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In vitro deletion analysis of *ARS* elements spanning the replication origin in the 5' non-transcribed spacer of *Tetrahymena thermophila* ribosomal DNA

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ABSTRACT

Two adjacent but non-overlapping restriction fragments that encompass the replication origin of the macronuclear copy of rDNA from Tetrahymena thermophila allow autonomous replication of plasmids in the yeast Saccharomyces cerevisiae; i.e. they function as autonomously replicating segments (ARS). Deletions generated in vitro into these fragments yield an 82 bp segment from each as the smallest sequence specifying ARS function. These 82 bp segments are at the 5' end of a 220 bp region of homology between the two original ARS restriction fragments. A 39 bp region of almost complete sequence identity between the two 82 bp fragments is suggested to be a core sequence element necessary for ARS function. This 39 bp sequence contains a region identical or nearly identical to the 11 bp yeast ARS consensus sequence (T/ATTTATPuTTTA/T) which is suggested to be essential for ARS function. Detailed comparisons of the 82 bp segments and of the 39 bp core with other ARS sequences reveal no extensive homologies aside from the consensus.

INTRODUCTION

Cloned genomic DNA fragments that act in cis to allow autonomous replication of a circular plasmid in the bakers yeast Saccharomyces cerevisiae contain sequence elements referred to as autonomously replicating segments (ARS) (1,2,3). An ARS element when present on a plasmid containing a selectable genetic marker confers on that plasmid the ability to transform yeast mutant for the marker at a high frequency. Extrachromosomal plasmid DNA can be isolated from transformed cells grown under selective conditions and transformants are unstable for the selectable marker when propagated in complete medium. In the context of this transformation assay, ARS elements derived from the yeast genome (1-8) function as origins of replication and are thus good candidates to be bona fide replicators on yeast chromosomes. A growing body of evidence from in vitro (9,10,11) and in vivo experiments (12,13) supports the hypothesis that the ARS1 sequence derived from chromosome IV of yeast is a chromosomal replicator.

The many yeast ARS elements sequenced to date share no extensive sequence homology except for a small 11 bp sequence referred to as the yeast ARS con-

sensus sequence (4-8). This sequence is also present on many ARS elements isolated from non-yeast DNA (14-17). Kearsey has shown that for the yeast HO-ARS fragment, a divergent copy of the consensus plus an additional flanking 3 bp is crucial for ARS function (5). Substitutions introduced into the divergent consensus by in vitro manipulation inhibit the ability of the element to generate Ars^+ phenotype in the assay. Another study showed that deletions extending into the consensus sequence of ARS1 abolished ARS function (18). More recent studies suggest that the consensus sequence plus 4 bp on either side is the minimum requirement for high frequency transformation with ARS1 (19). These results suggest that the consensus sequence may be essential for replicator activity of ARS elements but an additional domain may be required for efficient replication and its regulation through the cell cycle (20). The presence of a consensus sequence on a segment of DNA is, however, not necessarily predictive of ARS function (4,14, this study).

The yeast transformation assay has been used as an initial screen of non-yeast DNA fragments for the possible presence of replication origins (1,21-29). This is the case for the ciliated protozoan Tetrahymena thermophila for which a DNA-mediated transformation system does not exist.

In this communication we report results of in vitro deletion analysis of two 5' NTS ARS fragments of T. thermophila rDNA. We have identified a 39 bp sequence element which appears to be necessary for ARS function, and which contains the 11 bp yeast ARS consensus sequence. The 39 bp sequence is present at the 5' end of each copy of a 420 base pair tandem repeat that is thought to include the in vivo origin of rDNA replication (30,31).

MATERIALS AND METHODS

Plasmid Vectors, Strains, Transformation and DNA Isolation

Most plasmids and strains of both Escherichia coli and S. cerevisiae used in this study have been described previously (14,26). The yeast integrating vector YIP5 contains a dG:dC tailed 1.1 kbp HindIII fragment carrying the yeast URA3 gene inserted in the AvaI site of pBR322. Transformation of both E. coli and S. cerevisiae and isolation of DNA from these organisms were done as previously described (14).

DNA Manipulations

Restriction endonucleases, T4 DNA ligase and other DNA modifying enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim Canada Ltd. and PL Biochemicals and were used according to the supplier's specifications. Deletions generated in vitro using Bal31

nuclease were done essentially as described by Maniatis et al. (32) with modifications as described under Results. Electrophoresis of DNA fragments through horizontal agarose slab gels containing 5 μg/ml ethidium bromide or through polyacrylamide vertical slab gels was performed as described previously (14).

Nucleotide sequence analysis of DNA fragments from CsCl purified plasmid DNA was done according to the procedure of Maxam and Gilbert (33). DNA restriction fragments were isolated from horizontal agarose gels by electrophoresis onto ion exchange paper (NA 45 Schleicher and Schuell Inc.). Fragments were eluted from the paper in 1 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl pH 8 at 55-60°C for 45 min. followed by precipitation with 2.5 volumes 95% ethanol. Samples were reprecipitated with ethanol from 0.3 M sodium acetate and washed in 70% ethanol. Endpoints of *in vitro* deletions were determined by sequencing plasmid DNA prepared from 1.5 ml cultures by the dideoxy chain termination procedure of Sanger et al. (34) as modified by Wallace et al. (35). The 16 nucleotide 'Eco Rl' primer (5' GTATCACGAGGCCCTT, PL Biochemicals) complementary to bases 4434-4450 in plasmid pBR322 (36) was used in these experiments. α-^{32}P dNTP's (3000 Ci/mM) were purchased from New England Nuclear. Computer assisted analysis of DNA sequence data was performed as previously described (37). The Nucaln sequence alignment program of Wilbur and Lipman (38) was also used in these studies.

RESULTS

Construction of rDNA Recombinant Plasmids

We have recently shown that two adjacent but non-overlapping restriction fragments from the 5' NTS of the extrachromosomal palindromic rDNA of *T. thermophila* function as *ARS* in yeast (14) (see Fig. 1). These fragments are a *TaqI* - *XbaI* fragment (bp 63 - 720) and an *XbaI* - *XbaI* fragment (bp 720 - 1147), each in the vector pACYC184 and containing the yeast *HIS3* gene as the selectable marker (14). Because restriction sites in these plasmids are not well suited for the construction *in vitro* of deletions into rDNA sequences, rDNA containing fragments were subcloned into the yeast integrating plasmid YIP5. The *EcoRl* - *HindIII* fragment containing rDNA sequences from each of plasmids pRP174 and pRP141 (14) were ligated to the large fragment from *EcoRl* - *HindIII* digested YIP5. Plasmids pRP266 and pRP285 were obtained (Fig. 1). pRP266 contains the 657 bp *TaqI* - *XbaI* rDNA fragment whereas pRP285 contains the 427 bp *XbaI* - *XbaI* fragment. These plasmids all transform *S. cerevisiae* S277 at a high frequency, transformants are mitotically unstable when grown

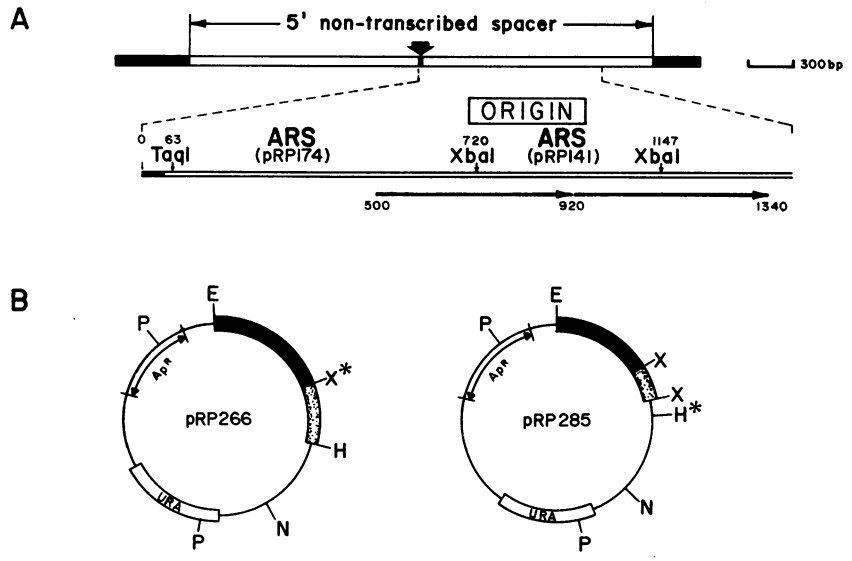


Fig. 1. (A) A section of the 5' NTS of T. thermophila rDNA showing the location of the in vivo origin of replication (30,31). ▼ : center of the molecule. ▬ : 29 bp non-palindromic sequence at the center of the molecule. Numbering of the rDNA sequence is as described previously, and begins at the non-palindrome/palindrome junction (37). ⟶: 420 bp sequence duplicated at the replication origin. The 657 bp TaqI - XbaI and 427 bp XbaI - XbaI fragments which we have shown previously to function as ARS in yeast are contained in plasmids pRP174 and pRP141 respectively (14). (B) Circular restriction map of plasmids pRP266 and pRP285. pRP266 contains the 657 bp TaqI - XbaI ARS fragment and pRP285 contains the 427 bp XbaI - XbaI ARS fragment. Restriction sites: X = XbaI; E = EcoR1; H = HindIII; P = PstI; N = NruI. ▨ : rDNA sequences; ▬ : pACYC184 sequences; ——— : pBR322 sequences; (*): restriction site at which Bal 31 deletions were initiated.

in the absence of selection and unrearranged plasmid DNA can be isolated from transformants. Transformants remain mitotically unstable and exhibit very rapid 1:0 segregation even when a yeast CEN sequence is present on the plasmid. This behaviour is similar to that for strains transformed with plasmids containing a yeast ARS4 and CEN sequence (39). Quantitative data (not shown) from these transformation experiments are identical to those obtained previously for plasmids pRP174 and pRP141 (14).

Construction and Analysis of Deletions in rDNA Sequences

Deletions were generated into rDNA sequences in each plasmid using nuclease Bal31 acting from a unique restriction site chosen so that the deletion required to reach rDNA sequences in one direction would not extend into either the β-lactamase or URA3 genes in the opposite direction. Deletions

were initiated from the XbaI site in pRP266 and from the HindIII site in pRP285. 0.5 units of Bal31 nuclease was used to digest 15 μg of linear DNA at a rate of approximately 50 bp per min. To ensure that all deletions had one common endpoint in vector sequences, DNA from deletions initiated at the XbaI site was cleaved with EcoR1 and the 5' overhang of this site repaired using the Klenow fragment of DNA polymerase I from E. coli prior to ligation. Those deletions initiated at the HindIII site were digested with NruI prior to ligation. The ligation mixes were used to transform E. coli R80 selecting for Ap^r Ura^+ transformants. The approximate extent of the deletions was analyzed by electrophoresis of HindIII plus PstI or XbaI plus PstI digested DNA obtained from 1.5 ml cultures of transformants grown to saturation in LB broth containing 50 μg ampicillin/ml. Endpoints of deletions were determined by DNA sequence analysis as described under Materials and Methods. Sequences at the rDNA/vector junction were determined by comparison with the known rDNA 5' NTS sequence (37).

ARS-Activity of Plasmids

Plasmid DNA containing deletions as well as DNA from RP266 and RP285 were isolated from 1.5 ml cultures, treated with RNase (50 μg/ml final concentration) for 15 min at 37°C and then with protease K (250 μg/ml final concentration) for 30 min at 37°C. All contained the same amount of predominantly supercoiled DNA as determined from the ethidium bromide staining intensity of samples following electrophoresis through an agarose gel. One half of each DNA sample was used to transform S. cerevisiae S277 spheroplasts selecting for Ura^+ transformants. Ars^+ and Ars^- phenotypes were scored based on high frequency of transformation or low frequency of transformation respectively (14,26). This assay defines sequences essential for autonomous replication of plasmids although not necessarily efficient and regulated replication (20,39).

Two plasmids which contained the smallest rDNA sequences conferring an Ars^+ phenotype were chosen for further analysis. One plasmid, designated pTA55, was obtained from deletion of XbaI-linearized pRP266 and carried rDNA sequences from bp 63 - bp 615. Another plasmid, designated pTA37, was obtained from deletion of HindIII-linearized pRP285 and carried sequences from bp 919 - bp 1147 (Fig. 2).

To further delimit ARS sequences, Bal31 deletions were generated from the unique HindIII site in pTA55 and from the now unique XbaI site in pTA37. These deletions were initiated into rDNA sequences from the end opposite to that used in obtaining the original deletions in pRP266 and pRP285 respective-

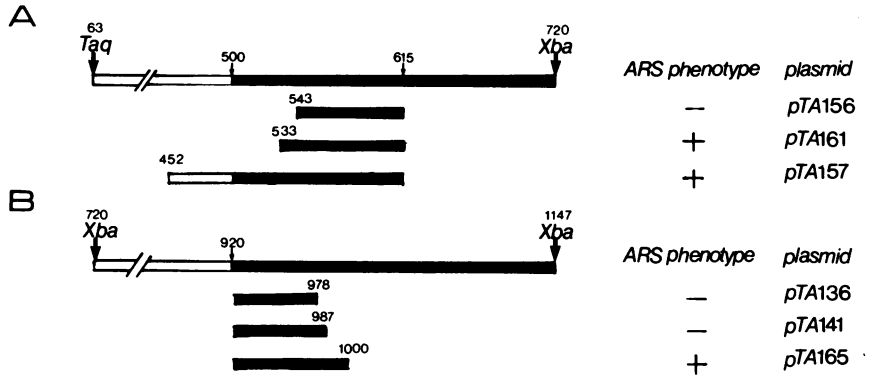


Fig. 2. Ars phenotype of some plasmids obtained by Bal 31-generated deletions from the HindIII site of pTA55 (A) and from the XbaI site of pTA37 (B). The rDNA sequences contained in all of these plasmids are indicated on the left to illustrate their relative positions on the 657 bp TaqI - XbaI and 427 bp XbaI - XbaI fragments. ▬: sequences of the 420 bp duplicated region.

ly. These experiments were done as described above generating deletions with a common filled in EcoRl site endpoint from pTA55 and with an NruI endpoint from pTA37. Transformation of E. coli, sizing and sequence determination of endpoints and transformation of yeast were carried out for these deleted plasmids as described above for the original deletions.

The exact rDNA sequences carried on plasmids as well as the Ars phenotype conferred by these plasmids are presented in Fig. 2. rDNA segments from bp 534 - bp 615 and from bp 919 - bp 1000 were the smallest sequences that allowed autonomous replication, and were contained in plasmids pTA161 and pTA165 respectively. As expected, these sequences are included in the 220 bp sequence common to the TaqI - XbaI and XbaI - XbaI rDNA restriction fragments. Plasmids pTA156 and pTA141 which do not replicate autonomously in yeast carry rDNA sequences from bp 543 - bp 615 and from bp 919 - bp 987 respectively. The rDNA sequences in pTA156 is 10 bp shorter than that of pTA161 whereas the rDNA sequence in pTA141 is 13 bp shorter than that of pTA165. Thus these 10 and 13 bp sequences are required for expression of an Ars^+ phenotype in pTA161 and pTA165 respectively.

Cloned rDNA sequences from the 5' NTS region of two other tetrahymenid species were also tested for ARS function. One plasmid, pRP233 contains the yeast URA3 gene and a 1.4 kbp KpnI - HindIII fragment spanning 90% of the 5' NTS of Tetrahymena pyriformis rDNA (40,41). Another plasmid, pRP411 contains the same URA gene fragment and a 6.5 kbp fragment carrying the entire 5' NTS

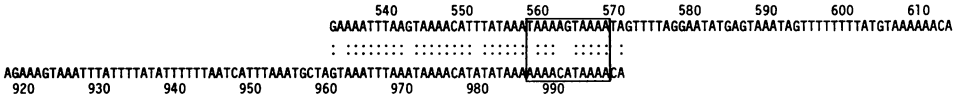


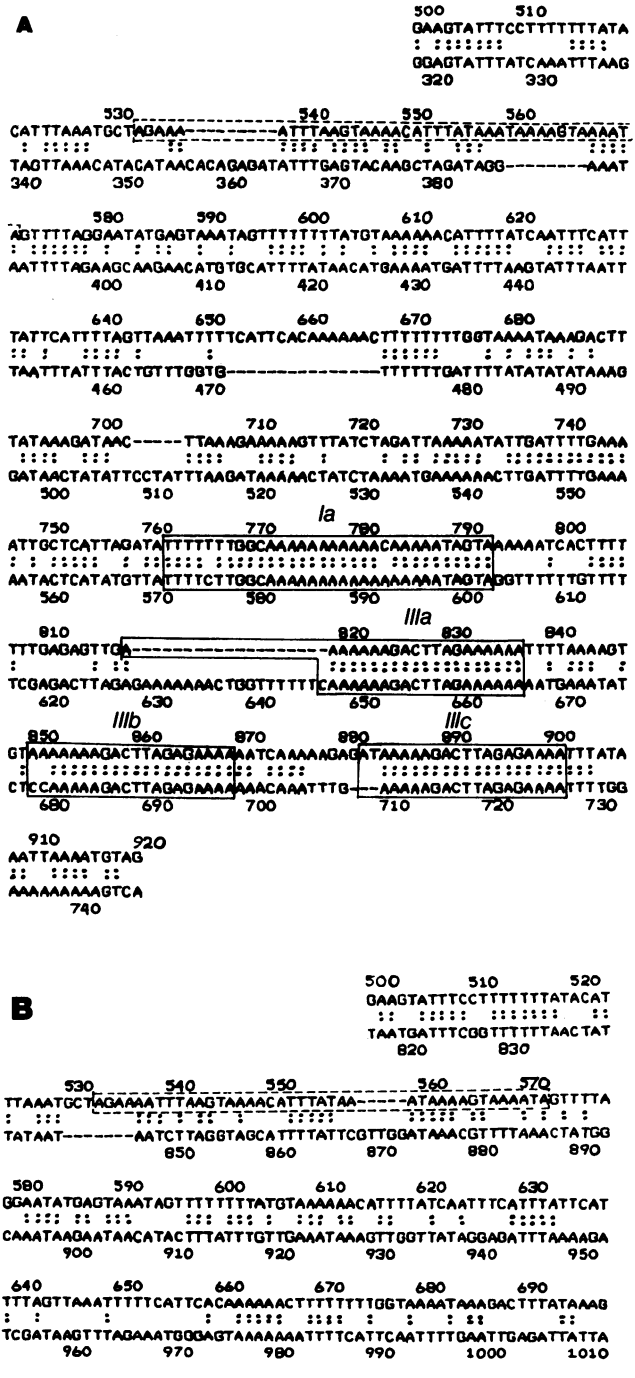
Fig. 3. A computer generated sequence alignment for maximum homology between the rDNA ARS elements contained in plasmids pTA161 (bp 919 to bp 1000) and pTA165 (bp 534 to bp 615) (see Fig. 2.). The 39 bp sequence common to both elements is from bp 534 to bp 572 in one case and bp 961 to bp 1000 in the other. [____]: the 11 bp A+T rich yeast ARS consensus sequence.

of Glaucoma chattoni rDNA (42). This 6.5 kbp fragment contains sequences from the 5' telomere to an internal BamHl site in rDNA. Both plasmids transformed S277 at a high frequency but transformants grew poorly, were extremely small and were mitotically unstable for the Ura$^+$ phenotype even when grown under selective conditions (data not shown). This is characteristic of a weak Ars$^+$ phenotype as described for a 260 bp fragment in the 3' NTS of T. thermophila rDNA (14) and domain B of ARS1 from yeast (6).

DNA Sequence Comparisons

Computer generated sequence alignment of the 82 bp ARS sequences from pTA161 and pTA165 reveals the presence of a 39 bp region, almost identical in the two plasmids (Fig. 3). These sequences, from rDNA bp 533 - 571 and bp 962 - 1000 define a core element for the rDNA 5' NTS ARS elements. Deletion of 10 or 13 bp from this core sequence as described above results in loss of ARS function. The core sequence contains in one case, a perfect copy of the yeast ARS consensus sequence (4) and in the other case, a slightly divergent copy of this consensus (Fig. 3).

We have also analyzed the homology shared between the 420 bp duplicated sequence element in the 5' NTS of T. thermophila rDNA and sequences spanning an analogous region of T. pyriformis rDNA. This region in T. pyriformis rDNA is not duplicated (30). An alignment generated by computer to give maximum homology between the sequences is presented in Fig. 4a. A similar alignment between the 420 bp T. thermophila sequence and the entire 5' NTS sequence of G. chattoni rDNA is presented in Fig. 4b. In both comparisons, extensive conservation of the 3' region of the 420 bp T. thermophila sequence is observed but the sequences diverge extensively in the 5' 222 bp of the 420 bp element. It is within this non-conserved 222 bp of T. thermophila rDNA that the 82 bp ARS elements are found. Neither the 82 bp ARS sequence nor the 39 bp ARS core sequence, as defined in T. thermophila rDNA, are conserved among these three quite closely related ciliates. The only conserved features in


A
B
Ia
IIIa
IIIb
IIIc

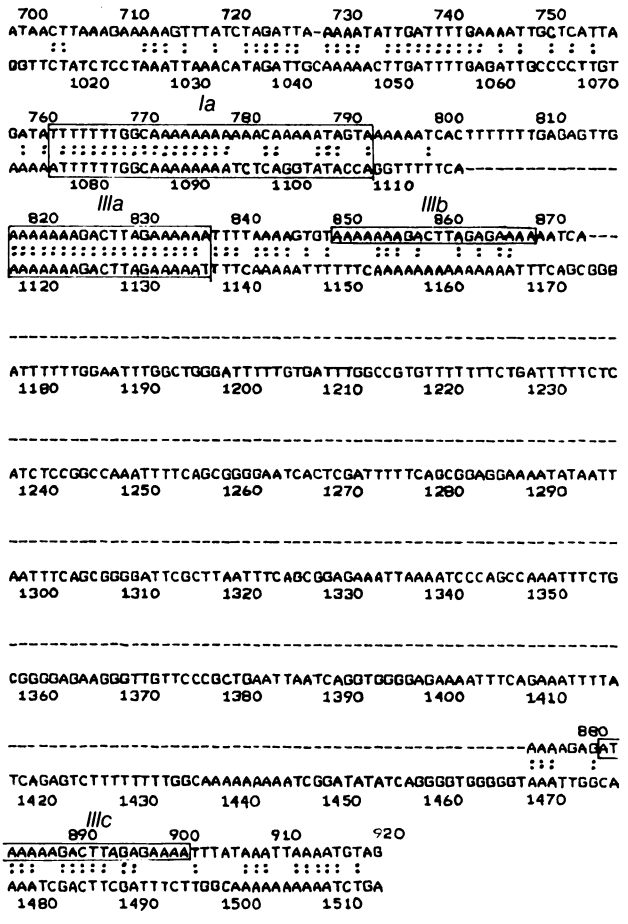


Fig. 4. (A) A computer generated nucleotide sequence alignment for maximum homology between sequences from bp 500 to bp 920 of T. thermophila rDNA and sequences from bp 1 to bp 800 of T. pyriformis rDNA. (B) A similar alignment but between sequences from bp 500 to bp 920 of T. thermophila rDNA and from bp 1 to bp 1545 of Glaucoma chattoni rDNA. In both comparisons, only the sequences which align with the 420 bp T. thermophila sequences are presented. [ ]: type I and type III sequences which we have described previously (37). [ ]: 39 bp sequence common to the T. thermophila rDNA ARS elements (see Fig. 3.).

this region of rDNA are the Type I and Type III repeated sequences we have described previously (37). These are found in the 3' portion of the 420 bp T. thermophila element and are boxed in Fig. 4a and 4b.

DISCUSSION

Deletion Analysis in vitro; ARS Function

We have used deletion analysis *in vitro* to identify an 82 bp sequence on each of two adjacent restriction fragments spanning the origin of replication of *T. thermophila* rDNA, which allow autonomous replication of plasmids in yeast. This analysis has allowed us to address questions regarding nucleotide sequence requirements for *ARS* function in *S. cerevisiae* as well as whether these *ARS* fragments are the *in vivo* origins of rDNA replication in *Tetrahymena*. Common to both 82 bp *ARS* elements is a 39 bp sequence which we refer to as the rDNA *ARS* core. The two 39 bp sequences differ by only 7 bp. Four of those seven changes do not alter the base pairs in the element. Removal of 10 and 13 bp from each of the core elements results in loss of *ARS* function. The core sequence, or at least most of it, is therefore necessary although likely not sufficient to specify *ARS* function in yeast.

ARS Function and the Origin of Replication in vivo in Ciliate rDNA

The 39 bp sequence element described above is present at the 5' end of a 420 bp sequence duplicated at the origin of replication of *T. thermophila* rDNA (14,30,37). In *T. pyriformis*, the origin of replication of the extrachromosomal rDNA is closer by approximately 200 bp to the center of the molecule than in *T. thermophila* (30,31). The origins in these two species do, however, occur in regions with some sequence homology, and it would be expected that in such closely related species a high degree of conservation would exist for sequences specifying an essential function such as the initiation of DNA replication. Neither the 82 bp *ARS* nor the 39 bp sequences of *T. thermophila*, however show more than minimal homology when compared with sequences encompassing the origin of replication of *T. pyriformis* rDNA (Fig. 4). Similarly, no homology is seen when these *T. thermophila* sequences are compared with the sequence of the entire 5' NTS of the extrachromosomal rDNA of the related ciliate *G. chattoni* (Fig. 4). The sequence homologies which do exist between these three species in the rDNA replication origin region are confined to the repetitive Type I and Type III elements (37).

An analysis of macronuclear rDNA chromatin from *T. thermophila* showed that the 5' 170 bp of the 420 bp duplicated sequence encompassing the origin of replication, is bound in a nucleosome whereas the remainder of the region is hypersensitive to treatment with a variety of nucleases (30). It was postulated that the hypersensitive region presents an open chromatin structure which would facilitate the entry of trans-acting factors required for the initiation of DNA replication at the origin. The same nucleosome pattern is

also seen at the replication origin region in the rDNA of T. pyriformis (30). No data on the structure of the rDNA chromatin of G. chattoni are available but a region does exist in G. chattoni rDNA with extensive sequence homology to the nuclease hypersensitive region of the two Tetrahymena species (37). A DNA fragment spanning the nuclease hypersensitive region of T. thermophila rDNA is not an ARS and neither are a fragment from T. pyriformis rDNA which includes the in vivo origin of replication nor a fragment which includes the entire 5' NTS of G. chattoni rDNA. We conclude from these data that sequences in T. thermophila rDNA which allow autonomous replication of plasmids in yeast are unlikely to be the sequences required for the initiation of DNA replication in vivo. Experiments in progress with replciation of Tetrahymena rDNA in vitro should allow us to address questions to determine the DNA sequence at the rDNA replication origin.

Common Features of ARS Elements

An 11 bp A/T rich sequence has been identified as a consensus sequence present on yeast (4-8) and on non-yeast (14-17) ARS elements. The 39 bp core sequences we have identified in T. thermophila rDNA contain this consensus sequence. This sequence now appears common to and an important component of sequences which allow the autonomous replication of plasmids in yeast. Srienc et al. have recently shown that a sequence of 19 bp from ARS1, of which 11 bp is the consensus, enables a plasmid containing CENIV of yeast to transform yeast at a high frequency (19). The transformants are, however, extremely unstable mitotically when compared to yeast transformed with a plasmid carrying the 838 bp EcoR1 - HindIII ARS1 fragment and CENIV. Kearsey has demonstrated the presence of a slightly variant copy of the consensus sequence on a 46 bp HO - ARS fragment (5). The consensus sequence is crucial for ARS function.

Despite the importance of the 11 bp consensus sequence, this sequence alone is not sufficient for ARS function in yeast (4). Specific auxillary sequences or domains are required along with the consensus to effect an efficient Ars^+ phenotype of ARS1 (19,20) and HO - ARS (5). Whatever the role of flanking domains is, it is not sequence specific because there is no homology between sequences flanking the consensus in the ARS elements described above. Data from three other experiments presented above support the idea that the 11 bp sequence is necessary, but not sufficient for ARS function in yeast. Plasmid pTA141 (Fig. 2) contains the 11 bp consensus sequence but does not allow high frequency transformation of yeast. 5' NTS sequences from the rDNA of T. pyriformis and G. chattoni both contain the 11 bp consensus

sequence (37,41) but plasmids containing these regions give a very weak Ars$^+$ phenotype on transformation of yeast. Broach et al. have also presented evidence that the presence of the 11 bp consensus sequence in a cloned DNA fragment is not necessarily diagnostic of ARS function (4).

Other Heterologous ARS Sequences

Sequences from a number of organisms other than S. cerevisiae and the ciliate rDNAs described in this paper allow autonomous replication of plasmids in yeast (15-17,21-25,27-29). For most of these, the nucleotide sequence of the element is not known. In cases where sequences have been determined, experiments have not been done to determine the smallest fragment retaining ARS function and so data from sequence comparisons with the minimal yeast and Tetrahymena sequences are difficult to interpret. We have, however, performed computer assisted sequence comparison and alignment between the 82 bp T. thermophila ARS and the 39 bp core sequence and other published ARS sequences (4-8,15-17,43) (data not shown). The only common feature among these sequences is the 11 bp consensus sequence generally present in an A + T rich environment. Some short inverted repeats are present but there is no consistent organization of these relative to the 11 bp sequence. Even the ARS fragments from the 3' NTS rDNA telomere regions of T. thermophila, two strains of T. pigmentosa and G. chattoni (26, Amin and Pearlman, unpublished observations) show no strong sequence conservation among themselves and, except for the 11 bp consensus sequence, none to the 5' NTS ARS of T. thermophila.

A number of other experiments also raise questions about the suitability of the yeast transformation assay as a tool to isolate non-yeast replication origins. Maundrell et al. (27). have shown that a majority of genomic DNA fragments from the fission yeast Schizosaccharomyces pombe which function as "self-identifiable" ARS when transformed into S. pombe do not function as ARS in S. cerevisiae. Furthermore, genomic fragments from S. pombe that are ARS in S. cerevisiae are not ARS in S. pombe. Roth et al. (28) have previously shown that mouse ARS elements fail to enhance the transformation efficiency of a plasmid in mouse cells. Also Vallet et al. (29) have reported that there is no correlation between the location of an ARS sequence and the site of one of the origins of replication (ori A) of Chlamydomonas reinhardii chloroplast DNA.

We suggest that the DNA sequences in the 5' NTS of T. thermophila rDNA which allows autonomous replication of plasmids in yeast are close to but are not the in vivo origin of rDNA replication. The sequences function in yeast because they contain the 11 bp ARS consensus sequence as well as additional

sequence which supplies some feature required to function in the yeast transformation assay. This requirement cannot be specified by nucleotide sequence alone but could involve some structural feature such as nucleosome organization or possibly DNA secondary and/or tertiary structure (44,45) in these generally A + T rich regions. In this regard, it is of interest that the replication origin region of T. thermophila rDNA shows extremely anomalous electrophoretic mobility on polyacrylamide gel electrophoresis (Levene, Amin and Pearlman, unpublished observations) which might indicate sequence directed DNA curvature in this region.

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