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Origin of replication in chloroplast DNA of *Euglena gracilis* located close to the region of variable size

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Chloroplast DNA (cpDNA), containing ~10% replicative molecules, was isolated 2 h after onset of the dark period from cultures of *Euglena gracilis* strain Z. The DNA was digested with the restriction enzymes *Pvu*II, *Sal*I, *Bam*HI, or *Eco*RI. Fragments that contained intact replicative loops were measured to determine the position of replicated sequences in relation to the restriction enzyme sites. It was found that replication starts at a unique position near one of the palindromic sequences I_2 (Koller and Delius, 1982a) which is located upstream (with respect to the direction of rRNA transcription) of the AT-rich region of variable size (Jenni *et al.*, 1981; Schlunegger *et al.*, in preparation). In the majority of cases DNA synthesis proceeds unidirectionally away from this region for ~5000 nucleotides before it starts in the other direction (in the same sense as the rRNA transcription) through the Z-region and the second palindromic sequence.
Key words: chloroplast DNA/origin of replication/Z-region/inverted repeat/*Euglena gracilis*

Introduction

The replicative DNA of the chloroplasts of *Euglena gracilis* (Manning and Richards, 1972) and pea and corn (Kolodner and Tewari, 1975a, 1975b) has been described. Pea cpDNA replicates by both Cairns type and rolling circle mechanisms, and synthesis starts at a defined position (Kolodner and Tewari, 1975b); but until now this position had not been mapped in chloroplast DNA. *E. gracilis* strain Z offers several advantages for the study of cpDNA replication. The cultures can be synchronised by a simple light and dark rhythm, with the highest rate of cpDNA synthesis occurring at the transition from the light to the dark period (Richards and Manning, 1974). In addition a large number of restriction enzyme maps are available for this cpDNA (for review, see Hallick, 1982). We therefore used this organism to determine the position of the origin of replication and to study some of its structural features.

Results

E. gracilis strain Z was grown in an environmental chamber providing a 12 h light and a 12 h dark period. The cells were harvested 2 h after onset of the dark period. The cpDNA extracted from these cells contained ~10-20% replicative molecules as judged by the presence of replicative loops in the circular cpDNA seen under the electron microscope.

Aliquots of the DNA were digested with either one of the restriction enzymes *Pvu*II, *Sal*I, *Bam*HI, and *Eco*RI and prepared for electron microscopy by cytochrome spreading. In these preparations containing the mixtures of restriction fragments all the molecules which contained an intact replication loop were selected for measurements of the fragment length and the position and extent of the replicated DNA region. In all of the four restriction enzyme digests only fragments belonging to one specific size class contained replicative loops. From their size these fragments could be identified as fragments *Pvu*II A, *Sal*I B, *Bam*HI B, and *Eco*RI B (Figure 1). Figure 1a is the electron micrograph of a *Pvu*II fragment. The size of the fragment and the long distance between the origin and the ends of the fragment allows the analysis of large replication loops. In the molecule shown, the replicated stretch is ~35 kbp with one of the forks approaching the end of the molecule. Figure 1b is an example of a replicative loop in fragment *Bam*HI B. In fragment *Sal*I B (Figure 1c) only small intact replicative loops were observed, because the origin is very close to the end of the fragment. Fragment *Eco*RI B (Figure 1d) was the smallest fragment analysed. Replication starts almost in the middle of the fragment. In most cases one of the two replicated DNA strands is connected to the fork *via* a single strand. The single-stranded regions may have a size of 2000 nucleotides or more as in the molecules shown in Figure 1b or Figure 1d.

Measurements of replicative loops obtained from these fragments are shown in Figure 2. Maps of individual molecules of fragment *Pvu*II A with replicative loops of various sizes are shown in Figure 2a. The maps are oriented with the shorter of the non-replicated stretches to the right. The smallest loops are located in a rather confined region on the molecules while the larger replicative loops are in most cases centred around this position as would be expected for a bidirectional synthesis. A histogram obtained from these molecules is shown in Figure 2b. The peak of the histogram has a slightly skewed shape, with a steeper edge on the left side and a flatter slope on the right side. This is more pronounced in the histogram obtained from the measurements on fragment *Sal*I B (Figure 2c), which includes only small replicative loops of <6000 nucleotides. Here the well-defined starting positions on one side of the replicated regions and the variable extension of the regions on the other side are clearly visible. In fragment *Bam*HI B (Figure 2d) and *Eco*RI B (Figure 2e) the replicative forks at the left of the small loops are again at a defined position whereas the larger loops have grown in both directions.

A preliminary arrangement of the four histograms relative to each other was carried out on the basis of the published restriction maps (Hallick *et al.*, 1978) placing the rRNA genes to the left of the positions of the inverted repeats I1 and I2 (Koller and Delius, 1982a) so that the direction of rRNA transcription is from right to left. Based on this arrangement there is only one possible orientation of the four histograms shown in Figure 2 in which all peak positions can be matched. A more precise alignment of the histograms is possible when the positions of two inverted repeats (I1 and I2) (Koller and Delius, 1982a) are taken into account. To determine the position of the inverted repeats, all four restriction fragment mixtures were denatured and briefly renatured. The positions of

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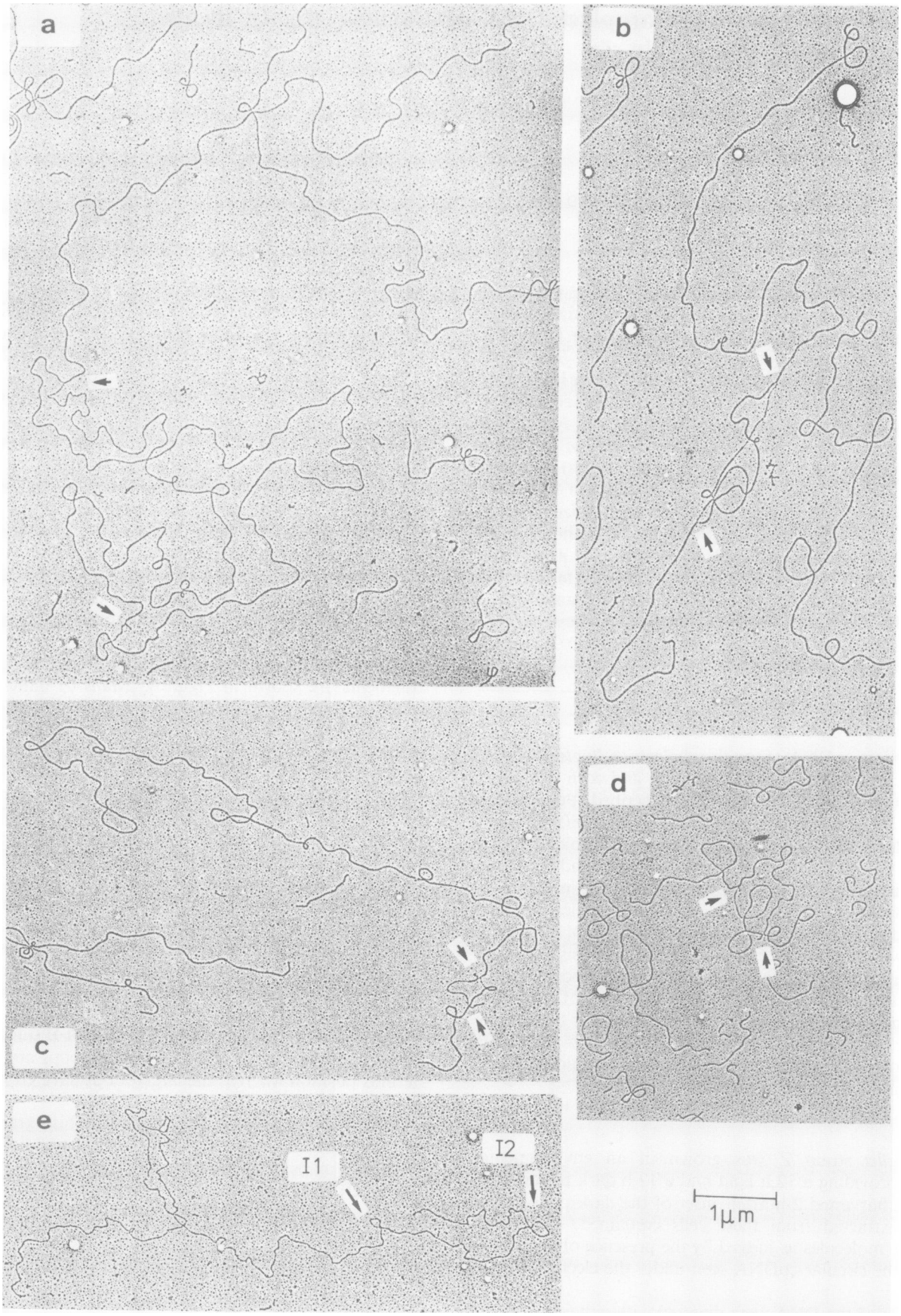

Fig. 1. Cytochrome spreadings of replicative and single-stranded restriction enzyme fragments from *Euglena* cpDNA. **(a)** *Pvu*II fragment A, showing a large replicative loop. On one side replication has almost reached the end of the fragment. **(b)** *Bam*HI fragment B, **(c)** *Sal*I fragment B, and **(d)** *Eco*RI fragment B. The short arrows point to the two replicative forks in each fragment. **(e)** A single-stranded molecule of fragment *Sal*I B showing the two inverted repeat structures I1 and I2.

the repeats I1 and I2 were then measured in the single strands. The inverted repeat I2 is very unstable and was observed in ~10% of the molecules. Figure 1e shows a single-stranded molecule of *Sal*I B with both inverted repeat structures visible. Maps of the positions of the inverted repeats in the single-stranded fragments were aligned with the histogram of replicative loops as shown for *Sal*I B in Figure 3. I1 was measured at a well-defined position, but the extension and, therefore, the position of I2 in the standardized maps was quite variable.

The maps with the positions of the inverted repeats were used to arrange the histograms in Figure 2b–e so that inverted repeat I1 is lined up in the vertical direction. The distances between I1 and the restriction enzyme sites, as well as the sites of the fragments as determined in the electron micrographs are indicated underneath each histogram in Figure 2. The resulting map positions for the restriction sites are in general agreement with the previously published restriction maps (Hallick *et al.*, 1978), although the sizes of the restriction fragments differ somewhat from the values obtained from gel analysis.

It is apparent that the steeper edge of the replicated areas in all four histograms coincides with the palindromic sequence at the right side of I2, and that there is a tendency for the DNA synthesis to proceed away from it to the right for ~5000 nucleotides before the loops are enlarged into the other direction. Only four of the 41 replicative loops shown in the *Pvu*II fragments could have started at the same site but in the other direction. Large replicative loops as measured on the *Pvu*II fragments show DNA synthesis in both directions.

Figure 4 summarises the measurements. The origin of replication is very close to the palindromic sequence of I2 which is upstream with respect to the direction of rRNA transcription. Using measurements for the distances between I1 and I2 and the extra 16S rRNA gene which were published previously (Koller and Delius, 1982a), it can be calculated that DNA synthesis starts ~6800 bp upstream of the 5′ end of the extra 16S rRNA gene, assuming that the loop formed by the inverted repeat I2 has an average size of 1750 bp. The accuracy of this value is limited to about ± 500 bp since the distance includes the region of variable length (Jenni *et al.*, 1981). A more precise position can be given using the *Sal*I restriction site to the right of I2 as a reference: the origin of replication (the left border of the replicative loops) measured in the *Sal*I restriction fragments is 7100 ± 350 bp away from the right end of the fragment.

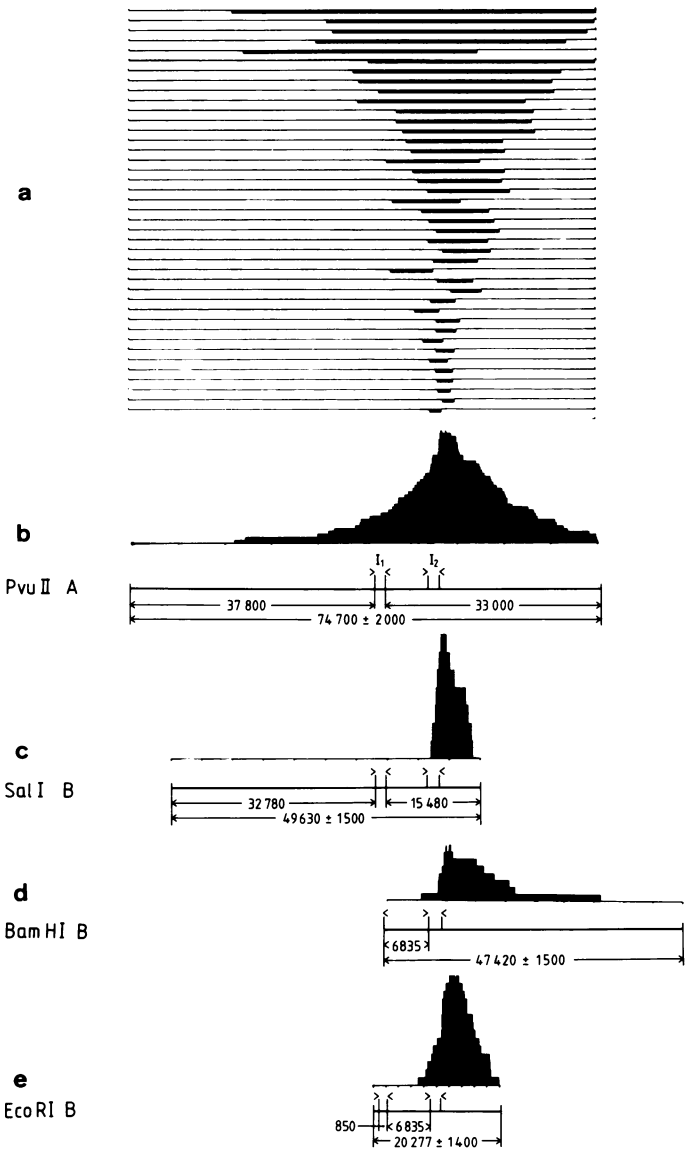

Fig. 2. Measurements of replicative loops on different restriction enzyme fragments. (**a**) Individual molecules of fragments *Pvu*II A. The thin line represents the non-replicated double-stranded ends of the fragment, the black bars the replicative loops. (**b**) A histogram derived from the measurements shown in (**a**), black areas represent the replicated regions. Histograms derived in the same way as in (**b**) are shown in (**c**) of *Sal*I fragment B, (**d**) of *Bam*HI fragment B, and (**e**) of *Eco*RI fragment B. The positions of the inverted repeats I1 and I2 within the restriction fragments are indicated in the maps below each histogram. The distances are given in number of base pairs. The fragments were arranged by lining up the positions of I1.

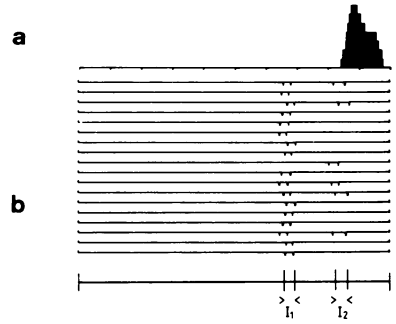

Fig. 3. Measurements of the positions of the inverted repeats I1 and I2 in the fragment *Sal*I B. The positions of the replicative loops summarized in the histogram (**a**) are compared with the maps of the individual single-stranded molecules shown in (**b**). The marks indicate the positions of the inverted repeat sequences.

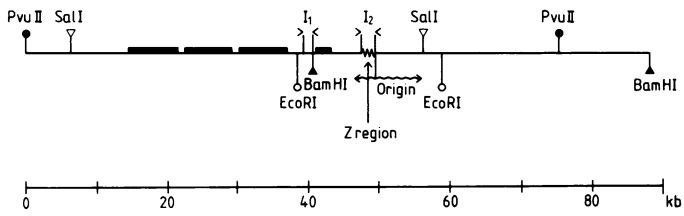

Fig. 4. Schematic drawing of the position of the origin of replication. The black bars indicate the positions of the three rRNA operons and of the extra 16S rRNA gene. The extensions of the loops formed by the inverted repeats I1 and I2 are indicated by arrows. Z-region designates the region of variable size.

Discussion

A region of heterogeneous size in cpDNA of *E. gracilis* had been described earlier (Jenni *et al.*, 1981) and had been mapped on a *Bgl*II fragment (called Z-fragment) which is in the middle of the *Eco*RI fragment B. The difference between the minimal and maximal size was estimated to be ~800 bp. In a recent analysis of two clones isolated from this region it was found that the variable sequence is flanked by the short inverted repeat I2 and that it is extremely AT-rich (Schlunegger *et al.*, in preparation). The origin of replication has now been mapped directly at, or very close to, the right one of the two palindromic sequences. It is not clear whether the fluctuation in size is a reflection of a heterogeneous chloroplast population or of unequal crossing-over within the chloroplasts.

In many organisms the origin of replication has been closely correlated with AT-rich sequences that are organised in tandem and inverted repeat structures, e.g., in *Escherichia coli* (Messer *et al.*, 1978) and in many plasmid, virus, and mitochondrial DNAs (for review, see Kasamatsu and Vinograd, 1974; Kolter and Helinski, 1979). Size variation of the AT-rich origin-associated sequences within one species has not been described before. It was found, however, that the variation of sizes of mitochondrial DNAs from different species of *Drosophila* is due to size differences in an AT-rich region ranging from ~400 to 4000 bp (Fauron and Wolstenholme, 1976). This region is near the origin of replication. In this case synthesis starts near the AT-rich region and proceeds unidirectionally around the molecule (Goddard and Wolstenholme, 1978). In other cases such as mouse L cell mitochondria (Robberson *et al.*, 1972) replication proceeds for about two-thirds of the molecule before synthesis of the second strand starts at another defined position.

In *Euglena* cpDNA even the smallest replicative loops identified (of a size around 1500 bp) showed double-stranded DNA on both arms of the replicative loops. The fact that a lag phase was observed for the extension of the replicative loops to the left might, therefore, point to a block in the DNA synthesis in this direction, rather than to a delay in the initiation of synthesis on the opposite strands of the replicative loops. Synthesis proceeds in both directions around the circle after an initial lag phase, corresponding to the synthesis of ~5000 bp, during which most of the replicative loops grow in the direction away from the rRNA genes. A few molecules do not follow that rule as can be seen in Figure 2a, and show synthesis originating at the same site but proceeding in the opposite direction.

A short single-stranded tail of varying length is often visible at the fork. It may have been spliced out by branch migration or it may represent an *in vivo* configuration in which the newly synthesised strand is kept single-stranded by the proteins of the replicative complex. Deproteinization may then result in the preferential re-annealing of the parental DNA strands.

In the case of *Euglena* cpDNA we did not obtain evidence for the initiation of DNA synthesis at a second start site as was suggested by the presence of two displacement loops in pea cpDNA (Kolodner and Tewari, 1975a).

Materials and methods

Preparation of cpDNA

E. gracilis strain Z was grown in Difco *Euglena* Broth in 500 ml cultures, in a growth chamber providing a 12 h light period at 22°C and a 12 h dark period at 18°C. 100 ml of the culture was diluted into fresh medium every 3 days. The cells were harvested 2 h after the onset of the dark period. The cells were lysed with a French pressure cell and the chloroplasts were purified with a Percoll gradient. The chloroplast lysate was passed over a Sepharose C1-2B column. Details of the preparation method have been described before (Koller and Delius, 1980, 1982b). The DNA eluted from the column was used directly for digestion with one of the restriction enzymes *Pvu*II, *Sal*I, *Bam*HI, or *Eco*RI (New England Biolabs, Beverly, MA).

Electron microscopy

After digestion with restriction enzymes, 10% SDS was added to a final concentration of 1%, and the sample of 100 µl was passed over a small Sepharose C1-2B column (45 x 5 mm) which was equilibrated in 10 mM Tris, 1 mM EDTA. The DNA was then spread with cytochrome. Electron microscopy and length measurements were performed as described before (Davis *et al.*, 1971; Koller and Delius, 1980).

For the determination of the position of the inverted repeats, the DNA was denatured by immersing the tube in boiling water for 60 s, incubated at 37°C for 10 min in the spreading mixture without the cytochrome, to allow renaturation and then spread as usual.

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