cellulose column chromatography and Southern blotting procedures. The origin is located at or near a 0.19-kb *Eco*R I fragment in the non-transcribed spacer region between the 25S and 18S rRNA genes. Identification of the origin was based on three criteria: (i) an enrichment of the 0.19-kb fragment in replicating rDNA from asynchronously dividing root meristematic cells, (ii) the scarcity of the 0.19-kb fragment in rDNA from non-dividing carbohydrate starved cells, and (iii) a 60-min periodic enrichment of the 0.19-kb fragment in replicating rDNA that temporally coincides with the sequential initiation of replication of replicon families in synchronized pea root cells.

## Introduction

In the previous paper we presented evidence that a family of replicons in synchronized root meristem cells initiates replication 45 to 60 min after the cells are released from the $G_1$/S phase boundary [24]. Replication initiation was detected by kinetic analysis of pulse-labeled DNA separated into replicating and non-replicating fractions by benzoylated naphthoylated DEAE-cellulose column chromatography (BND chromatography). These results, and those of others [2, 3, 14, 18], suggest that BND chromatography is a useful means to identify and eventually obtain isolated replicon origins. The findings described in this paper show that this suggestion is valid. The combination of restriction enzyme digestion, BND chromatography and Southern blotting procedures allowed us to identify the replicon origin containing segment of the 9-kb repeat size class of rDNA of pea cv. Alaska.

The rRNA genes of pea are amenable to replicon analysis because of their high copy number [5, 8], their tandem arrangement, and because they exist in clusters that replicate sequentially throughout the S phase [20, 23]. Digestion of pea rDNA with *Hin*d III shows that it exists as tandem repeats of 8.6 and 9 kb with the latter being the more abundant by a factor of 3 to 4 [6, 9, 25]. The tandem characteristic of the rDNA indicates that the replicons responsible for the replication of these sequences contain only the rRNA genes and their non-transcribed spacer [6, 9, 25]. Also, since the chromosomal replicons of pea range from 54 to 72 kb [16, 19] each replicon contains 6 to 8 repeats of rRNA genes and only one of these is expected to have a functional origin.

Here we present evidence that the replication origin of the 9-kb repeat size class of rDNA, to be called *ori-r9*, is located in the spacer region about 1.3 kb 3′ to the 25S gene and is associated with a

190-base *Eco*R I fragment that appears to have a transient *Eco*R I site [23].

## Materials and methods

The materials and methods used in these experiments are as given in preceding papers [23, 24] with one exception, namely the method used to isolate and digest the 9-kb and 8.6-kb *Hin*d III repeat size classes of rDNA.

The two repeat size classes were separated by electrophoresis in 0.6 or 1.0% agarose gels in TBE buffer [11] at 0.6 V/cm for 72 to 96 h. The DNA fragments were stained with ethidium bromide, sliced from the gel and electroeluted into dialysis tubing containing TAE buffer. Electroelution was done at 6.7 V/cm for 30 min after which the fragments were dislodged from the membrane by current reversal for 30 s at 6.7 V/cm. The fragments were precipitated from TAE buffer, made up to 2.5 M ammonium acetate, with 2 volumes of 100% ethanol, dissolved in TE and re-precipitated with 10 mM spermine pH 7.0. Finally, the pelleted DNA was dissolved in 20 $\mu$l TE buffer, dialyzed on a Millipore VSWP filter (pore size 0.025 $\mu$m) over *Eco*R I buffer for 1.5 h and digested with *Eco*R I enzyme.

All DNA digests run on agarose gels were transferred to Zeta-probe nylon membrane (Bio-Rad Laboratories, Richmond, CA) by the alkaline method of Reed and Mann [13] after a 5-min treatment with 0.25 N HCl. After overnight transfer the blots were neutralized at room temperature with a 10-min wash in 0.5 M Tris, 1 M NaCl, pH 7.5, followed by two 15-min washes with 2 × SSC and then dried at 80 °C for 2 h in a vacuum oven. Prehybridization, hybridization and all other washings followed the procedures of Gatti *et al.* [7].

## Results

### *Restriction map of the 9-kb and 8.6-kb* Hind *III rDNA repeat size classes of pea cv. Alaska*

Simple restriction maps of the two repeat size classes of rDNA are shown in Fig. 1. The maps are based on rDNA isolated from the meristematic tip of pea roots and they serve as guides showing the reader the location of the fragments of interest. The maps agree with those of others confirming

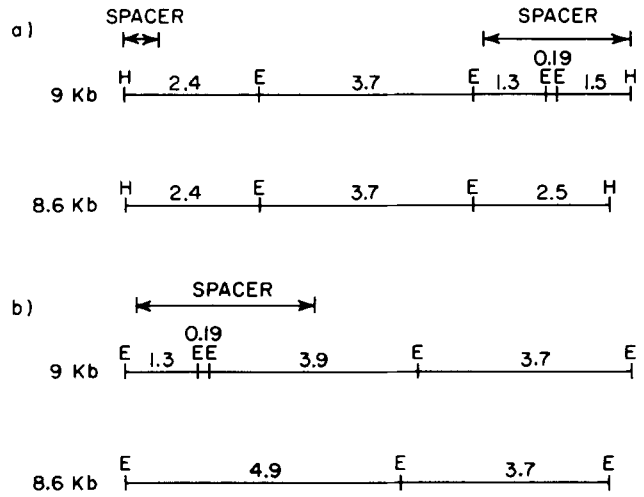


*Fig. 1.* Restriction maps of the 9-kb and 8.6-kb repeat size classes of rDNA isolated from root tip meristematic cells of *Pisum sativum* cv. Alaska. H, *Hin*d III sites; E, *Eco*R I sites; spacer, non-transcribed repeated sequences. (a), rDNA repeat size classes double-cut with *Hin*d III and *Eco*R I, (b), rDNA repeat size classes cut with *Eco*R I. For further details see ref. 9 and 25. The size of the fragments is noted in kb units and the 5′ end is to the left.

that rDNA from root meristematic cells has the same restriction sites as rDNA from other tissues [6, 9, 25]. Hybridization of radioactive cloned rDNA to *Eco*R I digests of isolated 8.6-kb and 9-kb *Hin*d III repeats shows that the 8.6-kb repeats have two *Eco*R I sites producing three fragments of 3.7, 2.5 and 2.4 kb (Fig. 2, lane 1), and that the 9-kb repeats have four *Eco*R I sites producing fragments of 3.7, 2.4, 1.5, 1.3 and 0.19 kb (Fig. 2, lane 2, left and the overexposed autoradiogram lane 2, right). The present experiments are concerned only with the 0.19-kb and 1.3-kb fragments of the 9-kb repeats. Both fragments are from the spacer region, each contains repeated sequences and each is detectable in single *Eco*R I digests of rDNA.

*Replication of rRNA genes in asynchronously dividing and carbohydrate starved root meristem cells*

Root meristems normally have asynchronously

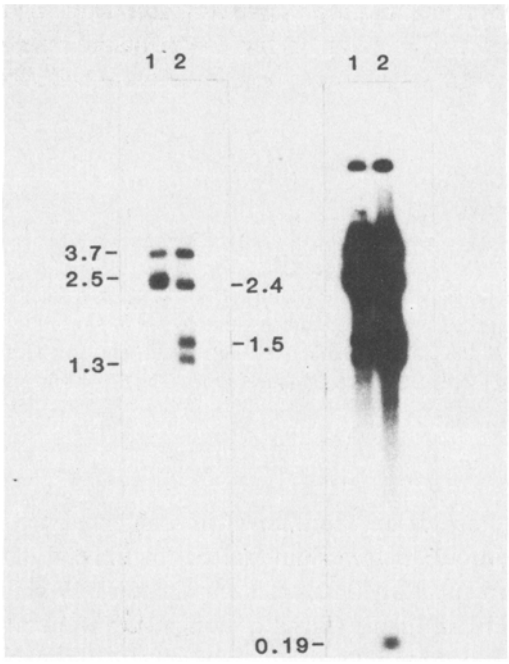


*Fig. 2.* Autoradiograms of isolated 8.6-kb (lane 1) and 9-kb (lane 2) *Hin*d III rDNA repeats digested with *Eco*R I and hybridized with cloned pea rDNA, pHA1, to show the different cleavage patterns of the two repeat size classes. The autoradiograms are of the same blot exposed for different lengths of time.

dividing cells, therefore, by definition their replicating rDNA has replication forks randomly scattered throughout the rRNA genes and their spacer sequences. The rDNA of asynchronously dividing cells, and all other rDNAs described in this paper, was digested with *Eco*R I and then fractionated by BND column chromatography into replicating rDNA – (+)rDNA – and non-replicating rDNA – (–)rDNA – before electrophoretic separation and Southern blotting hybridization. The results obtained with these procedures using DNA from asynchronously dividing cells are shown in Fig. 3a. The gel shown, as well as the others discussed subsequently in this paper, has 0.25, 0.125, and 0.062 μg of (–)DNA, respectively, in lanes 1, 2 and 3, and lanes 4, 5 and 6 have corresponding amounts of (+)DNA. The three autoradiograms to the right of the stained gel are of blotted rDNA. They are of the same blot exposed for increasingly longer times. Different exposure times aid in gauging the relative hybridization signals of the 0.19-kb and 1.3-kb fragments in both (+) and (–)rDNAs. The autoradiogram on the far right of Fig. 3a is most informative because it shows that the signals of the 0.19-kb and 1.3-kb fragments of (+)rDNA are enhanced compared to those of the corresponding fragments in (–)rDNA.

The identification of the rDNA fragment containing *ori-r9* is based on its periodic appearance in replicating rDNA isolated by benzoylated naphthoylated DEAE-cellulose chromatography from DNA of synchronized meristematic cells. Synchronization, however, involves several steps.

In the first step the excised roots are starved of carbohydrate for 48 h. After 48 h the meristems exhibit no mitoses and have only a few cells in late S phase [21, 22]. Consequently, their (+)rDNA is expected to have a reduced amount of the 0.19-kb and 1.3-kb *Eco*R I fragments, assuming, of course, that these fragments are associated with replication initiation. This expectation is met only partially, however (Fig. 3b). Though the hybridization signal of the 0.19-kb fragment in the (+)rDNA is barely detectable, that of the 1.3-kb fragment is not reduced (Fig. 3b, lanes 4, 5 and 6). Nevertheless, the weak signal of the 0.19-kb fragment in (+)rDNA is significant because its reduction corre-

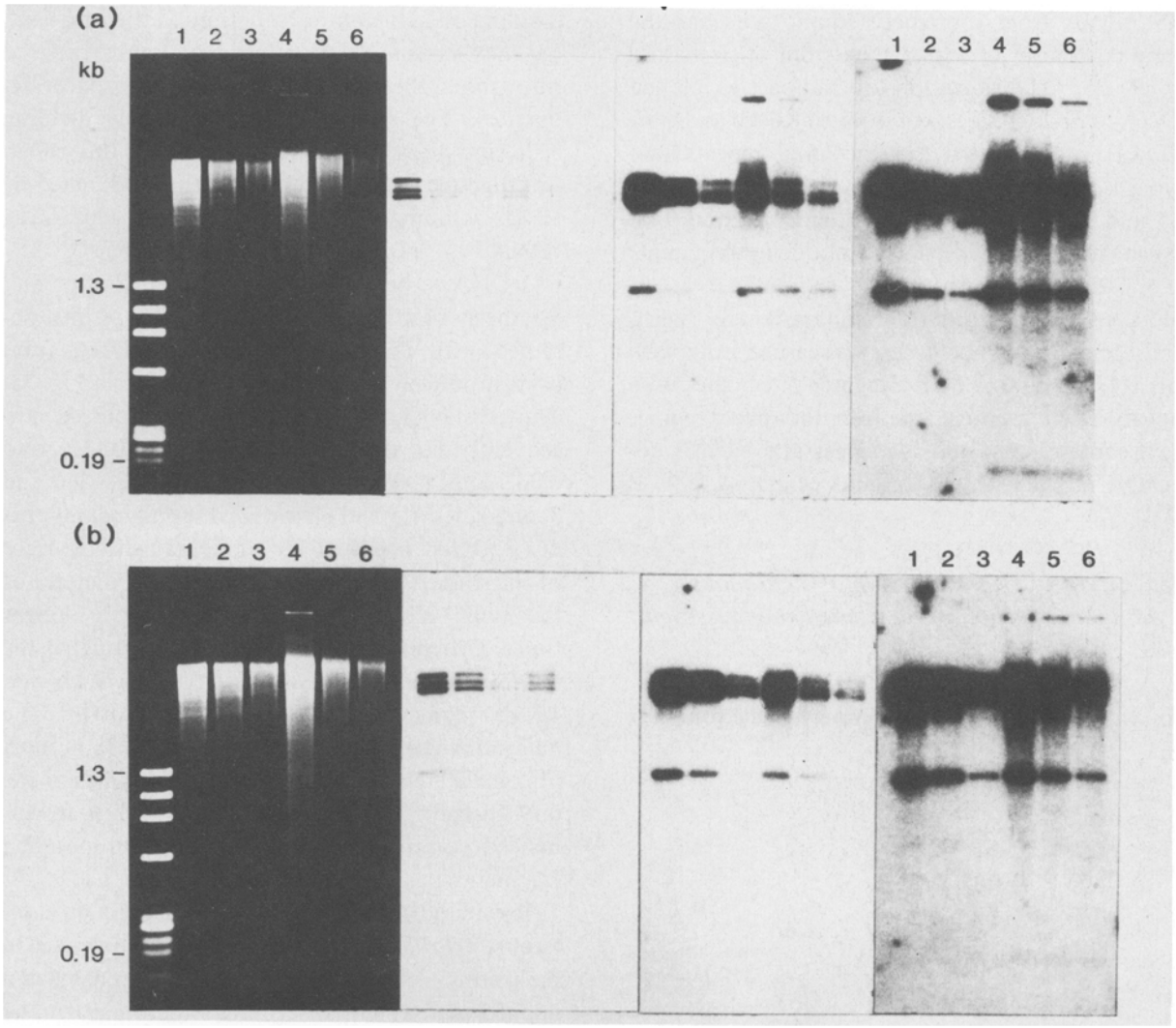


*Fig. 3.* Stained 1.5% agarose gels and autoradiograms of *Eco*R I digested non-replicating and replicating rDNA. (a), rDNA from asynchronously dividing pea root meristematic cells; (b), rDNA from meristematic cells starved of carbohydrate for 48 h. Lanes 1, 2 and 3, 0.25, 0.125 and 0.062 μg, respectively, of non-replicating DNA; lanes 4, 5 and 6, 0.25, 0.125 and 0.062 μg, respectively, of replicating DNA. The autoradiograms to the right of the gels are of the blotted DNA exposed for increasingly longer times. The *Eco*R I fragments of rDNA are identified by hybridization with radioactive cloned rDNA.

lates directly with the low frequency of replication initiations in the few cells replicating DNA in the starved meristems [24].

The next step in the procedure involves feeding the roots 2% sucrose and 1 μM each of 5-fluoro-2′-deoxyuridine and uridine for 12 h. In this medium cells in $G_2$ phase divide joining others blocked at or near the $G_1$/S phase boundary by the inhibitor. The blockage is not complete, however. Replication continues in the presence of 5-fluoro-2′-deoxyuridine so that by the end of the treatment the (+)rDNA is enriched in 0.19-kb and 1.3-kb fragments (Fig. 4a, lanes 4, 5 and 6).

Replicating rDNA sequences are still detected after the third synchronization step. This step, consisting of 12 h culture in fresh medium without either sucrose or the inhibitor, allows the cells to recover from the inhibitor treatment while retaining

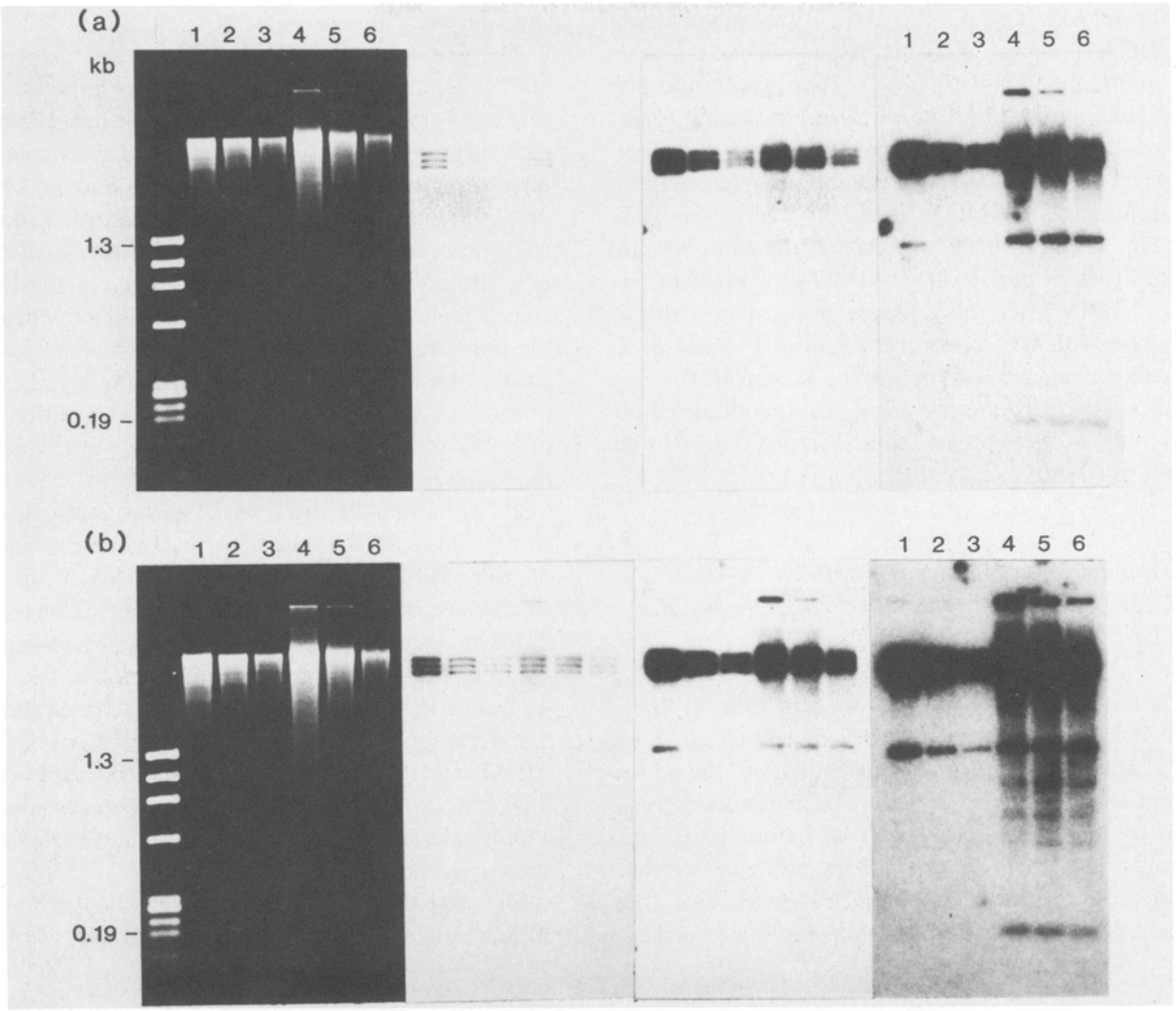


*Fig. 4.* Stained 1.5% agarose gels and autoradiograms of *Eco*R I digested non-replicating and replicating rDNA. (a), rDNA from meristematic cells treated with 1 $\mu$M each of 5-fluoro-2′-deoxyuridine and uridine and fed 2% sucrose for 12 h; (b) rDNA from meristematic cells allowed to recover from the inhibitor treatment for 12 h in medium without sucrose. Lanes 1, 2 and 3 non-replicating rDNA; lanes 4, 5 and 6 replicating rDNA. Other details are as given in the legend of Fig. 3.

the rDNA in a replicative conformation. In this DNA not only does the (+)rDNA have relatively high amounts of the 0.19-kb and 1.3-kb fragments but the probe hybridizes to other intermediate sized sequences as well (Fig. 4b, lanes 4, 5 and 6). The absence of these intermediate sized fragments in the (−)rDNA (Fig. 4b, lanes 1, 2 and 3) rules out the possibility that they are products of partial digestion, and suggests that their presence in (+)rDNA is due to a stalling or retardation of the replication forks in these sequences.

Finally, to release the cells from the $G_1$/S phase boundary, the roots are fed 2% sucrose. During this step the critical observations are made regarding the identification of the origin of the 9-kb rDNA repeats. The identification of *ori-r9* is based on three criteria, two of which are established in this experiment and one of which is derived from other replication measurements in pea. The first of these is the enhancement of the hybridization signal of

the 0.19-kb and 1.3-kb *Eco*R I fragments when rDNA is replicating. (This criterion is based on the results obtained with rDNA from asynchronously dividing cells shown in Fig. 3a.) The second is the weak hybridization signal of the 0.19-kb fragment when only a few rDNA sequences are replicating as seen in the (+)rDNA from cells starved for 48 h (Fig. 3b), and finally, the third is the 60 to 80 min cyclic appearance of the 0.19-kb fragment in (+)rDNA after the cells are released into the S phase. This last criterion is predicated on previous replicon and replication fork measurements that indicate the interval between replication initiations of serially active replicon families in pea ranges from 60 to 80 min [16, 19, 20].

### *Periodic changes in the 0.19-kb and 1.3-kb* Eco*R I fragments of the 9-kb rDNA repeat size class*

With these criteria in mind, we now turn to Fig. 5 which shows blots of rDNA from cells at 5 to 90 min after they were released from the $G_1$/S phase boundary. Noted above each autoradiogram is the time after release and, as before, lanes 1, 2 and 3 show hybridization of the probe to decreasing concentrations of (−)rDNA while lanes 4, 5 and 6 show the hybridization to the same decreasing concentrations of (+)rDNA.

The first autoradiogram in the series is that of rDNA isolated from cells at 5 min. The important feature of this blot is the intensity of the hybridization signal of the 0.19-kb fragment and the virtual absence of bands between the 0.19-kb and 1.3-kb *Eco*R I fragments in +rDNA (Fig. 5, lanes 4, 5 and 6). These bands, though present 5 min earlier (Fig. 4b), are faint at 5 min. Thus, the previously stalled replication forks resumed replication when the cells were supplied sucrose. The abundance of the 0.19-kb fragment, on the other hand, signifies that rDNA sequences are replicating. This pattern of hybridization is temporary, however. At 15 min the signal of the 0.19-kb fragments in (+)rDNA is weak while that of the 1.3-kb fragments remains strong. Taken at face value, the change in the signal of the 0.19-kb fragments in (+)rDNA within 10 min suggests that the replication forks no longer reside in these sequences. Meanwhile, the strong signal of the 1.3-kb fragments indicates that forks still reside in these sequences. If this interpretation is correct, then the hybridization signals of the 0.19-kb and 1.3-kb fragments should be reduced further in (+)rDNA isolated at 30 min because the additional time would allow more forks to move to other sequences.

The pattern of hybridization to rDNA isolated at 30 min validates this interpretation. In this DNA

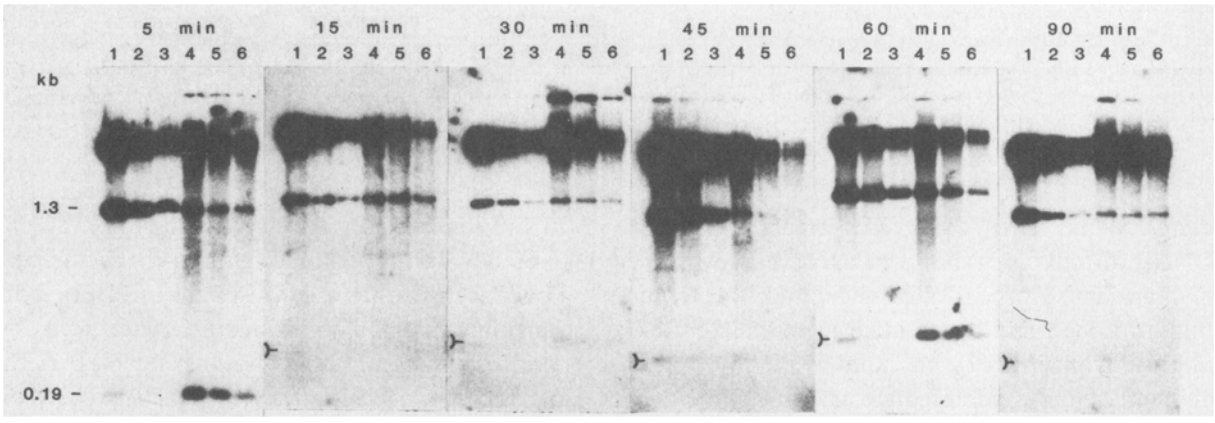


*Fig. 5.* Autoradiograms of non-replicating (lanes 1, 2 and 3) and replicating (lanes 4, 5 and 6) *Eco*R I digested rDNA from synchronized pea root meristematic cells extracted at 5 to 90 min after release from the $G_1$/S phase boundary. The amount of DNA loaded per well and the radioactive probe used are as given in the legend of Fig. 3. The )– marks the position of the 0.19-kb *Eco*R I fragment on the various blots.

the signal of the 0.19-kb fragment in (+)rDNA is low and that of the 1.3-kb fragment is reduced (Fig. 5, 30 min, lanes 4, 5 and 6). Moreover, fork movement from the 1.3-kb fragment continues so that at 45 min its presence in (+)rDNA is seen clearly only in lane 4, the lane with the highest amount of (+)rDNA (Fig. 5, 45 min).

A significant finding is that the reduction of the 0.19-kb and 1.3-kb fragments in (+)rDNA is temporary. At 60 min (Fig. 5, lanes 4, 5 and 6) their hybridization signals are enhanced, producing a pattern reminiscent of that seen in (+)rDNA isolated at 5 min. The similarity of hybridization patterns of (+)rDNA isolated at 5 and 60 min is strong evidence that the replication process is cyclic having a periodicity of about 60 min. Moreover, the results obtained with (+)rDNA isolated at 30 and 90 min argue favorably for this idea. If the presence of the 0.19-kb and 1.3-kb fragments in (+)rDNA has a 60-min periodicity, then the pattern of hybridization to (+)rDNA isolated at 90 min should correspond to that seen in (+)rDNA isolated at 30 min since both time points occur 30 min after the hybridization signal of the 0.19-kb fragment is most intense. The autoradiograms in Fig. 5 show that the pattern of hybridization of the probe to (+)rDNA extracted at these times is similar. In both blots the hybridization signal of the 0.19-kb fragment is weak and that of the 1.3-kb fragment is reduced. Thus, not only is the presence of the 0.19-kb fragment in (+)rDNA cyclic, being most abundant at 5 and 60 min, but the abundance of the 1.3-kb fragment is cyclic as well. The periodicity of the hybridization signal of the 1.3-kb fragment, however, is out of phase with that of the 0.19-kb fragment lagging behind that of the smaller fragment, a point to be considered in the Discussion.

## Discussion

The combination of BND chromatography, cell synchronization and Southern blotting procedures allowed us to identify the replicon origin, *ori-r9,* of the 9-kb repeat size class of rDNA of pea. *Ori-r9* is located in the non-transcribed spacer region between the 25S and 18S rRNA genes (Fig. 1). It is in or near the 0.19-kb *Eco*R I fragment a little less than 1.3 kb from the 3′ end of the 25S gene.

Like *ori-r9,* the replication origin of rDNA of other eukaryotes also is located in the spacer region of the rRNA genes. In *Tetrahymena,* the origin of the extrachromosomal rDNA is nearly centrally located in the palindrome in the spacer region of the molecule [4, 10]. Similarly, in *Drosophila* embryo nuclei the initiation of replication was traced by electron microscopy to the non-transcribed spacer region in transcribing rDNA genes [12]. The same method was used to show that replication bubbles are first seen in the non-transcribed spacer of rDNA in yeast [15]. Finally, Bozzoni *et al.* [1] used electron microscopy, specific radioisotopic labeling, and restriction enzymes to identify the origins of replication of rDNA of *Xenopus.* These workers found that *Xenopus* rDNA also initiates replication in the spacer region.

The procedures we used also can detect fork movement away from *ori-r9* provided the adjoining fragments are sufficiently separated from others in the digested DNA. Our results show that the 1.3-kb *Eco*R I fragment of replicating pea rDNA, like the 0.19-kb fragment, increases and decreases in (+)rDNA with a 60-min periodicity. This periodicity, however, is not in phase with that of the 0.19-kb fragment. It lags behind that of the 0.19-kb fragment by about 30 min. As seen in Fig. 1, the 1.3-kb fragment is located 5′ to the 0.19-kb fragment. Thus, when replication is initiated at or near the 0.19-kb fragment it is the first to appear and disappear from the (+)rDNA fraction. The 1.3-kb fragment is coincidently enriched in (+)rDNA but its disappearance from (+)rDNA trails that of the 0.19-kb fragment. We interpret this trailing aspect as evidence that replication begins at or near the 0.19-kb fragment and then moves bidirectionally with one fork replicating the adjoining 1.3-kb fragment and the other replicating the 3.9-kb fragment.

The reason why the 0.19-kb *Eco*R I fragment of the 9-kb rDNA repeat is seen transiently remains obscure. We describe it as transient because it is rare in (−)rDNA even when it is scarce in (+)rDNA (Fig. 5, 45 min). Earlier we proposed that the 0.19-kb fragment was transient because one

of the *Eco*R I sites is hemimethylated. At the time, the precise location of the hemimethylated site was unknown but we now know that it is in the spacer region 190 bases 3′ to the *Eco*R I site that is unmodified (Fig. 1b). This location presents a problem. If the proposed hemimethylated site is not cut by *Eco*R I a fragment of about 4.1 kb would result. Thus, in fractionated *Eco*R I digested rDNA, the (−)rDNA should have a 4.1-kb fragment while the (+)rDNA should have a 3.9-kb fragment. The blots in Fig. 4b, however, fail to support these expectations. The first autoradiogram to the right of the stained gel has three bands corresponding to fragments of 4.9, 3.9 and 3.7 kb in both (−)rDNA (lanes 1, 2 and 3) and (+)rDNA (lanes 4, 5 and 6). There is no evidence that the middle band of (−)rDNA is a fragment that is slightly larger than its counterpart in (+)rDNA even though the latter is rich in 0.19-kb fragments (Fig. 4b, far right autoradiogram).

Another possible explanation of this divergence from expectation is diversity among the 9-kb repeats. Jorgensen *et al.* [9] point out that the pea cultivar Alaska has other size classes of rDNA besides those of the 8.6-kb and 9-kb repeats. These workers cloned an 8.8-kb *Hin*d III rDNA repeat from Alaska in pACYC 184 constructing pHA1, the cloned rDNA probe used in these experiments. The insert, however, lacks the *Eco*R I site needed to produce a 0.19-kb fragment of spacer DNA. This finding shows that Alaska peas have other rDNA besides that of 8.6 kb which lack an *Eco*R I site in the spacer region. Given this diversity, it is possible that the number of 9-kb rDNA repeats with an additional hemimethylated *Eco*R I site is relatively low and that this low number reflects the fact that there is one replication initiation site per 54 to 72 kb in peas [16, 17, 19]. Replicons in this size range have between 6 and 8 rDNA repeats per replicon and only one of these has a functional origin. The frequency of repeats with an origin and an additional hemimethylated *Eco*R I site, therefore, is expected to be between 12 and 17%. To determine whether or not this view is valid requires further work.

Finally, a third possibility is that the 0.19-kb fragment has a conformation that produces a degree of single strandness sufficient for binding to the BND column. The presence of looped, hairpin or lollypop configurations can be the source of such single-stranded segments.

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