Structure of a *Cephalosporium acremonium* mtDNA replicator

Miguel A. Peñalva, Cristina Patiño and Víctor Rubio

Departamento de Genética Molecular, Antibioticos, SA, Bravo Murillo, 38, 28015 Madrid, Spain

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We have investigated the ARS (autonomously replicating sequence) activity of a 1.94 kb mitochondrial DNA fragment of *Cephalosporium acremonium* and found that several subfragments of this piece of mtDNA conferred the ARS phenotype. The nucleotide sequence of the fragment shows: (i) a high A+T content (72.5%); (ii) a perfect consensus ARS sequence (ATTTATATTTA) in the subfragment with the highest ARS activity; (iii) a large number of ARS consensus-related sequences in the other subfragments, even in one lacking ARS activity; (iv) several potential hairpin structures. One of them contains the perfect consensus ARS sequence.

Nucleic acid *Nucleotide sequence* *mitochondrial DNA* *Replication origin* (Cephalosporium acremonium)

1. INTRODUCTION

Autonomously replicating sequences (ARS) are segments of eukaryotic DNA which are capable of promoting self-replication of integrative yeast vectors [1]. They confer on the colinear DNA an easily selectable property: high frequency of transformation in yeast [2]. Several pieces of evidence which suggest that ARS sequences might represent chromosomal origins of DNA replication have been obtained, both in vivo [3–6] and in vitro [7], although conclusive data are lacking. Accordingly, ARS sequences have been identified in chromosomal DNA from several eukaryotes [8] which might be functional as replication origins in the homologous system. In addition to chromosomal ARS, these sequences have been found in extrachromosomal DNA [9–14]. On the basis that ARS sequences represent eukaryotic origins of DNA replication, it has been argued that organellar DNA replication origins might be identified because they should confer the ARS phenotype.

At least one DNA fragment, 1.94 kb long, which confers the ARS phenotype has been identified in the mtDNA of the filamentous fungus *Cephalosporium acremonium* [15,16]. To determine the structural characteristics that are responsible for its ARS phenotype, we have performed a detailed analysis of this fragment that has shown a large number of sequences related to that of the ARS consensus [17].

2. MATERIALS AND METHODS

2.1. *Strains*

C. acremonium ATCC 11550 was maintained in defined medium [18]. *E. coli* strains HB101 or DH1 were used for both transformations [19] and plasmid maintenance, while strain JM103 [20] was the host for M13 phages and derivatives. *S. cerevisiae* D483 (Mata, leu2-112, his4, can1) was the recipient strain in yeast transformations [21].

2.2. *DNA*

Mitochondrial DNA was purified from *C. acremonium* as described [22]. Plasmid DNA was obtained from *E. coli* by the alkaline lysis procedure [23] and purified by CsCl-EtBr centrifugation. Total yeast DNA was prepared according to

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Zakian [11]. DNA fragments were purified from either polyacrylamide or agarose gels [24]. Probes for Southern blot hybridization [25] were labelled by nick-translation [26].

Plasmid pMA2 is a yeast integrative vector that was constructed by ligation of a 2.2 kb yeast DNA fragment containing the *LEU2* gene [27] in the *Sal*I site of pBR325 (see fig.1). Insertion in its single *Pst*I site of the 1.94 kb *Pst*I DNA fragment from the mtDNA of *C. acremonium* allowed its propagation in yeast as an autonomous plasmid in agreement with results obtained by others [15,16]. This latter plasmid was named pCP1 (see fig.1).

The nucleotide sequence of the 1.94 kb mtDNA fragment was determined using the dideoxy method of Sanger et al. [28].

3. RESULTS AND DISCUSSION

To determine which part of the 1.94 kb *Pst*I fragment was responsible for the ARS activity, we obtained a detailed restriction map of this piece of DNA and cloned different subfragments in the yeast integrative plasmid pMA2. Purified plasmids containing different subfragments were used to transform yeast as an assay of its ARS activity. Fig.2A lists the plasmids obtained and the relevant inserts that they carried, as well as the transformation frequencies obtained in yeast. Surprisingly, all but one of the subfragments showed ARS activity (i.e. the plasmids transformed yeast at high frequency, see fig.2A). Plasmid pBB1 that contains the central 560 bp *Sau*3A subfragment transforms yeast with a frequency even higher than that obtained with plasmid pCP1, that carries the complete 1.94 kb mtDNA fragment. To confirm that these plasmids which transform yeast at high frequency replicate autonomously in yeast, we cultured *S. cerevisiae* clones transformed to the leu$^+$ phenotype by the plasmids described in fig.2A in minimal medium without leucine and isolated DNA from these cultures. Undigested total DNAs were run in an agarose gel, transferred to nitrocellulose and hybridized with ^{32}P-pBR325. As shown in fig.2B, all types of yeast leu$^+$ clones contained autonomously replicating plasmids of the expected size, confirming that the high frequency of transformation was correlated with self-replication of these plasmids in yeast.

To establish a correlation between the nucleotide sequence of the subfragments and their ARS properties we have sequenced the 1.94 kb mtDNA fragment. Fig.3 shows the complete nucleotide sequence of the fragment that contains several open

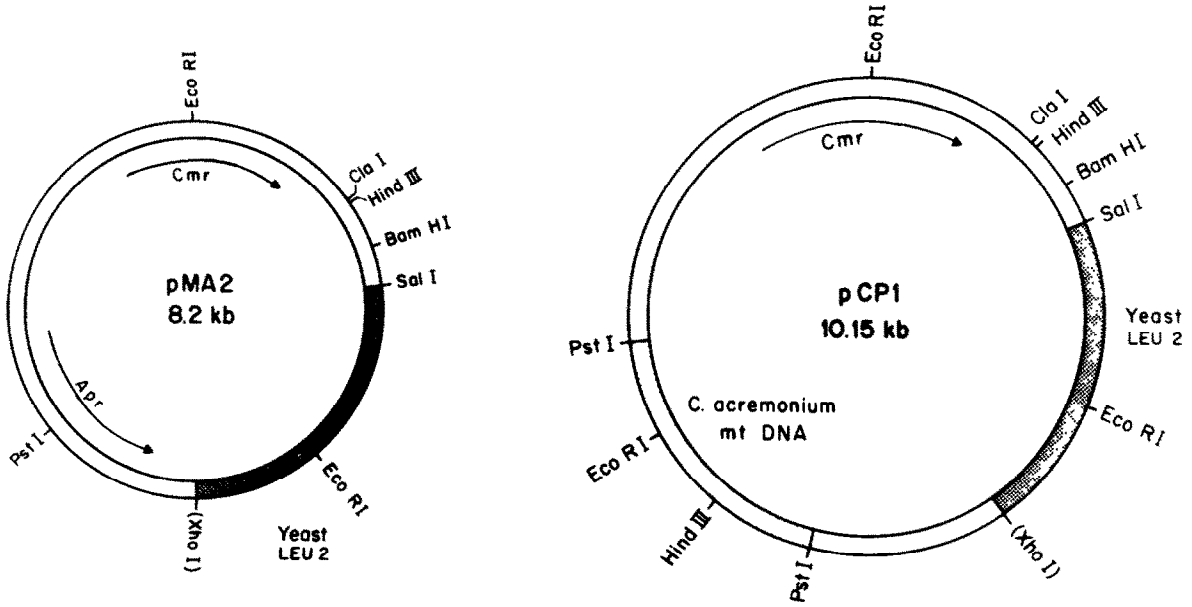
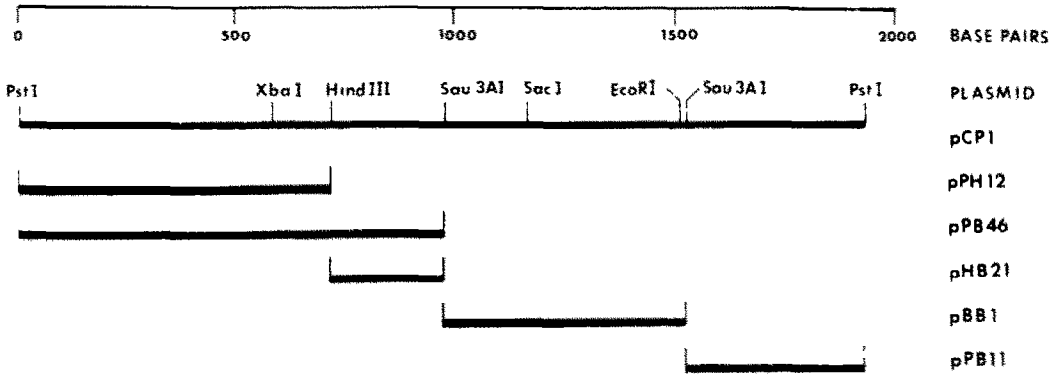


Fig.1. Restriction maps of plasmids pMA2 and pCP1. The latter was obtained by insertion in the single *Pst*I site of pMA2 of a 1.94 kb mtDNA fragment from *C. acremonium* with ARS activity.

A



PLASMID	mt DNA INSERT(Kb)	TRANSFORMATION FREQUENCY (Transformants/μg x 10^{-4})
pCP1	1.94	0.47
pPH12	0.72	0.3
pPB46	0.98	0.32
pHB21	0.26	0.28
pBB1	0.56	1.07
pPB11	0.42	<0.01
pMA2	–	<001

B

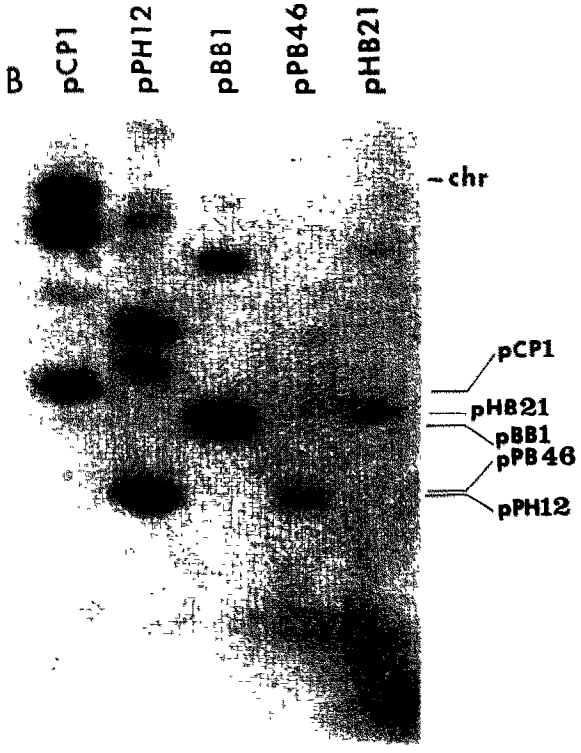


Fig.2. (A) Restriction map of the 1.94 kb mtDNA fragment of *C. acremonium*. The different subfragments shown under the restriction map were subcloned in the yeast integrative vector pMA2 (see section 2). The names of the recombinant plasmids obtained are shown to the right of the relevant inserts that they contain. Covalently closed circular plasmid DNA from each subclone was used to transform yeast spheroplasts. Transformation frequencies obtained with the different recombinant plasmids (i.e. ARS activity) as well as the approximate size of the inserts are shown in the table. (B) Total DNA was isolated from yeast leu^+ clones, named according to the plasmid used for transformation to the leu^+ phenotype, and samples run in a 0.8% agarose gel and blotted on a nitrocellulose filter which was hybridized with ^{32}P-pBR325 and autoradiographed. The electrophoretic mobilities of chromosomal DNA (chr) and of the ccc forms of the different plasmids isolated from *E. coli* are shown.

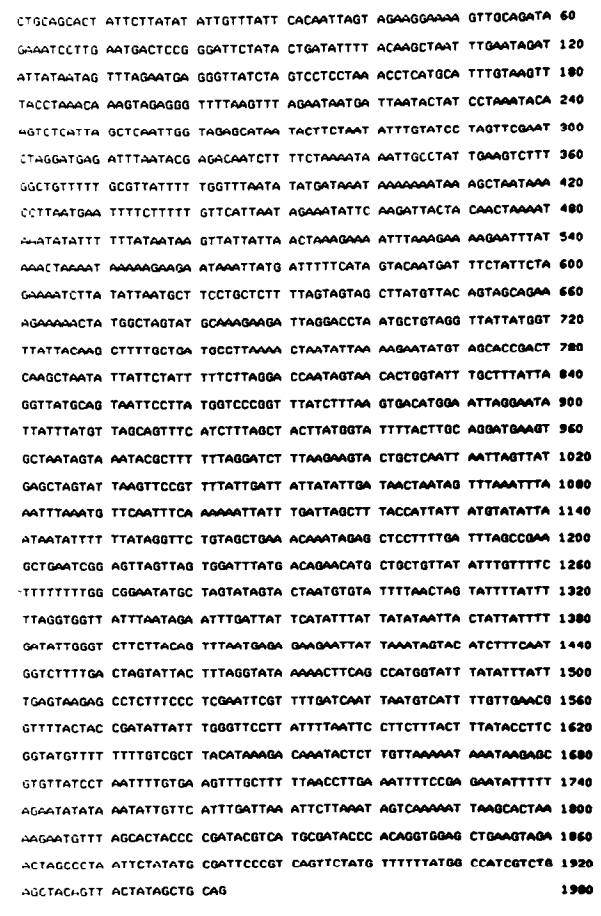


Fig.3. Nucleotide sequence of the 1.94 kb mtDNA fragment. The recognition sites for some restriction endonucleases, expressed by nucleotide number of the 5′-end are: *Eco*RI, 1523; *Hin*dIII, 728; *Sau*3A, 986, 1534; *Sac*I, 1187; *Xba*I, 597. A detailed list of other restriction sites may be obtained upon request.

reading frames. The only one with coding capacity for a protein with an M_r of more than 10 000 goes from ATG in position 568 to TAA in position 1645 and codes for a potential 359-amino-acid-long polypeptide. There is an open reading frame of unknown size that starts in position 1805 with no stop codons in this reading frame till the end of the fragment. A consensus sequence of 11 bp (A/TT-TTATPuTTTA/T) has been described [17,29] as a structural requirement present in most ARS sequences described so far. When we studied the nucleotide sequence of the 1.94 kb mtDNA fragment of *C. acremonium* (see fig.4), we found up to 43 11 bp sequences with two mismatches with the consensus sequence, 10 with one mismatch and only one sequence which perfectly resembled that of the consensus. Interestingly, only the 548 bp *Sau*3A fragment with the highest ARS activity contains the sequence (ATTTATATTTA) that completely matches the consensus sequence (boxed sequence in fig.4, positions 1487–1498 in fig.3).

This sequence not only corresponds to that of the ARS consensus but, moreover, matches perfectly the highly conserved 11 bp sequence found in the *1a* region of *S. cerevisiae ori1, ori5*, and *ori7* [30] and the ARS element of *C. utilis* mtDNA [10]. Overlapping this ARS site, in the strand complementary to that shown in fig.3, there is a 9-nucleotide-long sequence ATATAAATA which shows striking homology with an mtDNA transcription initiation signal in yeast – ATATAAGTA [31]. Transcriptional activity of this region of DNA causing a local melting of the double helix could facilitate the initiation of DNA replication. Some other interesting features are found in the 548 bp fragment as clusters of different ARS-like sequences (e.g., one may find 4 overlapping 11 bp consensus-like sequences from positions 1127 to 1159, or two adjacent ones from position 1349 to 1370). All the other subfragments which promote autonomous replication contain several consensus-related sequences that could account for its ARS activity. However, it has to be pointed out that 6 of these sequences are found in a subfragment which does not present the ARS phenotype (see fig.4). Consequently, there must be some other feature in a DNA fragment besides the consensus-like sequence for this to show ARS activity. In agreement with this interpretation, *MAT a* and *MAT α* loci of yeast contain the consensus sequence but do not show the ARS phenotype [17].

A second feature of ARS-containing fragments is high A + T content and, accordingly, the A + T content of the 1.94 kb fragment is high (72.5%). However, the A + T content seems not to be directly correlated with the ARS activity, since in *C. acremonium* mtDNA subfragments with almost identical A + T content show very different transformation frequencies (fig.4). It seems reasonable to believe that other factors are needed in an ARS element besides the consensus sequence and the high A + T content. If ARS elements represent the replication origins, the DNA must be easi-

Subfragment	PstI-HindIII (1) (728)	HindIII-Sau3A (728) (986)	Sau3A-Sau3A (986)(1534)	Sau3A-PstI (1534)(1943)
Transformation frequency/μgx10^{-4}	0.3	0.28	1.07	< 0.01
A+T%	74.3	68.7	74.67	68.05
ARS consensus-like sequences	TcTTATATaTT TTaTATATTgT AcTgATATTTT TTgTAaGTTTA TTTTAaGTTTA ATTTtTGgTTT TTTTtgGTTTA AaaTATATTTT ATaTATtTTTT TTTTATAaTaA AaTTATGaTTT ATTTcTATTcT TcTTATATTaA TaTTATGgTTT cTTTtTATTTT ATTTtaGTTTA cTTTAaATTTT AaaTATATTTA ATaTtTATTTT ATTTcTATTaA ATTaAgGTTTA cTTTATtTTTT TTTTtTATTTA AcTTgTATTTA	TaTTcTATTTT gTTTATcTTTA ATTTATGTTag TTTcATcTTTA	TaTTATATTgA gTTTAaATTTA ATTTAaATTTA ATTTAaATgTT TaTTATGTaTA ATgTATATTaA AaTaATATTTT TTTTATAggTT TgTTATATTTg ATaTtTGTTTT TTTTcTtTTTT ATgTgTATTTT TaTTtTATTTT TTTTATtTTTA ATTcATATTTA TTaTATAaTTA ATTTATATTTA TTTTATAccTA ATTTAaATTTA ATTTAaATTTA	TTcTATGTTTT ATTTATATaTT TTaTATATTcT TTaTtTATTTT ATTTATtTTTA ATTTgTcTTTA

Fig.4. Computer search of ARS consensus-related sequences along the 1943 bp mtDNA fragment. Only 11 bp sequences with two or less mismatches with the consensus sequence (A/TTTTATPuTTTA/T) are listed. Mismatches are represented in lower-case letters and subfragments correspond to those described in fig.2. ARS activity in yeast of each subfragment as well as its A + T% derived from the sequence is also shown.

ly denatured in these regions. Correlated with the A + T% of a given DNA fragment is the relative abundance of the sequence NTAN, which seems to be preferentially unwound by negative torsional stress [32]. This fact could account for the observed high A + T% of ARS-containing fragments.

Finally, there are several potential hairpin structures along the sequence which are presented in fig.5. Potential structure 'F', located in the subfragment with the highest ARS activity, contains the 11 bp consensus sequence. It has been shown [30] that replication origins in yeast mtDNA are associated with such structures.

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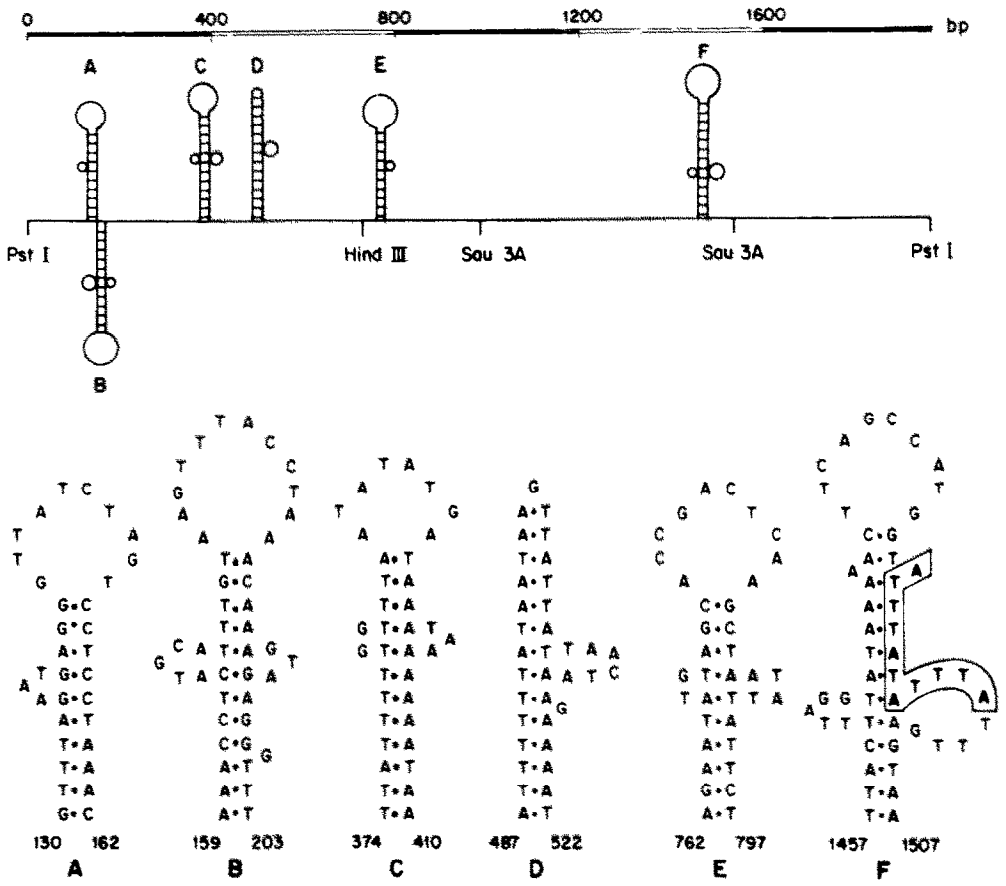

Fig.5. Potential secondary structures found in the 1.94 kb mtDNA fragment; approximate position of the hairpins is represented relative to its position in the restriction map. The consensus ARS sequence is boxed in structure F.

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