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Localization and sequence analysis of chloroplast DNA sequences of *Chlamydomonas reinhardii* that promote autonomous replication in yeast

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Four distinct chloroplast DNA segments from *Chlamydomonas reinhardii* of 400, 415, 730 and 2300 bp which promote autonomous replication in yeast have been mapped on the chloroplast genome. Plasmids carrying these chloroplast DNA fragments are unstable in yeast when the cells are grown under non-selective conditions. Sequence analysis of three of these chloroplast ARS regions (autonomously replicating sequences in yeast) reveals a high AT content, numerous short direct and inverted repeats and the presence of at least one element in each region that is related to the yeast ARS consensus sequence A/T TTTATPuTTT A/T. These three chloroplast regions share, in addition, two common elements of 10 and 11 bp which may play a role in promoting autonomous replication.

Key words: ARS elements/*Chlamydomonas reinhardii*/ chloroplast DNA/DNA replication origin

Introduction

Yeast ARS sequences (autonomously replicating sequences) are defined by their ability to promote autonomous replication of plasmids in yeast (Struhl *et al.*, 1979). These plasmids transform yeast at a high frequency and they are mitotically and meiotically unstable (Stinchcomb *et al.*, 1979; Hsiao and Carbon, 1979). Several observations suggest that ARS elements represent nuclear origins of replication. They occur about once every 30–40 kb in the yeast genome (Beach *et al.*, 1980; Chan and Tye, 1980), a frequency which is similar to that found for origins of DNA replication (Newlon and Burke, 1980). The ARS site of yeast 2μ circles (Broach and Hicks, 1980; Broach *et al.*, 1981) appears to overlap the origin of replication whose location has been determined both *in vivo* (Newlon *et al.*, 1981) and *in vitro* (Kojo *et al.*, 1981; Celniker and Campbell, 1982).

Eukaryotic, but not *Escherichia coli* DNA segments have been shown to act as ARS elements in yeast (Stinchcomb *et al.*, 1980). It was also found that a 2.2-kb mitochondrial DNA restriction fragment of *Xenopus laevis*, containing the origin of DNA replication, promotes autonomous replication in yeast (Zakian, 1981). Several ARS sequences have been found in the mitochondrial DNA from yeast (Blanc and Dujon, 1981; Hyman *et al.*, 1982) and at least one sequence of this sort has been detected in the mitochondrial genome of *Cephalosporium acremonium* (Tudzynski and Esser, 1983). These findings raised the question of whether other organellar DNA replication origins may be active in yeast. Since chloroplast DNA consists of large circular molecules with a size ranging between 130 and 190 kb (Bedbrook and Kolodner, 1979), it is not an easy task to map their DNA replication origins. Only in the case of *Euglena* has one origin of replication been accurately mapped (Koller and Delius, 1982; Ravel-Chapuis *et al.*, 1982). Screening for chloroplast ARS sequences in yeast would provide a rapid test for these origins of replication. On the other hand, chloroplast ARS sequences may bear no relationship to chloroplast DNA sites involved in the initiation of DNA replication. In any case, a comparative sequence analysis with authentic yeast ARS elements may provide new insights into the sequence specificity of yeast ARS sites. It therefore seemed of interest to search for ARS elements in the chloroplast genome of *Chlamydomonas reinhardii*. Here we describe the isolation, localization, properties and sequence analysis of several of these sequences.

Results

Strategy for isolating chloroplast ARS sequences

In *C. reinhardii* the *arg7* locus codes for arginino succinate lyase, the last enzyme in the arginine biosynthetic pathway which converts arginino succinate into arginine and fumarate (Gillham, 1965; Loppes and Matagne, 1972). In yeast and *E. coli* the corresponding loci are *arg4* and *argH*, respectively. We have deliberately chosen arginine-independent growth as a selective marker because of the possibility of using it in *C. reinhardii* (Rochaix and van Dillewijn, 1982), yeast and *E. coli* (Clarke and Carbon, 1978). Accordingly, a 2.7-kb yeast nuclear *Hind*III fragment containing the *arg4* locus was isolated from plasmid pYearg4 (Clarke and Carbon, 1978). This fragment was made flush-ended with DNA polymerase and deoxynucleotide triphosphates and it was inserted by blunt-end ligation into the *Eco*RI site of pBR322 whose ends had also been filled. A plasmid pJD2 was recovered that contains the *arg4* locus and is resistant to ampicillin and tetracycline (Figure 1). The *Eco*RI site has been reconstructed in this plasmid, presumably because the two terminal bases at the *Hind*III site were digested during the construction of the plasmid.

Chloroplast DNA was digested with *Hind*III or *Mbo*I and the fragments were inserted into the *Hind*III or *Bam*HI site of pJD2. Recombinant plasmids were recognized by their resistance to ampicillin and their sensitivity to tetracycline. Pools of 300 plasmids containing chloroplast *Hind*III fragments and of 400 plasmids containing chloroplast *Mbo*I fragments were prepared and the DNA was used to transform a yeast *arg4* strain, selecting for arginine-independent growth (cf., Materials and methods).

Because the pJD2 plasmid does not replicate in yeast, only recombinant plasmids carrying chloroplast ARS sequences should be able to transform yeast at a high frequency. Indeed, a large number of yeast transformants were obtained with the *Hind*III and *Mbo*I plasmid pools. In each case, 100 clones were grown separately and pooled for DNA extraction. The DNA of the plasmids was introduced into *E. coli* by transformation and the plasmid DNA of individual clones was examined. Eight *Hind*III clones and eight *Mbo*I clones

were arbitrarily chosen for further study.

Localization of ARS sequences on the chloroplast genome of C. reinhardii

The DNA of the recombinant plasmids able to replicate autonomously in yeast was labelled by nick translation and hybridized to chloroplast DNA digested with *Eco*RI and *Bam*HI which had been transferred after gel electrophoretic separation onto nitrocellulose filters (Figure 2; Southern, 1975). These hybridizations revealed four distinct classes of chloroplast ARS sequences. When plasmid pCM2, a representative of the first class 01, is cleaved with *Sau*3A (an isoschizomer of *Mbo*I), a 400-bp DNA fragment is released that hybridizes to chloroplast *Eco*RI fragment R2 and *Bam*HI fragment Ba5 (Figure 2, lanes a1, b1). The location was verified on the corresponding cloned R2 fragment (Figure 2, lane c1). The three other ARS sites 02, 03 and 04 are represented by plasmids pCH8, pCM3 and pCA1, respectively.

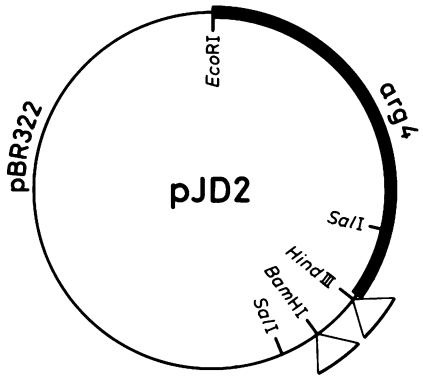

Fig. 1. Map of pJD2 plasmid. The yeast *arg4* locus is indicated by a thick line. The sites used for cloning are marked by triangles.

The ARS activity of the pCA1 plasmid was found accidentally while we were searching for *C. reinhardii* DNA sequences promoting autonomous replication in this alga. A detailed description of its isolation will be published elsewhere. Plasmid pCM3 contains a 2.2-kb *Bam*HI fragment that hybridizes to the chloroplast *Eco*RI fragments R25, R18 and R02 (Figure 2, lane a3) and to the *Bam*HI fragment Ba7 (Figure 2, lane b3). The same plasmid hybridizes to the cloned R18 and R02 fragments (Figure 2, lane d3). The pCH8 and pCA1 plasmids contain a 3.2-kb *Hind*III (which co-migrates with the internal 3.2-kb *Hind*III fragment of R18) and a 415-bp *Sau*3A fragment, respectively, both of which hybridize to the chloroplast fragments R18 and Ba7 (Figure 2, lanes a2, a4, b2, b4). These hybridizations were confirmed with the cloned chloroplast DNA fragments R18 and the 2.2-kb internal *Hind*III fragment of R18 (Figure 2, lanes d2, e4 respectively). No cross-hybridization is detectable between the inserts of these four ARS plasmids (data not shown). After repeated shuttling between yeast and *E. coli* no structural alteration was detectable in the chloroplast DNA inserts of these plasmids. The location of these chloroplast ARS elements on the chloroplast genome of *C. reinhardii* is shown in Figure 3.

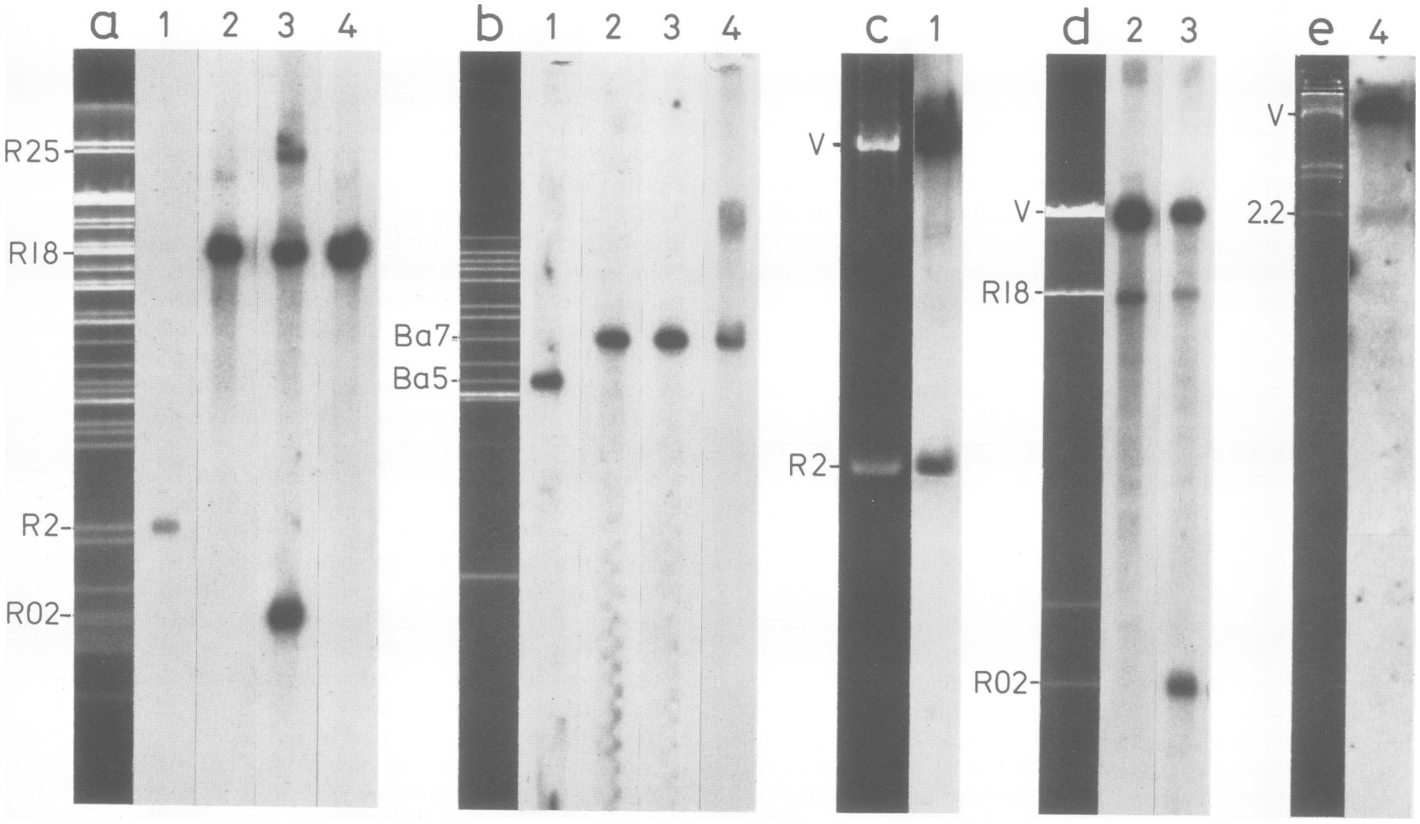

Fig. 2. Hybridizations of plasmids containing chloroplast ARS elements to authentic and cloned chloroplast DNA restriction fragments of *C. reinhardii*. Agarose gel electrophoretic patterns of chloroplast DNA digested with *Eco*RI **(a)**, *Bam*HI **(b)** and of *Eco*RI-digested plasmids containing the chloroplast *Eco*RI fragments R2 **(c)**, R18 and R02 **(d)**. **Lane e** shows a *Hind*III-*Eco*RI digest of a plasmid containing the chloroplast *Eco*RI fragment R18. The upper band V represents the vector plasmid pCRI (Covey *et al.*, 1976). **Lanes 1, 2, 3, 4** represent autoradiograms of Southern hybridizations of the DNAs described above with the ^{32}P-labelled plasmids pCM2, pCH3, pCM3 and pCA1, respectively. The band marked 2.2 in **lane 4** corresponds to the 2.2-kb *Hind*III fragment of R18 (cf., Figure 5).

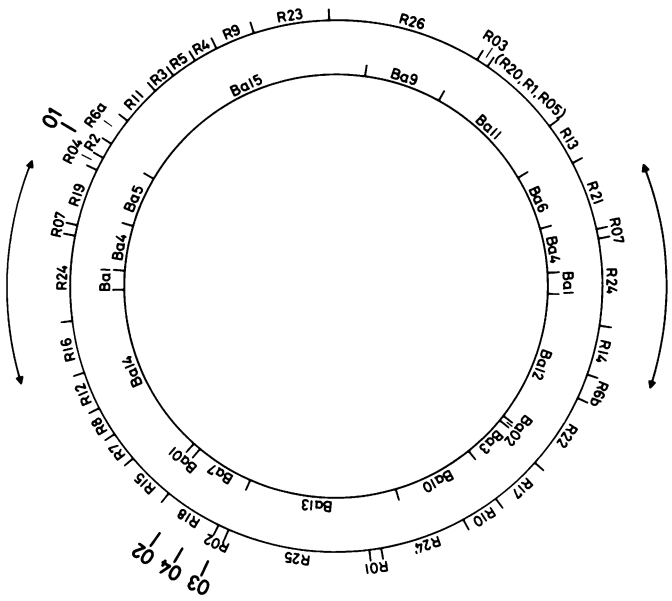

Fig. 3. Location of the chloroplast ARS elements 01, 02, 03 and 04 on the physical map of the chloroplast genome of *C. reinhardii*. The outer and inner circles represent the *Eco*RI and *Bam*HI fragments, respectively. Arrows on the outside indicate the inverted repeats containing the chloroplast rRNA genes (Rochaix, 1978).

Table I. Properties of chloroplast ARS sequences

Plasmid	ARS site	Transformants/μg	T_0 (h)	Stability	
				A	B
pCM2	01	200	8.5	95.5	100
pCH8	02	1920	3.0	79	98
pCM3-HH916	03	830	3.9	80	98
pCA1	04	8	7.9	32	64

T_0 is the doubling time in minimum medium without arginine. The untransformed strain 2072 *arg4* has a doubling time of 2.7 h in minimum medium with arginine. Columns A and B indicate the percentage of cells unable to grow in the absence of arginine after 6 and 30 generations of non-selective growth, respectively. pCM3-HH916 is a recombinant plasmid between pJD2 and the 916-bp *Hind*III fragment of pCM3.

Properties of chloroplast ARS sequences

With the exception of pCA1, the other plasmids pCM2, pCH8 and pCM3 transform yeast at a high frequency as expected for autonomously replicating plasmids (Table I). The doubling times of these plasmids range from 3.0 to 8.5 h under selective growth conditions. Table I also shows that these plasmids are lost rapidly when the yeast cells are grown under non-selective conditions, except pCA1 which is slightly more stable.

Sequence analysis of the chloroplast ARS sequences 01, 03 and 04

A map of the 1.9-kb chloroplast *Eco*RI fragment R2 is shown in Figure 4. The 01 sequence is contained within a 400-bp *Bam*HI-*Sau*3A fragment which was cloned. Digestion of the recombinant PCM2 plasmid with *Sal*I and *Bam*HI generates a 675-bp fragment (Figure 4), which was isolated, cut with *Sau*3A, labelled at its 3′ or 5′ ends, strand separated and sequenced. A striking feature of this sequence is its high AT content (75%) with a short 21-bp GC-rich island (62%) and the presence of numerous short 8–10 bp direct and inverted

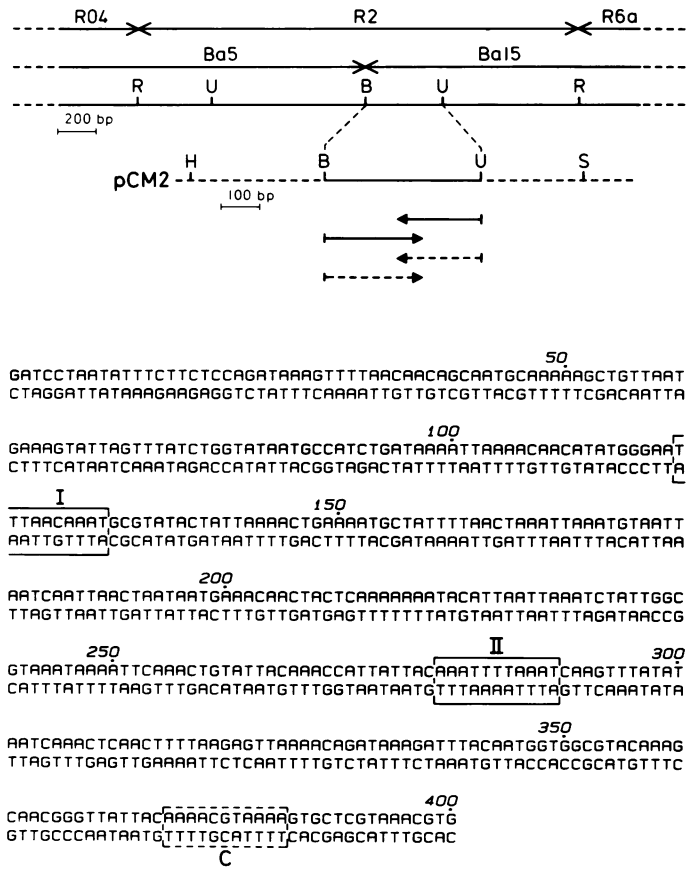

Fig. 4. Organization and sequence of the chloroplast DNA region containing the ARS element 01. The restriction maps of this region in the chloroplast genome and in plasmid pCM2 are shown in the upper part of the figure. The sequencing strategy is indicated. 5′ and 3′ end-labelled fragments are marked by ⊢⟶ and ⊢ — → respectively. The common regions I and II are framed (cf., text). Sequences related to the yeast ARS consensus sequence A/T TTTATPuTTT A/T are indicated by dashed line boxes C. Restriction endonuclease sites are indicated by B, *Bam*HI; H, *Hind*III; R, *Eco*RI, S, *Sal*I; U, *Sau*3A.

repeats (cf., Figure 7 and Discussion).

A restriction map of the chloroplast DNA region containing the 03 sequence is shown in Figure 5. This ARS sequence was first located on a 2.2-kb *Bam*HI fragment of the pCM3 plasmid. To localize this sequence more precisely, the two internal *Hind*III fragments of 916 and 330 bp and the two outer *Bam*HI-*Hind*III fragments of 680 and 240 bp were subcloned into pJD2 and the new recombinant plasmids were used to transform a yeast *arg4* strain. Only the 916-bp fragment promotes autonomous replication. Digestion of this fragment with *Sal*I generates two fragments of 730 and 186 bp. Subcloning of these fragments into the Y1p5 plasmid (Struhl *et al.*, 1979) and transformation of a yeast *ura3*$^-$ strain reveals that the ARS sequence is contained within the larger fragment. The sequence of the 916-bp *Hind*III fragment was determined using the strategy shown in Figure 5. This fragment is 74% AT-rich and it contains several 10-bp direct repeats and several 10-, 12- and 16-bp inverted repeats (cf., Figure 7 and Discussion). It is noteworthy that the entire sequence of the 916-bp *Hind*III fragment corresponds to a portion of an unidentified open reading frame of 307 amino acids (data not shown).

The chloroplast ARS sequence 04 is contained within a 415-bp *Sau*3A-*Bam*HI fragment which has been inserted into

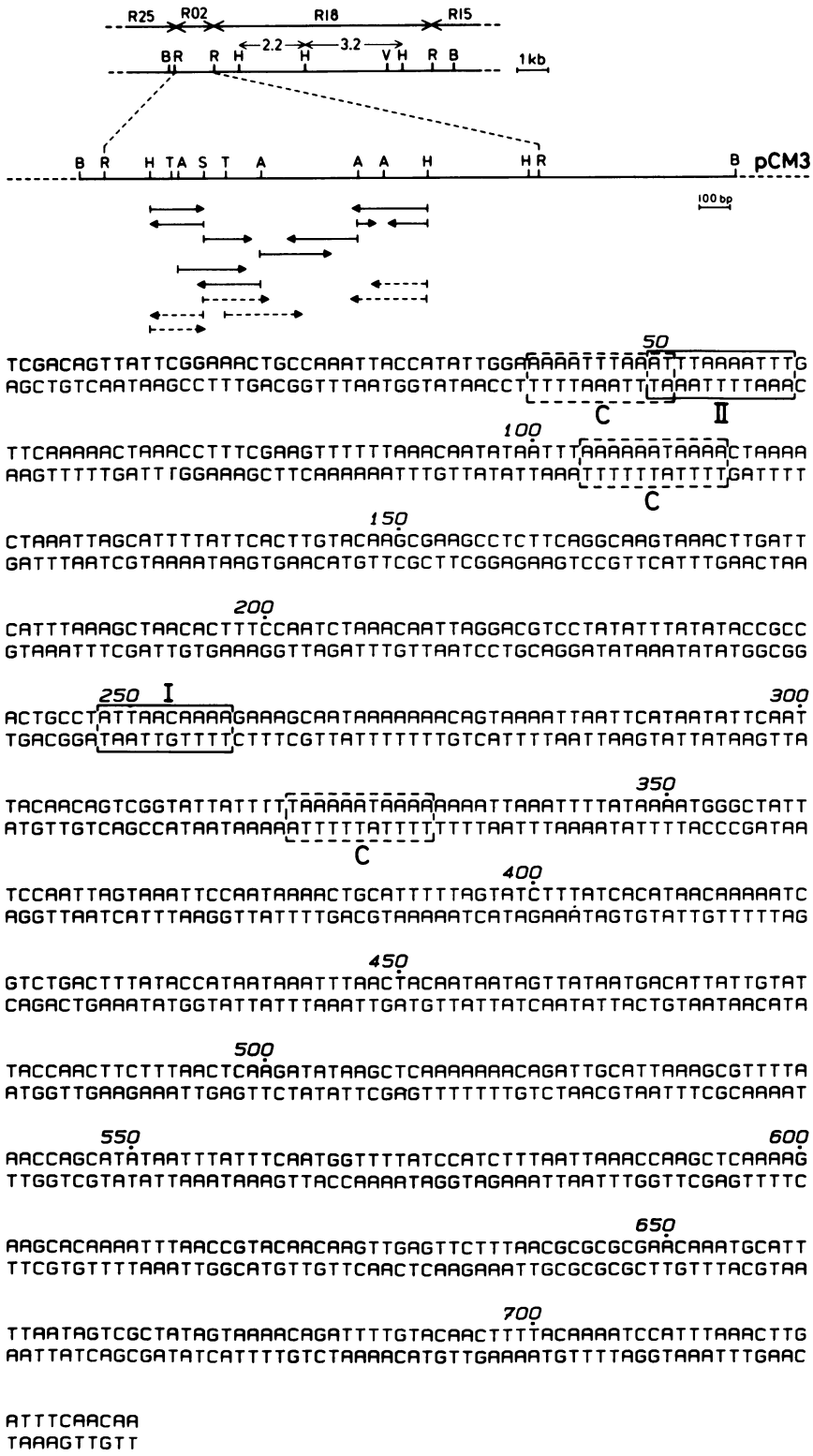

Fig. 5. Organization and sequence of the chloroplast DNA region containing the ARS element 03. The restriction maps of this region in the chloroplast genome and in plasmid pCM3 are shown in the upper part of the figure. Restriction endonuclease sites are indicated by A, *Alu*I; T, *Taq*I, V, *Eco*RV. Other symbols are as in Figure 4.

the *Bam*HI site of pJD2 (Figure 6). A 760-bp *Hind*III-*Bam*HI fragment was isolated from the recombinant plasmid, recut with *Taq*I, end-labelled at the 5′ or 3′ ends, strand separated and sequenced. The sequence is 74% AT-rich and contains several 8–9 bp direct repeats. A striking feature is the presence of a 26-bp inverted repeat and of smaller inverted repeats of 8–10 bp (cf., Figure 7 and Discussion).

Discussion

It is well documented that chloroplast genomes display

Fig. 6. Sequence of the chloroplast DNA region containing the ARS element 04. Restriction map and sequencing strategy for this region are given. Symbols are as in Figure 4. The 26-bp inverted repeats are indicated in the sequence by arrows.

several prokaryotic features. Chloroplast rRNA sequences are remarkably related to their bacterial counterparts (Schwarz and Kössel, 1981; Edwards and Kössel, 1981). This homology is also valid for some chloroplast protein genes which have been identified by using *E. coli* gene probes (Watson and Surzycki, 1982, 1983). Similarly, sequences preceding and following chloroplast genes contain elements which strongly resemble prokaryotic promoters and terminators (Whitfeld and Bottomley, 1983). However, the presence of introns in chloroplast genes coding for rRNA (Rochaix and Malnoe, 1978), tRNA (Koch *et al.*, 1981) and proteins (Stiegler *et al.*, 1982; Erickson *et al.*, 1983) is a distinctive eukaryotic character. The ability of certain chloroplast fragments to promote autonomous replication in yeast appears to be another eukaryotic feature since this property is shared by eukaryotic, but not by *E. coli* DNA (Stinchcomb *et al.*, 1980). However, Goursot *et al.* (1982) have recently shown that plasmids from *Staphylococcus aureus*, with a broad host range, are able to replicate in yeast. It remains to be demonstrated whether the same origins of replication are used in the prokaryotic and eukaryotic hosts.

At least four distinct chloroplast ARS sequences from *C. reinhardii* have been found. Whereas the 01 sequence is located on the *Eco*RI fragment R2, the other three ARS sequences are located close to each other on the *Eco*RI fragments R02 and R18 within a region of 7 kb. The three ARS segments that we sequenced, 01, 03 and 04 are contained within regions that are significantly richer in AT (74%) than

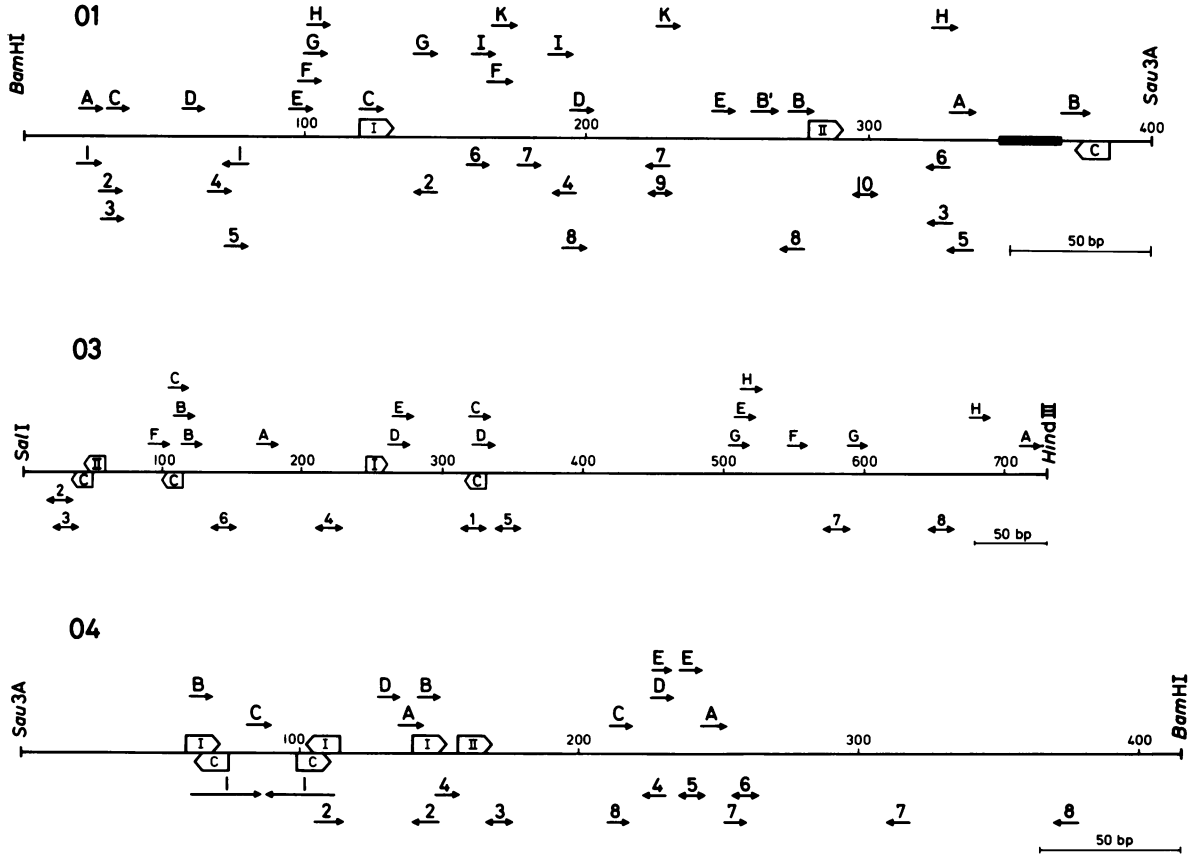

Fig. 7. Sequence organization of the chloroplast DNA regions containing the ARS elements 01, 03 and 04. 01 contains direct repeats of 9 bp (marked by A, B), 10 bp (B'), 8 bp (C–K) and inverted repeats of 9 bp (1) and 8 bp (2–10). The GC-rich island is indicated by a thick line. 03 contains direct repeats of 11 bp (A), 10 bp (B–H) and inverted repeats of 16 bp (1), 12 bp (2, 3, 4, 5) and 10 bp (6, 7, 8) with a dyad symmetry. 04 contains direct repeats of 9 bp (A), 8 bp (B–E) and inverted repeats of 26 bp (1), 10 bp (2) and 8 bp (3–8). The two common sequences I and II and the elements C related to the yeast ARS consensus sequence are indicated.

Table II. Relatedness of chloroplast ARS elements to the yeast ARS consensus sequence

Yeast consensus	5'A/T TTTATPTTT A/T
01	5' T TTTAcGTTT T
03	5' T TTTATtTTT A
	5' T TTTATtTTT T
04	5' A TTTATATTT g
	5' A TTTATATTT g

P stands for purine. Nucleotides that differ from the consensus sequence are shown in lower case letters.

the average chloroplast DNA (63%) (Sueoka *et al.*, 1967). Other structural features include the presence of multiple direct and inverted repeats (Figure 7). The 26-bp inverted repeat in the chloroplast ARS sequence 04 is especially noteworthy. Inverted repeats have been found near the mitochondrial DNA replication origin in HeLa cells (Crews *et al.*, 1979) and in yeast (de Zamaroczy *et al.*, 1981). The ARS1 and ARS2 sequences from yeast also contain inverted repeats (Tschumper and Carbon, 1980, 1982) although this property does not appear to be common for all yeast ARS sequences (Broach *et al.*, 1982).

Yeast ARS sites have been localized on sequences as short as 57 bp (Kearsey, 1983). Comparison on 10 yeast ARS sequences has revealed an 11-bp consensus sequence 5' A/T TTTATPuTTT A/T located in a high AT-rich region (Stinchcomb *et al.*, 1981; Broach *et al.*, 1982). Each of the chloroplast ARS sequences 01, 03 and 04 contain at least one 11-bp element which differs by only one bp from the yeast consensus sequence (Table II, marked by C in Figures 4, 5, 6 and 7). The sequence 04 contains two of these elements which fit best to the consensus and which are located within the 26-bp inverted repeat (Figures 6 and 7). Surprisingly pCA1, the plasmid containing this 04 sequence, transforms yeast at a significantly lower efficiency than the other chloroplast ARS sequences. It is possible that the long inverted repeat interferes with replication to some extent and/or that the ARS core sequence is not the only important determinant.

Comparison of the three sequenced chloroplast regions containing the ARS sites 01, 03 and 04 reveals two conserved sequences 5' ATTAACAAAT and 5' PuATTTTAAAT (boxed in Figures 4, 5 and 6 and also indicated in Figure 7). The first sequence I is present once in 01 and 03 and three times in 04, of which two copies are in the inverted repeat. The second sequence II appears once in each of the three sequences. There is no apparent conserved spatial relationship between these two elements in the three sequences examined. It can be calculated that elements of this type would occur once every 15 and 42 kb, respectively on a random basis in a DNA with 74% AT. Further subcloning of these chloroplast ARS regions will be required to test critically whether these conserved sequences play a role in promoting autonomous replication in yeast.

The relationship between chloroplast and mitochondrial ARS elements and authentic origins of replication is not yet clear. While the same mitochondrial DNA fragment which contains the origin of replication also carries an ARS element in *X. laevis* (Zakian, 1981), this does not hold for rat mitochondrial DNA (Zakian and Kupfer, 1982). Even in the former case the identity between the two elements has not been demonstrated. It will be of interest to examine whether the chloroplast origin of replication of *Euglena* which has been mapped (Koller and Delius, 1982; Ravel-Chapuis *et al.*, 1982) also acts as an ARS element, and conversely, whether the mapped ARS elements of *C. reinhardii* coincide with one or several chloroplast origins of replication.

Materials and methods

Strains

The *Saccharomyces cerevisiae* strains S2072A (*a, arg4, leu1, trp1, gal2*) and S-150-213 (*a, leu2-3, trp289, his3-1, ura3-52*) were obtained from the Yeast Genetic Stock Center (Berkeley, CA) and from P. Malnoe (Biogen), respectively. The *E. coli* strain C600 (*thr6, leuB, hsr*$^-$, *hsm*$^-$) was used for transformation.

Enzymes

Restriction endonucleases were from Genofit (Geneva) and Bethesda Research Laboratories Inc. and were used as recommended by the supplier. Polynucleotide kinase and *E. coli* DNA polymerase I and T4 ligase were from Genofit, DNA polymerase (Klenow fragment) was from Boehringer. β-Glucuronidase was from Pharmindustrie (Villeneuve la Garenne).

DNA

Plasmid DNA for large and small scale preparations were prepared as described by Katz *et al.* (1973) and Birnboim and Doly (1979), respectively. Yeast DNA was isolated according to Davis *et al.* (1980). Chloroplast DNA from *C. reinhardii* was prepared as described (Rochaix, 1980). Uncloned chloroplast DNA was cleaved with *Mbo*I while cloned chloroplast DNA was digested with the isoschizomer *Sau*3A. Cloning, *in vitro* DNA labelling and hybridizations were performed as described (Dron *et al.*, 1982). DNA sequencing was carried out by the chemical cleavage method of Maxam and Gilbert (1980). The DNA sequence analysis was performed on a Hewlett Packard computer, model 9845.

Transformation

Yeast protoplasts and intact cells were transformed with plasmids as described by Hinnen *et al.* (1978) and by Ito *et al.* (1983), respectively.

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