Transcription through the yeast origin of replication ARS1 ends at the ABFI binding site and affects extrachromosomal maintenance of minichromosomes

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ABSTRACT

When the function of origins of replication in yeast was compromised by placing ARS sequences downstream of strong promoters, ARS activity might have been affected either by transcription or by an altered chromatin configuration induced by the construct. To distinguish between these possibilities, derivatives of the yeast TRP1ARS1 minichromosome were constructed that contained either the DED1 or the PET56 promoter firing against ARS1 (DEDARS and PETARS constructs). PETARS constructs transformed yeast at high frequencies and were maintained as minichromosomes consistent with efficient ARS1 function, but DEDARS constructs transformed at low frequencies and had to be rescued as minichromosomes by insertion of a second ARS (H4-ARS). Chromatin analysis revealed that the ARS1 regions in PETARS and H4-DEDARS constructs were indistinguishable from the ARS1 region of the host TRP1ARS1 circle showing a nuclease sensitive region flanked by a nucleosome. However, RNA-analysis in the ARS region showed high and low levels of transcripts in H4-DEDARS and PETARS, respectively. Transcription elongated through the A, B1, and B2 elements and ended in B3, the binding site for ABFI. We conclude that transcription through ARS1 and not an altered chromatin structure affected ARS activity in these constructs.

INTRODUCTION

In yeast *S.cerevisiae*, autonomously replicating sequences (ARS) serve as origins of replication (1) (reviewed in (2)). DNA containing functional ARSs confers high frequencies of transformation and extrachromosomal maintenance of the DNA (3). A well known ARS is ARS1 which is located between the 3' end of the TRP1 gene and the 5' end of the GAL3 gene on chromosome IV (4, 5) (For convenience, we refer to nucleotides of the published sequence as map units, MU). The 1453 bp long

TRP1ARS1–EcoR1 fragment, when circularized, exists as a high copy number plasmid in yeast which is efficiently replicated, packaged into nucleosomes (6) and served as an ideal substrate to study chromatin structure and function (Fig. 1; for reviews (7, 8)).

ARS1 consists of an 11 bp A-element (Fig.2) which is essential for function, and additional sequences (B1, B2, B3-elements) which support function and bind proteins of the origin of replication complex (ORC) and ABFI (9, 10, 11, 12, 13), reviewed in (14)). The B region contains a stretch of bent DNA (15). Chromatin studies at low resolution revealed that the A-element is located at the edge of a nucleosome (for convenience called ARS1 nucleosome), while the other elements are located in a nuclease sensitive region (16). An alternative structure which was suggested for that region (17) could not be confirmed. In contrast, a large series of constructs with insertions in the HindIII site and NaeI site (Fig.1) did not affect the local chromatin structure of the ARS1 region (18, 19, 20, 21, 22, 23).

Not all ARSs are active as origins of replication in chromosomes which is consistent with the idea that ARS function is subjected to a kind of position effect (reviewed in (2)). ARS activity can be affected by chromatin structure. When the A element of ARS1 was shifted towards the center of a nucleosome by manipulation of outside sequences, the copy numbers of the constructs were reduced consistent with an inhibition of replication (24). Hence presence of a nucleosome might have prevented interactions with the ORC proteins. Moreover, this experiment dramatically elucidated the functional significance of nucleosome positioning in the ARS1 region and suggested that altered chromatin structures might be a reason for inactivated ARSs in the chromosomes.

Alternatively, it was tested whether ARS activity can be affected by transcription. This was done on plasmids by placing ARSs into close proximity of the strong actin promoter (25) or close to the GAL1-10 promoter (26, 27) and recording plasmid stability as a function of transcription. In a similar approach, replication of plasmids in human cells was correlated with the transcriptional activity of inserted promoter elements (28).

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Although transcription appears to be the likely cause for that effect, the following points needed to be reconsidered: (i) Two papers reported that transcription was going through the origin of replication (25, 26), while in another case transcription was blocked in front of ARS1 (27). Hence, it is not obvious how transcription should affect activity of ARS1 without transcribing through the elements that are essential for binding the ORC. (ii) The proximity of the promoter might have changed the chromatin structure in the ARS region and thereby prevented binding of the ORC components. It is well established, that chromatin structures can be altered by boundary effects of flanking sequences (19, 23) (reviewed in (7, 8)). This was in particular shown for the GAL1–GAL10 promoter region (29, 30) which was used in the studies described above.

We report here that constructs in which the DED1 promoter fires transcription against ARS1 prevent high frequencies of transformation and extrachromosomal maintenance. Rescue of one of those constructs as a minichromosome showed that inactivation of ARS function was not due to an altered chromatin structure, but due to transcription through the ARS1 region.

MATERIALS AND METHODS

Enzymes and chemicals

Restrictions enzymes, Micrococcal nuclease (MNase) and Proteinase K were from Boehringer Mannheim. Zymolyase-100T was from Seikagaku Kogyo Co. Ltd.

Yeast strains and plasmids

The host strain was Sc-3 (Mat alpha, ura3-52, his3-1, trp1, gal2, gal10, cir^o) (31). FTY61 is Sc-3 containing PETARS-817, DHY8 is Sc-3 containing H4-DEDARS. Constructs and strains are shown in Figure 1 and Table 1. Yeast strains were grown at 30°C in selective minimal media containing 2% glucose, 0.67% yeast nitrogen base without amino acids, uracil (20 mg/l), tryptophan (20 mg/l) (32).

Constructions

Constructions were made using standard procedures. pUC-PETARS-817 (previously called pUCFT817 (22)) is PETARS-817 inserted as an EcoRI fragment in the EcoRI site of pUC8. pUC-DEDARS-814 is the same as pUC-PETARS-817 but with the HIS3–BamHI fragment in the opposite orientation (orientation L, Fig. 1B). The yeast sequences of pUC-DEDARS-814 were purified as an EcoRI fragment, circularized by ligation, cut at the XbaI site and inserted in a pUC19 vector with a destroyed EcoRI site to give plasmid pDH2. pAB9 is a pUC8 containing the 374 bp long H4-ARS fragment in the BamHI site (33). The HindIII site of pAB9 was converted to an EcoRI site. The H4-ARS was purified as an EcoRI fragment and inserted into the EcoRI site of pDH2 to give pUC-H4-DEDARS. The yeast sequences were purified as EcoRI or XbaI fragments, circularized by ligation and introduced into yeast *Saccharomyces cerevisiae* Sc-3 by transformation (34). Colonies were selected on minimal media plates (32).

Plasmid loss assay

To test whether the DNA was integrated or maintained extrachromosomally, individual colonies were grown in non-selective media (YPD, (32)) for 30 hours and aliquots of the culture containing 300–500 cells were plated on non-selective

YPD-plates and on selective minimal media plates. Cells that lose the plasmid during growth on YPD can not develop colonies on selective plates.

Copy number determination

Cells were grown in 25 ml selective media, total DNA was extracted according to a Yeast Mini-Prep protocol (32), cut with EcoRI, fractionated on 1% agarose gel, blotted to nitrocellulose and hybridized to a radioactive TRP1ARS1 DNA (16). The 1453 bp long genomic TRP1ARS1 EcoRI fragment served as a standard for a single copy genomic sequence (4).

Chromatin preparations and mapping of nucleosome positions

The procedures have been published elsewhere (18). Briefly, plasmid containing yeast strains were grown on selective media, converted to spheroplasts by Zymolyase. Spheroplasts were lysed in 0.2% Triton X-100, the genomic chromatin was pelleted by centrifugation and the minichromosomes were separated from small molecular contaminants by fractionation on a Sephacryl-S300 column (Pharmacia). Elution buffer A was 20 mM Tris–HCl (pH 8), 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 1 mM PMSF. Chromatin and deproteinized DNA were digested in buffer A with different amounts of micrococcal nuclease (0, 2, 10, 50, 250 units/ml for chromatin; 0.4, 2, 10 units/ml for DNA) in the presence of 5 mM CaCl₂ for 5 minutes at 37°C. DNaseI digestions of chromatin were in 5 mM MgCl₂ using between 0.5 and 125 units/ml for 5 minutes at 37°C. The digestions were stopped with 1% SDS, 10 mM EDTA, 10 µg Proteinase K. After incubation for 2 hours at 50°C, the DNA was purified by phenol extractions, treated with RNase A (100 µg/ml) for about 30 minutes, and further purified by phenol extractions and ethanol precipitation. The DNA was dissolved in TE (10 mM Tris–HCl (pH 7.6), 1 mM EDTA). For mapping nucleosome positions by indirect endlabelling, the DNA was cut to completion with a restriction endonuclease as indicated in the figure legend, fractionated according to size on 1% agarose gels, blotted to nitrocellulose and hybridized to radioactively labelled probes. Template DNAs for probes were subcloned fragments of the TRP1 region (XbaI–EcoRV, Fig. 1). Radioactive probes were made by random priming using a kit (Pharmacia). A DNA ladder consisting of multiples of 256 bp was used for calibration (16). Radioactive bands were detected using Fuji X-ray films and enhancer screens.

RNA analysis

Cells were harvested during exponential growth. Total RNA was extracted (35), run on 1% agarose gels containing formaldehyde (36), blotted on Biodyne A membrane according to manufacturer's instructions (Pall) and hybridized to the appropriate probes (see figure legend). The membranes were exposed on Fuji RX films with intensifier screens. For S1-mapping of the 3' ends, a single stranded HindIII–BamHI fragment labelled at the BamHI 3' end was prepared from pBRAT2 according to (37). pBRAT2 is a pBR322 derivative containing the TRP1ARS1 sequence in the EcoRI site and a BamHI linker in the NaeI site of TRP1ARS1 (1070 MU (18)). S1-mapping was done according to standard methods (38). DNA sequencing for comparison with S1 protected fragment was done according to Maxam and Gilbert (39) with minor modification. Instead of cleaving the purine residues of end-labeled DNA with piperidine formate, we used 0.5M acetic acid and incubated samples at 45°C for 20 min.

RESULTS

In a series of constructs originally designed to study nucleosome positioning, we have observed that when the HIS3-BamHI fragment was inserted into the TRP1ARS1 circle in orientation R with PET56 facing ARS1 (referred to as PETARS constructs; Fig.1A), the frequency of transformation was high (several hundred colonies/ μ g DNA) and the DNA was maintained extrachromosomally as yeast replicating plasmids (21, 22). However, when insertion was in orientation L with DED1 facing ARS1 (referred to as DEDARS constructs), the frequency of transformation was low (no or a few colonies/ μ g DNA) and the DNA was integrated as examined by plasmid loss assays. Since high frequency of transformation and extrachromosomal maintenance are accepted criteria for ARS function, the results showed that DEDARS constructs had impaired ARS1 function. These observations were largely independent of various additional inserts made in the TRP1 gene, such as the 5'- and 3'-end, or

the central part of the URA3 gene, and they were also independent on whether bacterial vector sequences (pUC8 or a pBR322 derivative) were present in the EcoRI site.

To investigate whether an alteration in chromatin structure or transcription through ARS1 was responsible for differential ARS activity, one pair of constructs was studied in more detail (Fig. 1B; Table 1). pUC-DEDARS-814 is a pUC8 derivative that contains the TRP1ARS1-circle in the EcoRI site and the HIS3-BamHI fragment in orientation L. (A sequence element of URA3 (Umid) inserted in the HindIII site of TRP1 is not relevant for this work). pUC-PETARS-817 is the same as pUC-DEDARS-814, but with the HIS3-BamHI fragment in orientation R. While transformation of Sc-3 with pUC-DEDARS-814 did not yielded colonies, pUC-PETARS-817 transformed with about 1600 colonies/ μ g DNA.

Since procaryotic vector sequences might interfere with chromatin structure and transcription, the vector sequences were removed by EcoRI cleavage and the yeast sequences were

Figure 1. Constructs and relevant chromatin structures. **A.** The TRP1ARS1 circle is a circularized 1453 bp long EcoRI fragment of yeast *Saccharomyces cerevisiae* and consists of the TRP1 gene, an origin of replication (ARS1, black box) and a sequence upstream of the GAL3 gene (4, 5). Four nucleosomes were found on the TRP1 gene (wavy circles) and three on the GAL3 upstream region (hatched circles I, II, III). One nuclease sensitive region was found spanning the end of the TRP1 gene (arrowhead) and ARS1 (NS-ARS1). A second nuclease sensitive region includes the EcoRI site (NS-R1) (16). A BamHI linker was inserted at the NaeI site (N, (18)). PET56-HIS3-DED1 is a 1764bp long BamHI fragment containing the HIS3 gene flanked by the PET56 and DED1 promoters (43). The chromatin structure is shown as described (41). H4-ARS serves as an origin of replication (33). The relevant restriction sites are: H, HindIII; N, NaeI; RV, EcoRV; X, XbaI. **B.** The relevant constructs are shown. TRP1ARS1 sequences (black and white), PET56-HIS3-DED1, pUC vectors (thin line), H4-ARS (white box). All constructs contain a fragment of the central part of the URA3 gene inserted in the HindIII site of TRP1 (stippled box, (22)). This insert is irrelevant for this work. **C.** Copy number estimation. Total DNA of host strain Sc-3 (lanes 10–13) and of FTY61 (lanes 6–9) and DHY8 (lanes 2–5) containing the plasmids PETARS-817 and H4-DEDARS, respectively, was purified, restricted with EcoRI (lanes 3–5, 7–9, 11–13), run on a 1% agarose gel, blotted on a nitrocellulose membrane and hybridized to a radioactively labeled TRP1ARS1 sequence. Loading was 20 μ l (about 200 ng DNA, lanes 3,7,11), 10 μ l (lanes 4,8,12) and 2 μ l (lanes 5,9,13). Unrestricted DNA was loaded (about 100 ng DNA, lanes 2,6,10). The lower band is the 1453 bp long genomic TRP1ARS1 fragment. G is uncut genomic DNA, I is supercoiled plasmid DNA, II is nicked plasmid DNA, III is linearized plasmid DNA. The plasmid DNA in that particular DHY8 preparation was nicked (form II, lane 2) and the EcoRI digests were partial (lanes 3–5). A 256 bp ladder was used as a size marker (lane 1 (16)).

Table 1.

1	2	3	4	5	6
Yeast Strain	Construct	Vector	H4-ARS	Transformation	Extrachromosomal
FTY61	pUC-PETARS-817	pUC8		++	+
	pUC-DEDARS-814	pUC8		-	-
	PETARS-817	-		++	+
	DEDARS-814	-		-	-
DHY8	pUC-H4-DEDARS	pUC19	+	++	+
	H4-DEDARS	-	+	++	+

Column 5, Transformation efficiencies: > > 100 colonies/ μ g DNA (++) , no or few colonies/ μ g DNA (-).

Column 6, Extrachromosomal: Plasmid loss on nonselective media (+), no plasmid loss (-).

circularized before transformation to give DEDARS-814 and PETARS-817, respectively (Fig. 1B, Table 1). PETARS-817 transformed Sc-3 with about 240 colonies/ μ g ligated DNA, while no transformants were obtained using DEDARS-814. All colonies of cells transformed with PETARS-817 showed a loss of the HIS3 marker when grown in nonselective YPD media, demonstrating that PETARS-817 was maintained as an extrachromosomally replicating plasmid. This strain was called FTY61 (Table 1) (22). The result was confirmed by DNA analysis of FTY61 hybridized to a TRP1ARS1 sequence in a Southern blot (Fig. 1C). The bands representing the characteristic supercoiled form I DNA of PETARS-817, the nicked form II, traces of linearized form III DNA were observed (lane 6). The genomic TRP1ARS1 sequence hybridized with the bulk genomic DNA (G).

Rescue of extrachromosomal maintenance by insertion of a second ARS

In order to be able to analyse transcription and chromatin structure of the DEDARS constructs, the DEDARS-814 constructs were rescued as minichromosomes by insertion of a second origin of replication (the H4-ARS) to give pUC-H4-DEDARS and H4-DEDARS, respectively (Fig. 1B, Table 1). The H4-ARS was inserted into the EcoRI site which is located in a nuclease sensitive region (16). This site was previously shown to accept various kinds of inserts without affecting ARS1 function (20, 21, 23). When yeast was transformed with pUC-H4-DEDARS and H4-DEDARS about 106 and 310 colonies/ μ g DNA were obtained, respectively, demonstrating efficient transformation. All tested colonies showed a high plasmid loss when incubated in nonselective media (YPD) for several hours. Hence, insertion of the H4-ARS was sufficient to provide extrachromosomal maintenance to a DEDARS construct. The yeast strain was called DHY8 (Table 1).

To estimate the copy numbers, Southern blots of total DNA which has been cut by EcoRI were hybridized to a TRP1ARS1 probe (Fig. 1C). They revealed a weak band for the genomic TRP1ARS1 sequence, which occurs as one copy per haploid genome, and a stronger band corresponding to the TRP1ARS1 sequences in the minichromosomes. Based on the serial dilution, we estimated that PETARS-817 (in strain FTY61) and H4-DEDARS (in strain DHY8) occur in about ten and six copies per cell, respectively, which is an order of magnitude lower than the copy number of the host TRP1ARS1 circle (6).

Transcription proceeds through ARS1

RNA was extracted from DHY8, FTY61 and the host strain Sc-3. When the RNA was run on an agarose gel, blotted to a nylon

Figure 2. A schematic drawing of the ARS1 region in PETARS-817 and H4-DEDARS. The ARS1 region and the essential DNA elements A, B1, B2, B3 are shown (11). Ellipses represent nucleosome positions. Hatched ellipses indicate ARS1 nucleosomes. Arrows represent RNA. Rulers show Map Units (MU) in bp starting from the EcoRI site for PETARS-817 and H4-DEDARS (top) and for TRP1ARS1 (bottom). ($MU_{TRP1ARS1} = MU_{PETARS-817} - 546$)

membrane and hybridized to an ARS1-probe (PstI–NaeI, Fig.2). DHY8 showed a strong band of approximately 700 nucleotides and a minor band of about 550 nucleotides (Fig. 3A, lane 3). In contrast, FTY61 showed a weak band of about 550 nucleotides (lane2). No RNA was detected in the host strain indicating that there was no transcription through the genomic ARS1 (lane 1). (About twice as much RNA was loaded in DHY8 compared with FTY61 as shown by hybridization with an ACT1 probe (lanes 4–6)). The ARS1–RNA levels in DHY8 were approximately five times higher than in FTY61. To investigate whether transcription proceeds through ARS1, the 3' ends of the transcripts were determined by S1-mapping (Fig. 3B). In DHY8, the major transcripts terminated within the B3-element and close to it (at 765 MU of the TRP1ARS1 sequence), while the shorter and weaker transcripts ended 28 base pairs in front of the A-element (at around 896 MU). In FTY61 the amounts of transcripts were lower (faint bands) but the same 3' ends were mapped for the longer and the shorter transcripts (better visible on longer exposures, not shown). The differences in transcript lengths

Figure 3. RNA analysis by Northern blots and S1-mapping. **A.** RNA was extracted from SC-3, FTY61, DHY8, run on an agarose gel, blotted to a membrane and hybridized with a radioactively labelled ACT1 probe (lanes 4,5,6) or with a radioactively labelled ARS1 probe (PstI–NaeI; lanes 1,2,3). **B.** S1-mapping of the 3' end is shown. A HindIII–BamHI fragment labelled at the BamHI 3' end was used for hybridization with the RNA. Different amounts of S1 were used 250 U (lanes 1,4), 500 U (lanes 2,5), 1000 U (lanes 3,6). Sequencing lanes are shown (G,GA,TC,C). Arrows indicate 3' ends or processing sites of RNA at 896MU_{TRP1ARS1} (lower arrow) and 765 MU_{TRP1ARS1} (upper arrow). No S1 protection was recorded with Sc-3 RNA (not shown). DNA restricted at the BglII site (852MU_{TRP1ARS1}) and full length input DNA are shown (lanes 7, 8).

between DHY8 and FTY61 as observed in the Northern analysis (Fig. 3A) originate from the different start sites in the DED1 and PET56 promoters, respectively (Fig. 2A). Hence, when transcription occurred, the majority of transcripts proceeded through the ARS1 region including the A-, B1-, and B2-elements and terminated (or were processed) in the B3 box. This result strongly suggests that transcription might interfere with the initiation of replication e.g. by stripping of essential factors from the DNA.

No alterations in chromatin structure of ARS1

To investigate whether an altered chromatin structure in the ARS1 region might have led to the inactivation of replication in the DEDARS constructs, H4-DEDARS and PETARS-817 were partially purified as minichromosomes from DHY8 and FTY61 cells, respectively, and the chromatin structures were analysed by digestion with micrococcal nuclease (MNase). MNase preferentially attacks linker DNA between nucleosomes and leads to double stranded cuts (40).

When the double stranded cutting sites for MNase were displayed at low resolution by indirect endlabelling, the structures of the ARS1 region of H4-DEDARS and PETARS-817 appeared to be very similar. Both constructs showed a nuclease sensitive region and a flanking nucleosome (hatched boxes, Fig. 4) which are the characteristic features of the ARS1 chromatin structure in the TRP1ARS1 circle (16) and in many other derivative constructs (19, 20, 21, 22). Figure 4B shows a direct comparison on the same gel and the marker lane used for mapping.

Outside of the ARS1 nucleosome, the structures were remarkably different. Three positioned nucleosomes were found between the ARS nucleosome and the nuclease sensitive DED1

Figure 4. Chromatin structure of the ARS1 regions at low resolution. **A.** Chromatin (C lanes) and DNA (D lanes) were digested with MNase and double strand cuts were mapped from XbaI (Fig. 1) using a probe generated from an XbaI–EcoRV fragment. Regions protected against double strand cutting by MNase of 140 to 200 bp indicate positioned nucleosomes (boxes). The nucleosome flanking the ARS1 region (ARS1 nucleosome) is indicated (hatched box). H4-DEDARS (lanes 1–5), PETARS-817 (lanes 6–8). MNase digestion conditions were for lanes 1 to 8: 0.4, 10, 2, 50, 10, 2, 50 (in units, see Materials and Methods). **B.** A direct comparison of PETARS-817 (lane 3) with H4-DEDARS (lane 4) and naked DNA of H4-DEDARS (lane 2) is shown. A 256 bp ladder was used as a size marker (lane 1) (16).

promoter in H4-DEDARS. Two of them were tightly packed covering about 290 bp of DNA. Tight packaging of nucleosomes is not an unusual feature in yeast chromatin. It has been observed in the HIS3 gene (41) (see also Fig. 4 top part), in the URA3 gene on plasmids (19) and in the genome (Thoma, unpublished results), as well as in artificial constructs (20). In PETARS-817, two well spaced, but positioned nucleosomes were formed between the ARS1 nucleosome and the nuclease sensitive PET56 promoter. Hence, despite of these differences in flanking structures, no obvious effect on the chromatin structure of ARS1 was observed when the orientation of the HIS3–BamHI fragment was changed. In particular, a shift of the ARS1 A-element towards the center of a nucleosome as one would expect for ARS1 inactivation (24) was clearly not observed in the H4-DEDARS construct.

DISCUSSION

In contrast to PETARS constructs, the DEDARS constructs transformed yeast at very low frequencies demonstrating impaired ARS1 activity. The constructions were made outside of the ARS region and did not directly affect the DNA elements required for ARS1 function (10, 11, 12, 13). Furthermore, the same construction site in the TRP1ARS1 circle has been used for many other constructs without effect on ARS activity (18, 20, 21, 22). Hence, it was likely that transcription or an altered chromatin structure might have inactivated ARS1.

Using standard chromatin analysis by micrococcal nuclease digestion and indirect endlabelling (16, 18, 23, 24, 29), the ARS1 chromatin structures were found to be very similar in the H4-DEDARS, PETARS-817, and in the host TRP1ARS1 circle

(16), namely consisting of a nuclease sensitive region (NSR) and a positioned nucleosome ('ARS nucleosome'). A weak band in the ARS1 nucleosome of PETARS-817 which we had observed on longer exposures (not shown) suggested that a minor fraction of the ARS1 nucleosome may occupy alternative positions. The NSR contains the B1, B2, and B3 elements of ARS1, while the A-element is located at the edge of the ARS1 nucleosome. The location of the ARS elements and multiple positions for the ARS1 nucleosomes were confirmed by nucleotide resolution mapping (Tanaka and Thoma, in preparation). In order to obtain a strong effect on ARS activity, however, a shift of the A-element towards the center of a nucleosome is required (24). This situation was very clearly not observed. Hence, a chromatin effect imposed by placing the DED1 or PET56 promoters close to ARS1 can be ruled out.

Based on our previous manipulations of chromatin structures in the TRP1ARS1 circle, we have proposed a 'hierarchical' mode of nucleosome positioning, which implies that some positions are stronger than others (18). It is interesting to note that the chromatin structures of the flanking DED1 and PET56 regions were clearly different with respect to the arrangement of nucleosomes. Despite of these differences, the ARS1 region was not affected which further substantiates a dominant role of the ARS1 structure.

Transcription elongation disrupts chromatin structures and may lead to a local destabilization, rearrangement or loss of nucleosomes (reviewed in (42)). These effects may be seen in genes that are heavily transcribed such as the galactose regulated genes in yeast (e.g. (30) and refs. cited therein) or may be undetected in genes that are rarely transcribed such as the URA3 gene (19). Since nucleosome positions were very clearly detected in the DED1 and PET56 region, we infer that these structures reflect the transcriptionally inactive population of minichromosomes. Hence, the inactive DEDARS and PETARS constructs do not impose a deleterious structure on ARS1.

Since the effect of chromatin structure was ruled out, but transcripts were detected in the ARS region, we conclude that transcription through ARS1 affected ARS activity. RNA analysis showed that the ARS1 region was transcribed from the DED1 and PET56 promoters. Roughly five times more RNA was detected in the DEDARS strain compared with the PETARS strain. This result is comparable to the RNA levels produced by the DED1 and PET56 genes (43) and suggests that the DED1 promoter is stronger than the PET56 promoter. Although a minor stop or processing site of transcription was detected in the ARS nucleosome, the majority of the transcripts ended in the B3 element (Fig. 2A). This means that the RNA-polymerase must have transcribed on the top strand through the A, B1, and B2 regions. Since these elements were shown to be functionally important with respect to plasmid stability and high frequency of transformation (13) and since they are known to interact with proteins of the ORC *in vivo* (11) and *in vitro* (12), our results suggest that transcription affects ARS activity by disturbing the interaction of ORC proteins with DNA.

Our results are consistent with and further extend previous observations on ARS activity and transcription (25, 26, 27). Kipling and Kearsey reported that activity of an HO-ARS was negatively affected when it was placed in the intron of the actin gene just 200 bp downstream of the constitutive promoter (25). Murray and Cesareny observed replication inhibition in a 2μ derivative, when transcription was driven by a GAL1-10 promoter through the origin of replication (26). In a similar study,

plasmid stability was affected when transcription was driven by a GAL1-10 promoter against ARS1 in yeast replicating plasmids (27). Since none of these studies was substantiated by chromatin analysis, it could not be firmly decided whether transcription or a construction dependent alteration in chromatin configuration affected ARS activity. This is in particular important for the GAL1-10 promoter constructs, since several examples are known which demonstrated that the GAL1-10 promoter can affect nucleosome positioning in the flanking regions and that chromatin structures might alter when the promoter is activated (29, 30, 44). Furthermore, while transcription was reported to elongate through the HO-ARS and the 2μ ORI (25, 26), transcripts driven against ARS1 apparently stopped in front of ARS1, in region C (region C corresponds to the ARS nucleosome) (27). Our results clearly demonstrate transcription through ARS1.

The different RNA results might be based on a technical problem, since Snyder and coworkers used Northern analysis, while our results were complemented by S1-mapping. Alternatively, one might consider that chromatin structure was altered in a way that favored termination in front of ARS1 when the promoter was activated as previously shown (29, 30, 44) or that chromatin structure is altered, since the region between ARS1 and the EcoRI site is part of the upstream region of the GAL3 gene (5).

We have observed an average of approximately ten copies of the PETARS-817 per cell, which is considerably lower than 100 to 200 copies/cell originally reported for the TRP1ARS1 circle (6). In view of our transcription results, we think that the reduced copy number is a direct effect of low transcription rates when the PET56-promoter fires against ARS1. In the DEDARS constructs, an approximately five fold higher transcription is sufficient to extinct ARS-function.

In yeast, no example of an origin within a transcription unit has been found (2). Hence, transcription through origins of replication appears to be avoided. It is important to note that the 3'ends of transcripts mapped within B3, which is the binding site for ABFI (13). ABFI is an abundant protein which might play multiple roles within the cell besides being involved in replication (i.e. (9, 45)). Although we do not know whether the 3'end of the RNA indeed reflects the end of transcription or a processing site, our observations suggest that ABFI might act in termination of transcription. Whether the ABFI binding site is sufficient for termination and whether it works bidirectionally remains to be investigated. In the chromosome and in the TRP1ARS1 circle, ABFI or other proteins binding to the ABFI site might protect the ARS1 region against transcription from the TRP1 gene and ensure its ability to function.

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