

ARS ACTIVITY IN THE YEAST MITOCHONDRIAL GENOME OF LOW-COMPLEXITY PETITE MUTANTS

Daniel Delouya¹ and Francisco G. Nóbrega²

ABSTRACT

The reiterated fragments of five low-complexity rho⁻ genomes, mapped in the apocytochrome b region of mitochondrial DNA from *Saccharomyces cerevisiae*, were cloned in the integrative yeast vector YIp5, yielding eight different constructions. Their capacity for autonomous replication (ARS) was subsequently assayed in yeast. One repeat unit from four genomes was sufficient to confer an ARS positive phenotype but only two repeat units from one of them conferred activity. The results support the hypothesis that the ARS 11-nucleotide sequence motif could be the *cis*-active region responsible for the initiation of DNA replication in rho⁻ genomes devoid of the putative canonical ori sequences.

INTRODUCTION

Plasmids containing autonomous replicating sequence (ARS) elements, are maintained as extrachromosomal DNA in yeast (Newlon, 1988). Direct evidence for the role of the 11-nucleotide ARS consensus sequence in DNA replication was provided by Brewer and Fangman (1987). We have previously shown that restriction fragments obtained from the mitochondrial apocytochrome b (COB) region, which encompasses a large segment of the mitochondrial DNA (mtDNA) from *Saccharomyces cerevisiae* D273-10B, exhibit a variable degree of ARS-activity (Delouya *et al.*, 1987). This activity has maximal values in two classes of recombinants (Figure 1): those containing 1660 bp of a segment belonging to the 5' half of the first exon up to the glutamyl-tRNA gene

¹Departamento de Imunologia, Instituto de Ciências Biomédicas and ²Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, Caixa Postal 11461, 05422-970 São Paulo, SP, Brasil. Send correspondence to F.G.N.

located upstream (Mbo I fragment 2 from ρ^- mutant DS400/A12) and those containing a 2110 bp fragment located downstream to the last exon of the COB gene (Mbo I fragment 1 from DS400/A12). The downstream region carries ori 6, one of the mtDNA regions homologous to the canonical origin of the replication motif studied by Blanc and Dujon (1980) and De Zamaroczy *et al.* (1981). The ARS-activity of the upstream region was shown to be the strongest, as measured by the mitotic stability of the ura-independent phenotype in cells under selective pressure (Delouya *et al.*, 1987). The presence of many stable and well-characterized petite mutants of low complexity, with genomes common to the upstream region, suggested a more biologically meaningful way of analysing the *cis*-dominant activity of a DNA segment in the ARS assay: instead of using an arbitrary segment defined by a restriction endonuclease site, we decided to analyse the repeat units of some cytoplasmic petite mutants of low-complexity. It is shown that all low-complexity petites studied are able to confer replicative ability to the integrative vector YIp5 although the relative strength of the ARS-activity varies over a wide range.

MATERIALS AND METHODS

Yeast strains, plasmids and media

DS400/A12, DS400/N1, DS400/N7, DS400/M8, DS400/N9, DS400/N24 are ρ^- mutants derived from *S. cerevisiae* D273-10B-A21 (Nóbrega and Tzagoloff, 1980a). *S. cerevisiae* YNN27 is *trp1-289, ura3-52, gal2* (Stinchcomb *et al.*, 1980) and was used in yeast transformation experiments. *E. coli* K-12 strains JM83 (Vieira and Messing, 1982) and RR1 (Bolivar *et al.*, 1977) were used as bacterial hosts for plasmid construction and rescue. Plasmids pBC200 and pBC283 are the two possible YIp5 derivatives containing ρ^- DS400/A12 MboI fragment 2 cloned into the BamHI site (Delouya *et al.*, 1987). The plasmid YRp17 was obtained from R.W. Davis and is identical to YRp12 (Scherer and Davis, 1979) except for the loss of one EcoRI site (Celniker *et al.*, 1984). YIp5 has been described by Struhl *et al.* (1979) and the pUC family of vectors by Vieira and Messing (1982). Culture media for yeast and bacteria have been described by Delouya *et al.* (1987).

DNA preparation, manipulation and analysis

mtDNA and plasmid DNA preparation and purification were carried out as described in Nóbrega and Nóbrega (1986). DNA purification, dephosphorylation, ligation, *E. coli* transformation with CaCl_2 , Southern blots and preparation of nick-translated probes followed the standard procedures described by Maniatis *et al.* (1982). Yeast transformation by the LiCl procedure, transformant characterization and

preparation of plasmid DNA from yeast transformants were as described by Delouya *et al.* (1987).

Construction of mtDNA recombinant plasmids

DS400/N24 mtDNA was cut in the unique *Hinf*I site (Figure 1) and thereafter treated with 1U of S1 nuclease for 20 min at room temperature, and precipitated with ethanol. The blunt-end fragments were ligated to *Sma*I digested and dephosphorylated pUC9, yielding pUN24, which carries one repeat unit (170 bp) and pN24x2, which includes two repeat units of the mtDNA of this ρ^- . The cloned fragments were excised (*Eco*RI-*Hind*III) and transferred to the *Eco*RI-*Hind*III sites of YIp5, generating the recombinants pN24 and pN24x2. The mtDNA from ρ^- mutants DS400/N1, DS400/M8 and DS400/N9 was cut in the unique *Mbo*I site (+320) and cloned into the *Bam*HI site of YIp5, giving rise to pN1, pM8 and pN9S respectively, each containing only one repeat unit (430 bp, 1440 bp and 910 bp respectively) of the corresponding mtDNA. The reiterated fragment of DS400/N7 has an internal deletion, eliminating the *Mbo*I site common to DS400/N1, DS400/M8 and DS400/N9 (Figure 1). We therefore used the unique *Hae*III site (+766 bp) for cloning the genome repeat unit (720 bp) at the *Hinc*II

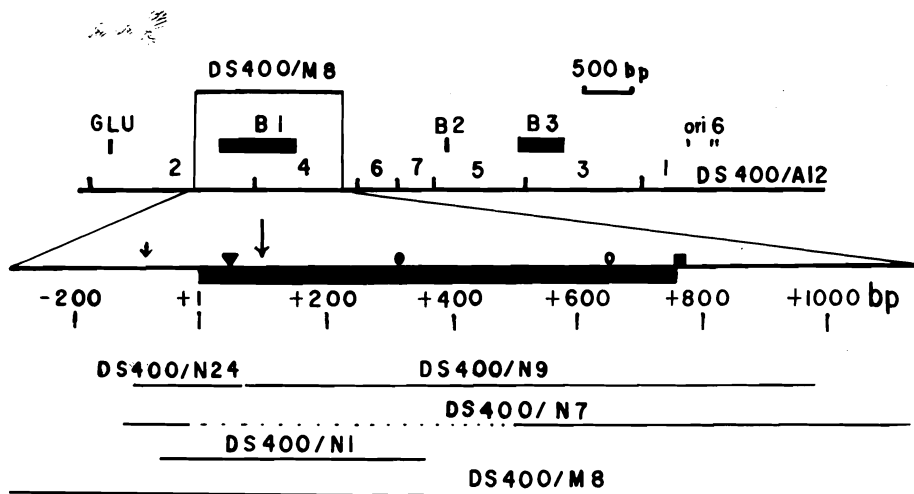


Figure 1 - Location of the conserved segments of mtDNA in the ρ^- mutants studied. The three exons of the apocytochrome b gene (B1, B2 and B3) and the glutamyl-tRNA gene (GLU) are indicated above the line that represents ρ^- DS400/A12 and its seven *Mbo*I restriction fragments. The expanded part of the figure shows the region occupied by the ρ^- DS400/M8 and other four smaller petites: DS400/N9, DS400/N7, DS400/N1 and DS400/N24. The vertical arrow indicates the location of the 11-nucleotide ARS consensus sequences mapped previously (Delouya *et al.*, 1987). The symbols used to denote the restriction sites are: *Hinf*I (∇), *Mbo*I (\circ), *Eco*RI (\circ), and *Hae*III (\blacksquare).

site of pUC7. The insert from the corresponding construct (pUN7), was excised (BamHI), and cloned into the BamHI site of YIp5 yielding pN7 and pN7x2 (containing two inserts in tandem). Using the same HaeIII site, alternative recombinants derived from DS400/N9 were generated: the fragment (910 bp) was cloned into the HincII site of pUC7 originating pUN9. This insert was subsequently cloned into the BamHI site of YIp5 yielding pN9. The orientation of the cloned segments in the vector (Table I) was determined by the appropriate diagnostic restriction endonuclease digestions (Figure 2).

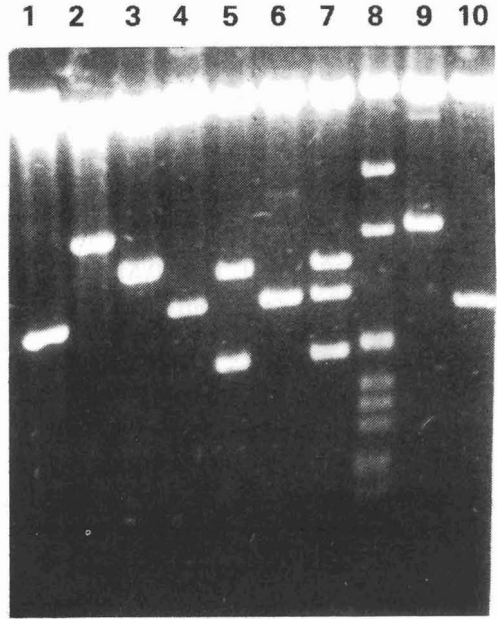


Figure 2 - Agarose gel (1%) electrophoresis of YIp5 plasmid derivatives containing the conserved segments of low-complexity rho⁻ mutants. 1, pM8; 2, pN9; 3, pN9S; 4 and 5, pN7; 6 and 7, pN7x2; 8. known-size DNA fragments derived from plasmid pCL778/44 digested with HincII are, from the uppermost band (in kb): 5.5, 1.6, 1.0, 0.6, 0.5, 0.4, 0.3, 0.22 and 0.15; 9, pN1 and 10, YIp5. Restriction enzyme digestions were EcoRI (lanes 1 to 3), BamHI (lanes 4 and 6) and EcoRI/SalI (lanes 5, 7, 9 and HincII (lane 8).

RESULTS

The rho⁻ mutants studied were all characterized during the study of the mtDNA region that encodes the apocytochrome b gene in yeast. Their complexity and deletion end-points were defined by DNA sequencing (Nóbrega and Tzagoloff, 1980a,b). The conserved segments of the rho⁻ studied overlap considerably (Figure 1) over the first exon of the "short" apocytochrome b gene. The recombinant clones constructed show a group with high ARS-activity as shown by the mitotic stability under selective conditions: pN1; pM8; pN9S and pN9 (Table I). In particular, recombinant pN1 could be regarded as a natural subclone of the MboI fragment 2, shown previously (Delouya *et al.*, 1987) to be

Table I - Transformation efficiency and mitotic stability of YIp5 derivatives of yeast rho⁻ mitochondrial genomes.

Plasmid ^a	Transformants ^b	Selective stability ^c	Non-selective stability ^d	Size of insert ^e
YIp5	0	-	-	-
YRp17	410	9.3	1.4	-
pBC283	370	10.0	8.0	1660
pBC200 ^f	740	4.5	4.0	1660
pN1	650	11.0	9.0	430
pM8	900	9.8	2.0	1440
PN9S ^f	800	6.7	0.8	910
pN9	500	5.5	0.6	910
Pn24x2	1060	0.4	< 0.1	2x170
Pn24	560	< 0.1	< 0.01	170
PN7x2 ^f	160	< 0.1	< 0.01	2x720
pN7 ^f	g	-	-	720

a: YIp5 derivatives containing one or two repeat units of the rho⁻ genomes as inserts (see Materials and Methods).

b: Number of yeast transformants per µg of plasmid DNA.

c: Percentage of ura⁺ cells in a population growing under selective conditions.

d: As in c but after five generations in rich medium.

e: Size of mtDNA insert in base pairs.

f: Orientation of insert (cyt b gene) is opposite to the tet gene in YIp5. For the remaining constructs the orientation is the same as the tet gene.

g: Abortive transformants.

(Values are the average from three experiments).

the most active in the ARS assay (Table I, pBC283 and pBC200). The short genome of this rho⁻ (DS400/N1) is almost completely contained within MboI fragment 2, derived from the complex rho⁻ DS400/A12 (Nóbrega and Tzagoloff, 1980a,b) and encompasses only 430 bp, with a ARS consensus at position +110 (Delouya *et al.*, 1987). In this group the percentage of cells carrying the recombinant plasmid ranges from 11 to 5.5%. The low activity group is represented by the recombinants containing the repeat units of DS400/N24 (170 bp) and DS400/N7 (720 bp). They have a mitotic stability below 1% (Table I). Reiteration increased the stability of these low ARS-activity DNA segments as shown in a comparison between pN24x2 and pN24. In the case of pN7, recombinants

with a single copy of the repeat unit gave only abortive transformants that failed to grow when re-inoculated onto selective medium. The recombinant pN7x2, with two copies in tandem, gave a weak but consistent ARS-activity. Recombinant plasmids identical to the original constructs (Figure 2) were rescued in *E. coli* from single colony isolates of yeast transformants grown on selective medium. This was possible from all constructs except pN7.

DISCUSSION

The high stability constructs (pN1; pM8 and pN9) derive from ρ^- mutants that overlap (Figure 1) and define a common region of 292 bp, from position +74 to position +365 in the apocytochrome b gene (Nóbrega and Tzagoloff, 1980b). Within this region Delouya *et al.* (1987) located a rDNA-like ARS consensus (Kearsey, 1984) at position +110. The unit segments retained in DS400/N24 and DS400/N7 yielded recombinants about ten times weaker in terms of mitotic stability (Table I). DS400/N7 had to be present as two copies to exhibit a very weak ARS-activity. The reiteration effect on the ARS activity was demonstrated for a 64 bp petite genome by Zeifel and Fangman (1990). In this case the unit segment had to be present eight or more times for the ARS activity to appear. The retained segments of DS400/N24 and DS400/N7 have no overlap with the 292 bp region common to the other three ρ^- . Delouya *et al.* (1987) located a putative ARS consensus sequence common to DS400/N24 and DS400/N7 at position -85. A comparison of the relative stabilities of genetic markers in ρ^- mutants DS400/N1; /M8, /N9 and /N7, showed that the first three exhibited a higher stability upon continuous subculturing, when compared to DS400/N7 (results not shown). The correlation between ARS-activity and replication/maintenance of ρ^- genomes was suggested initially (Delouya *et al.*, 1987) by the finding of this activity in mtDNA fragments common to low complexity petite mutants. No activity was found in fragments from regions where no low-complexity, exclusive and stable petites were described, despite the presence of genetic markers that allow for a targeted harvest of ρ^- mutants (De La Salle *et al.*, 1982). A further study dissected the ori 6 region located downstream from the apocytochrome b gene (Delouya and Nóbrega, 1991) and the ARS-active segments identified always had a homologous counterpart in a collection of neutral or suppressive petites derived from different ori sequences, described previously by De Zamaroczy *et al.* (1981) and Baldacci *et al.* (1984). The results presented here expand this finding to five low complexity ρ^- derived from a mtDNA region that lacks described ori or rep sequences. This suggests that a positive ARS assay indicates replicative potential inside the organelle and supports the idea that nuclear and mitochondrial DNA replication systems share functional similarities. Recently Zweifel and Fangman (1991) described a yeast nuclear gene (MGT1) that is required for the displacement of the ρ^+ genome in zygotic clones

derived from mating with a hypersuppressive ρ^- strain. The authors found that the maintenance of the ρ^- mtDNA is independent of the presence of the MGT1 allele. In consequence, Zweifel and Fangman postulated an alternative mechanism to replicate ρ^- genomes and suggested the A+T-rich regions of mtDNA as the possible cis-acting targets. In fact Fangman *et al.* (1989) provided conclusive evidence that petites containing only A+T base pairs in their mtDNA are capable of autonomous replication in yeast. Besides the alternate replication system proposed by Zweifel and Fangman (1991), it is also reasonable to imagine a solution that uses, instead of specialized gene products, the compartmental isolation of the ρ^- genome. In this unique system, natural hierarchies among high-affinity (for example, ori regions) or low-affinity (for example A+T-rich mtDNA regions) would become evident. This will happen when low-affinity regions, in the absence of competition from canonical replication targets, act as surrogate or secondary cis-acting sites for the usual replication machinery. We also suggest that the prime candidate for this cis-acting target is the 11-nucleotide ARS consensus sequence, a ubiquitous motif among sequenced petite genomes (Delouya *et al.*, 1987 and Delouya and Nóbrega, 1991).

ACKNOWLEDGMENTS

We thank José Lino and Cristina Keiko Takahashi for technical help. Financial support was provided by FAPESP, CNPq, FINEP/PADCT and BID/USP.

Publication supported by FAPESP.

RESUMO

Foram obtidos oito clones recombinantes construídos no vetor integrativo Y1p5 para os segmentos unitários repetidos que constituem o genoma mitocondrial de cinco petites de baixa complexidade e que pertencem à região correspondente ao gene do apocitocromo b no genoma mitocondrial de *Saccharomyces cerevisiae*. O aparecimento da capacidade de replicação autônoma (ARS) nessas construções foi examinada após transformação da levedura. Para quatro genomas a presença de uma única unidade de repetição foi suficiente para conferir a capacidade replicativa ao vetor. Em um dos casos foi necessária a presença de duas unidades de repetição para expressão do fenótipo ARS-positivo. Os resultados são compatíveis com a hipótese de que a sequência representada pelo consenso ARS de 11 nucleotídeos poderia atuar como uma região ativa em cis na replicação de genomas petite que não possuem as origens de replicação consideradas canônicas.

REFERENCES

- Baldacci, G., Cherif-Zahar, B. and Bernardi, G. (1984). The initiation of DNA replication in the mitochondrial genome of yeast. *EMBO J.* 3: 2115-2120.

- Blanc, H. and Dujon, B. (1980). Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. *Proc. Natl. Acad. Sci. USA* 77: 3942-3946.
- Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977). Construction and characterization of new cloning vehicles. II A multipurpose cloning system. *Gene* 2: 95-113.
- Brewer, B.J. and Fangman, W.L. (1987). The localization of replication origins on ARS plasmids in *S. cerevisiae*. *Cell* 51: 463-471.
- Celniker, S.E., Sweder, K., Srien, F., Bailey, J.E. and Campbell, J.L. (1984). Deletion mutations affecting autonomously replicating sequence ars 1 of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 4: 2455-2466.
- Delouya, D., Bonjardim, C.A. and Nóbrega, F.G. (1987). ARS activity along the yeast mitochondrial apocytochrome b region: correlation with the location of petite genomes and consensus sequences. *Curr. Genet.* 12: 583-589.
- Delouya, D. and Nóbrega, F.G. (1991). Mapping of the ARS-like activity and transcription initiation sites in the non-canonical yeast mitochondrial ori 6 region. *Yeast* 7: 51-60.
- De La Salle, H., Jacq, C. and Slonimski, P.P. (1982). Critical sequences within mitochondrial introns: pleiotropic mRNA maturase and cis-dominant signals of the box intron controlling reductase and oxidase. *Cell* 28: 721-732.
- De Zamaroczy, M., Marotta, R., Faugeron-Fonty, G., Goursot, R., Mangin, M., Baldacci, G. and Bernardi, G. (1981). The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity. *Nature* 292: 75-78.
- Fangman, W.L., Hcnly, J.W., Churchill, G. and Brewer, B.J. (1989). Stable maintenance of a 35-base-pair yeast mitochondrial genome. *Mol. Cell. Biol.* 9: 1917-1921.
- Kearsey, S. (1984). Structural requirements for the function of a yeast chromosomal replicator. *Cell* 37: 299-307.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory-Cold Spring Harbor, NY.
- Newlon, C.S. (1988). Yeast chromosome replication and segregation. *Microbiol. Reviews* 52: 568-601.
- Nóbrega, M.P. and Nóbrega, F.G. (1986). Mapping and sequencing of the wild-type and mutant (G116-40) alleles of the tyrosyl-tRNA mitochondrial gene in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 261: 3054-3059.
- Nóbrega, F.G. and Tzagoloff, A. (1980a). Assembly of the mitochondrial membrane system. Complete restriction map of the cytochrome b region of mitochondrial DNA in *Saccharomyces cerevisiae* D273-10B. *J. Biol. Chem.* 255: 9821-9827.
- Nóbrega, F.G. and Tzagoloff, A. (1980b). Assembly of the mitochondrial membrane system. DNA sequence and organization of the cytochrome b gene in *Saccharomyces cerevisiae* D273-10B. *J. Biol. Chem.* 255: 9828-9837.
- Scherer, S. and Davis, R.W. (1979). Replacement of chromosome segments with altered DNA sequences constructed *in vitro*. *Proc. Natl. Acad. Sci. USA* 76: 4951-4955.
- Stinchcomb, D.T., Thomas, M., Kelly, J., Selker, E. and Davis, R.W. (1980). Eukaryotic DNA segments capable of autonomous replication in yeast. *Proc. Natl. Acad. Sci. USA* 77: 4559-4563.

- Struhl, K., Stinchcomb, D.T., Scherer, S. and Davis, R.W. (1979). High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. *Proc. natl. Acad. Sci. USA* 76: 1035-1039.
- Vieira, J. and Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19: 259-268.
- Zweifel, S.G. and Fangman, W.L. (1990). Creation of ARS activity through iteration of non-functional sequences. *Yeast* 6: 179-186.
- Zweifel, S.G. and Fangman, W.L. (1991). A nuclear mutation reversing a biased transmission of yeast mitochondrial DNA. *Genetics* 128: 241-249.

(Received August 14, 1992)