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How to cite

VALLET, Jean-Marie Philippe, ROCHAIX, Jean-David. Chloroplast origins of DNA replication are distinct from chloroplast ARS sequences in two green algae. In: Current Genetics, 1985, vol. 9, n° 4, p. 321–324. doi: 10.1007/BF00419962

This publication URL: https://archive-ouverte.unige.ch/unige:150768
Publication DOI: 10.1007/BF00419962

Current Genetics (1985) 9:321–324

Current Genetics

Chloroplast origins of DNA replication are distinct from chloroplast ARS sequences in two green algae

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Summary. A 5.3 kb chloroplast restriction fragment of *Chlamydomonas reinhardii* containing an origin of DNA replication and a sequence capable of promoting autonomous replication in *C. reinhardii* (ARC sequence) also carries an ARS sequence (autonomous replication in yeast). The ARC and ARS elements have been physically mapped and shown to be distinct from the origin of DNA replication. Similarly, restriction fragments containing the origin of chloroplast DNA replication from *Euglena gracilis* are unable to promote autonomous replication in yeast.

Key words: ARS sequences – Chloroplast – Origin of DNA replication – *Chlamydomonas reinhardii*

Introduction

ARS elements are defined as DNA segments capable of promoting autonomous replication in yeast (Struhl et al. 1974). Plasmids containing these elements transform yeast very efficiently and they are mitotically and meiotically unstable (Stinchcomb et al. 1979; Hsiao and Carbon 1979). Although several features are shared between ARS sites and origins of DNA replication in yeast, their identity has not yet been proven. ARS sequences occur at a frequency which is compatible with the spacing of origins of replication observed by electron microscopy (Beach et al. 1980; Newlon and Burke 1980; Chan and Tye 1980). Initiation of DNA replication of the yeast 2μ circles has been shown to occur both in vivo (Newlon et al. 1981) and in vitro (Kojo et al. 1981; Celniker and Campbell 1982) at or very near the ARS site (Broach and Hicks 1980; Broach et al. 1981). While ARS elements have been found in several eukaryotic DNAs (Stinchcomb et al. 1980), they have not been detected in prokaryotic DNAs except in a plasmid from *Staphylococcus aureus* (Goursot et al. 1983).

Several mitochondrial and chloroplast DNAs also contain ARS sequences (Zakian 1981; Blanc and Dujon 1981; Hyman et al. 1981, Tudzynski and Esser 1983; Loppes and Denis 1983, Uchimiya et al. 1983, Vallet et al. 1984). At least seven ARS elements have been isolated and mapped on the chloroplast genome of *Chlamydomonas reinhardii* (Loppes and Denis 1983; Vallet et al. 1984, cf. Fig. 1). Three of these have been sequenced (Vallet et al. 1984) and found to contain elements that are related to the 11 bp yeast ARS core consensus sequence (Stinchcomb et al. 1981; Broach et al. 1982).

Recently DNA segments from *C. reinhardii* have been isolated that are able to promote autonomous replication in this alga (Rochaix et al. 1984). Four of these ARC elements (autonomous replication in Chlamydomonas) have been characterized and shown to map at four distinct sites of the chloroplast genome (Fig. 1). They contain two semi-conserved AT rich sequences I and II of 19 and 12 bp. One of these ARC elements has been localized on the 5.3 kb chloroplast EcoRI fragment R13 which has been shown to contain one of the origins of DNA replication by Waddell et al. (1984). Here we present a fine structure analysis of this region which shows that the ARC elements and the origin of replication are located 1.5 kb from each other. During the course of this work we found that the R13 fragment contains an ARS sequence (indicated by 08 in Fig. 1) which has been localized in a region which overlaps neither the ARC site nor the origin of replication.

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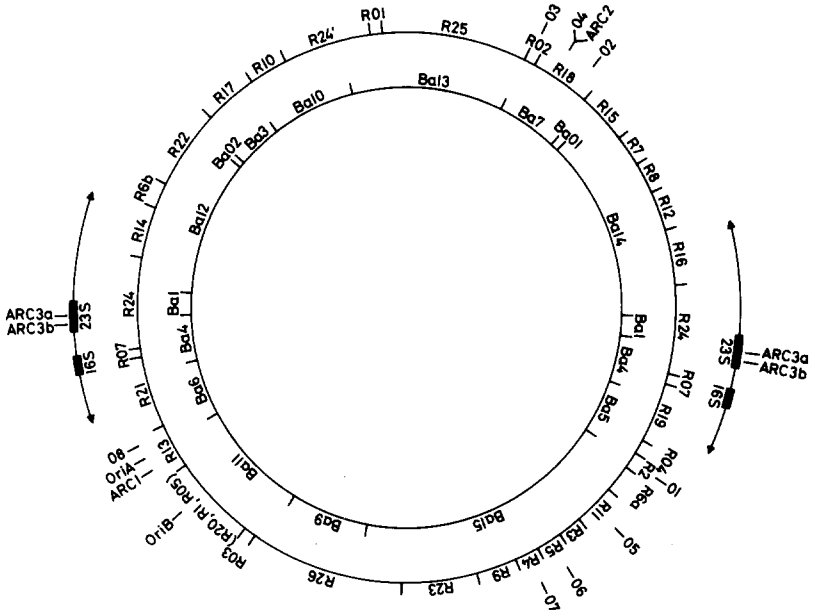

Fig. 1. Location of ARS and ARC sites on the chloroplast genome of *C. reinhardii*. The outer and inner circles represent the EcoRI and BamHI restriction maps, respectively (Rochaix 1978). The inverted repeats containing the chloroplast ribosomal RNA genes are shown on the outside. ARS sites are marked by 01 to 08 (Loppes and Denis 1983; Vallet et al. 1984). The ARC sites (Rochaix et al. 1984) and the two origins of replication OriA and OriB (Waddel et al. 1984) are indicated

Materials and methods

Enzymes. Restriction endonucleases, T4 ligase and DNA polymerase (Klenow), were purchased from Genofit (Geneva) and Biofinex (Praroman) and were used as recommended by the supplier.

Cloning. In order to map the ARS site of R13 the following plasmids were constructed (cf. Fig. 2 for the location of the fragments). The EcoRI-ClaI fragment I was first introduced into the corresponding sites of pBR322 and the HindIII fragment of plasmid pYeARG4 containing the yeast ARG4 gene (Clarke and Carbon 1978) was put into the unique HindIII site of the recombinant plasmid containing fragment I. The EcoRI-BamHI fragment II was inserted into the corresponding sites of plasmid pY1p5 containing the yeast URA3 gene (Struhl et al. 1979). The ClaI-BamHI fragment III and the ClaI-ClaI fragment IV were inserted into the corresponding sites of pJD2. The Bam-EcoRI fragment V was cloned into the corresponding sites of pBR322 producing plasmid pR13B. The yeast ARG4 gene was cut as a SalI-EcoRI fragment from pJD2 (Vallet et al. 1984), made flush-ended and inserted into the flush-ended SalI site of pR13B.

Transformation. Selections with the ARG4 and URA3 markers were performed in the *Saccharomyces strains* S2072A (*a, arg*4, *leu*1, *trp*1, *gal*2) and S-150-123 (a, *leu*2-3, *trp*289, *his*3-1, *ura*3-52) obtained from the yeast genetic Stock Center (Berkeley) and from P. Malnoe (Biogen), respectively. Yeast protoplasts were transformed as described by Hinnen et al. (1978) and transformants were selected on minimal medium with the required nutrients. ARS activity of plasmids was recognized by the high transformation efficiency (more than 500 transformants per μg of DNA) and by the fact that the intact plasmid could be recovered from the transformed yeast. The pJD2 and Y1p5 plasmids did not give rise to transformants under the same conditions.

DNA. Plasmid DNAs were prepared as described by Katz et al. (1973) or by Birnboim and Doly (1979). DNA sequencing was performed by the chemical cleavage method of Maxam and Gilbert (1980).

Results and discussion

One of the plasmids capable of replicating autonomously in *C reinhardii,* pCA2, was found previously to carry a 153 bp Sau3A fragment which hybridizes to the chloroplast EcoRI fragment R13 (Rochaix et al. 1984). In order to map the ARC element on R13, the fragment was cut with BamHI and hybridized with the pCA2 plasmid. Only the larger EcoRI-BamHI fragment of R13 (cf. Fig. 2) produced a signal (data not shown). Mapping of the Sau3A sites of this fragment (Fig. 2) revealed the presence of a fragment of equal size as the insert of pCA2. Sequencing of the 153 bp Sau3A fragment of R13 showed that its sequence is identical to that of the insert of pCA2 which was established previously (Rochaix et al. 1984). It can be concluded that no sequence rearrangements have occurred in this fragment during its propagation in *C. reinhardii,* a feature which had already been observed with another ARC element (Rochaix et al 1984).

Recently Waddell et al. (1984) have been able to localize two origins of replication OriA and OriB on the chloroplast genome of *C. reinhardii* by observing replication forks on chloroplast restriction fragments in the electron microscope. One of the origins OriA was mapped in the middle of fragment R13 in the central clal fragment (Wang et al. 1984; Fig. 2). Our mapping results indicate that the ARC element and the origin of replication are distant from each other by about 1.5 kb. The close distance between these two elements is intriguing and it remains to be seen whether this ARC element plays a role in the initiation of chloroplast DNA replication. Wang et al. (1984) have compared the chloroplast DNA regions con-

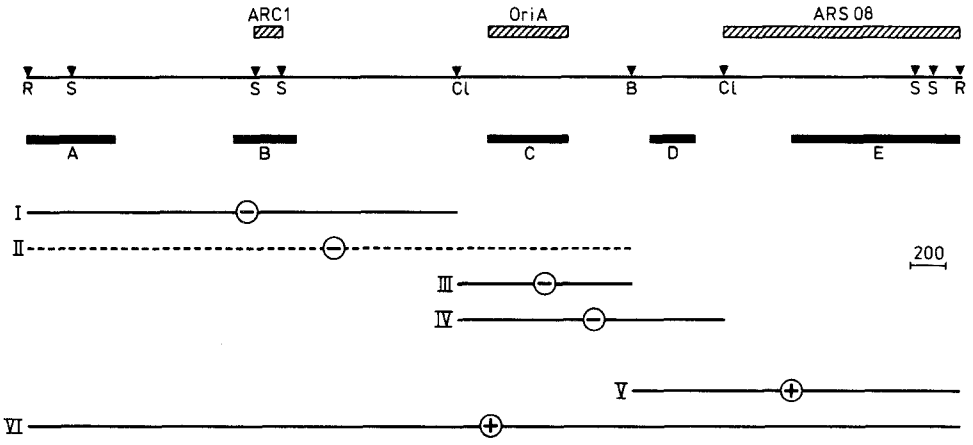

Fig. 2. Physical and functional map of the chloroplast EcoRI fragment R13 of *C. reinhardii*. Restriction sites are indicated by R, EcoRI, S, Sau3A; Cl, ClaI, B, BamHI. The ClaI sites were determined by Wang et al. (1984). Fragments drawn with continuous and discontinuous lines were cloned into plasmids containing the yeast *ARG4* locus (Vallet et al. 1984; Clarke and Carbon 1978) and into Y1p5 containing the yeast *URA3* locus (Struhl et al. 1978), respectively. Fragments able and unable to promote autonomous replication in yeast are marked with + and −, respectively. Homologous regions between *C. reinhardii* and *Chlamydomonas* strain WXM are indicated by A, B, C, D, E (Wang et al. 1984). Region C contains the origin of replication OriA (Wang et al. 1984). Region B contains the ARC1 sequence. The ARS 08 sequence is located on the 1.3 kb ClaI-EcoRI fragment

taining the origin of replication of *C. reinhardii* and *Chlamydomonas* strain WXM, whose chloroplast DNA restriction patterns differ markedly. Five regions of homology were detected which are indicated by A to E in Fig. 2. While region C contains one origin of replication in both species (Wang et al. 1984), it is interesting to note that the ARC I element is contained within the 0.35 kb of region B. Schlunegger and Stutz (1984) have recently found that a large portion of the conserved element II of ARC I is present very near the chloroplast origin of replication of *Euglena gracilis*.

In order to test for the presence of ARS elements in fragment R13, it was inserted into the single EcoRI of plasmid pJD2 which consists of pBR322 and the ARG4 locus of yeast (Vallet et al. 1984). This new plasmid was found to replicate autonomously in yeast (Fig. 2) while pJD2 does not. The ARS element of R13 was localized more precisely by subcloning several fragments of R13 in the pJD2 and Y1p5 plasmids and by testing for autonomous replication in yeast (cf Materials and methods for the construction of these plasmids). The results are summarized in Fig. 2. It can be seen that the ARS sequence is located within the 1.3 kb Cla-EcoRI fragment and is therefore clearly distinct from the origin of replication oriA.

The origin of replication has also been mapped in the chloroplast genome of *Euglena gracilis* (Koller and Delius 1982; Ravel-Chappuis et al. 1982). Plasmids pEgcH2 and pEgcB6 containing a 6.1 kb HindIII and a 6.3 BglII chloroplast DNA fragment from *E. gracilis*, respectively, were constructed by Schlunegger et al. (1983). The overlapping region of these two fragments contains the origin of replication. The fragments were inserted into the HindIII and BamHI sites of the pJD2 plasmid, respectively. In spite of several attempts, no evidence for autonomous replication of those recombinant plasmids in yeast could be obtained. We conclude that in *C. reinhardii* and in *E. gracilis* the origins of replication of chloroplast DNA do not have ARS activity. Since the yeast ARS consensus sequence is highly AT rich (Stinchcomb et al. 1981; Broach et al. 1982) it is possible that the large number of chloroplast ARS elements in *C. reinhardii* is a consequence of its high AT content. It is striking that ARS elements appear at a considerably lower frequency in the GC rich nuclear genome of *C. reinhardii* (Loppes and Denis 1984). Our results demonstrate that it is hazardous to draw firm conclusions on the location of authentic origins of replication when heterologous systems are used for assaying DNA replication.

Acknowledgments. Plasmids pEgcH2 and pEgcB6 were kindly provided by Dr. E. Stutz. We thank J. Erickson and M. Goldschmidt-Clermont for helpful comments and O. Jenni for drawings and photography. This work was supported by grants 3.258.082 and 3.587.084 from the Swiss National Foundation.

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Communicated by H. Kössel

Received October 23, 1984