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The silent *P* mating type locus in fission yeast contains two autonomously replicating sequences

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ABSTRACT

We show that in fission yeast two DNA fragments at the silent *P* mating type locus provide plasmids with the capability of autonomous replication. Bacterial vectors containing these sequences replicate in a polymeric form in fission yeast very much like plasmids with the commonly used replication sequence *ars1*, do. There are, however, several differences between the two new *ars* sequences. The percentage of cells containing the plasmid during selection, the plasmid copy number and the plasmid segregation during mitosis are all dependent on the choice of the *ars* sequence. A DNA fragment with *ars* activity from the left side of the silent *P* cassette represses the expression of the marker gene, *ura4*$^+$, at least three hundred fold compared to plasmids containing only the other new *ars* sequence or only *ars1*. The importance of replication in this promoter independent transcriptional regulation is further substantiated by the fact that the repression is partially released in the presence of *ars1* on the same plasmid.

INTRODUCTION

There are several examples in eukaryotic cells where transcription of a gene is dependent on its location in the genome. Two cases of this position effect have been known for tens of years (1). One of the two mammalian X chromosomes in females is unexpressed (2) and eye color determining genes in Drosophila variegate their expression when placed close to heterochromatin (3,4). Only recently has it become possible to analyse the mechanistic details of positional repression especially through studies on a third example, that of the mating type gene silencing in yeast (1).

A budding yeast cell switches between two mating types. The mating type information is stored at two unexpressed loci, which get expressed through transposition to a third, transcriptionally active locus on the same chromosome (5). Although the exact mechanism of silencing remains to be solved, there are several indications for the involvement of replication (1). The DNA elements responsible for repression at the two silent sites all contain autonomous replication sequences (*ARS*) (6,7). A disrupted silencer function can be re-established only by passage through the S phase in the cell cycle (8). One of the silencer elements is a chromosomal replication origin (9), and cis mutations in this element can be suppressed by overexpression of a gene believed to be involved in initiation of replication (10). This evidence, together with recent findings about the co-localization of an origin of replication and a negative regulatory element in Dictyostelium (11) suggest that replication commonly participates in long range transcriptional repression. That is why we decided to test if there is a coupling between replication and mating type gene repression in another genetically definable, but only remotely related yeast, *Schizosaccharomyces pombe*.

In fission yeast the mating type system also consists of three DNA cassettes, one active and two silent (12). One of the silent cassettes, the *mat2-P* cassette, cloned onto a plasmid, is repressed through four cis elements, two on each side of it (13). In addition, at least two trans acting factors are involved in the repression of the plasmid borne cassette (14). In screens for factors, which influence mating type switching in fission yeast, two mutations affecting the expression of chromosomal silent mating type cassettes were isolated (15, 16). These results indicate the involvement of protein–DNA interactions in the repression of transcription from these genes. It is not known if these interactions are modified through replication.

The fission yeast genome contains DNA sequences capable of supporting plasmid replication very much like those in budding yeast (17, 18). These *ars* sequences are found approximately once in 20 kb and share an 11 bp sequence, but this sequence is not essential for *ars* activity (17). In strong contrast to budding yeast, where *ars* sequences are about 100 bp long, the minimal *ars* sequences in fission yeast are over 500 bp long. The single chromosomal fission yeast origin studied so far has two *ars* elements several kb from each other and an initiation zone for replication between these elements (19). The defined *ars* sequences consist of at least 70% AT base pairs compared to a 60% AT content on average in the fission yeast genome.

We find that the silent *P* mating type region contains two *ars* elements, one on either side of the *P* mating type promoter. These DNA fragments differ significantly from each other in their effects on the plasmids carrying them. The left side sequences, unlike the right side sequences, strongly repress *ura4*$^+$ gene transcription on the plasmids. More importantly, the magnitude of this repression is influenced by the presence of another *ars*

element (*ars1*) on the same plasmid. Possible mechanisms for the involvement of replication in transcriptional repression in fission yeast are discussed.

MATERIALS AND METHODS

Strains and media

The strain *Eg328, mat1-M smt0 ura4-D18* was used as a *S.pombe* transformation host. The *E.coli* strain HB101 was used as a bacterial host for the shuttle vectors. Yeast was grown on rich YE medium, or minimal media with ammonium as a nitrogen source (20).

Plasmids and cloning procedures

A 3.9 kb Bgl II/Hind III fragment of the *mat2-P* locus was cloned into the polylinker of the non-replicating vector pDW228 (21). This vector is pGEM3 (Promega) with a *S.pombe ura4*$^+$ gene cloned in the PvuII site. The direction of *ura4*$^+$ transcription is towards the polylinker (fig.1) (21). Different subclones were obtained by deleting DNA in the insert with restriction enzymes, as indicated in the figures, followed by filling of DNA ends by Klenow fragment (Amersham), and ligation with T4 DNA ligase (Amersham).

Transformations

The lithium chloride method for fission yeast transformation was used (22). Bacterial transformations were done according to the standard procedures (23).

Southern analysis

Standard methods were used for Southern analysis (23). The bacterial vector, pGEM3 labelled by nick translation (Amersham) was used as a radioactive DNA probe for the rearrangement analysis. Copy number analyses were done by cutting total DNA with restriction enzymes that allowed separation of the plasmid band from the chromosomal, followed by Southern blotting and probing with RNA probes targeted at the plasmid inserts. Several different exposures were scanned using a BioRad model 620 video densitometer. The ratio between the two bands was taken as a measure of the total copy number. Each result is an average of at least three independent measurements.

Mitotic stability and loss rate measurements

The fraction of plasmid containing cells during selection (mitotic stability) was measured as previously described (13). Each value is an average of at least five measurements. Loss rate measurements in the absence of selection were made according

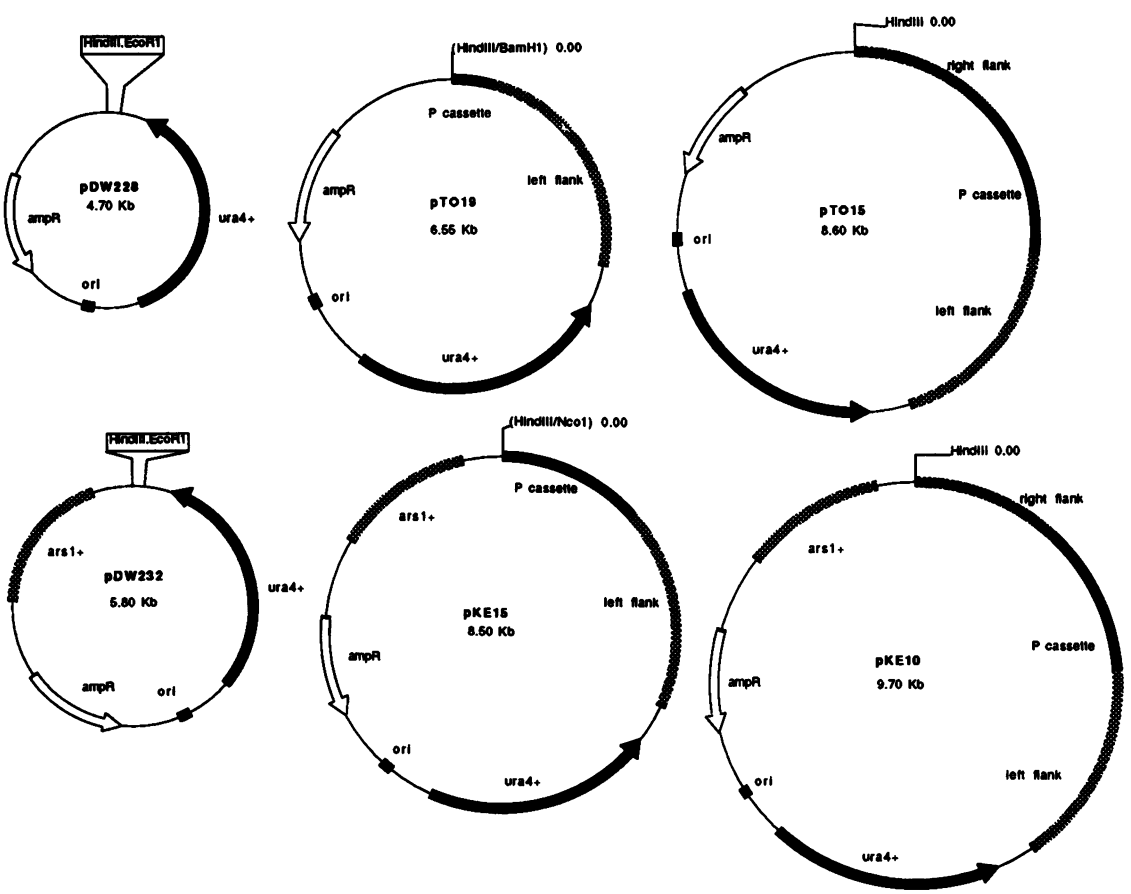

Figure 1. Schematic presentations of some of the most central plasmids used in this study (drawn to scale). All inserts are cloned in the same direction. *Ars1* is a previously isolated fragment of *S.pombe* DNA with *ars* activity. The ori sequence refers to the bacterial origin of replication. Plasmids pTO15 and pKE10 contain the 3.9 kb BglII/HindIII fragment from the silent *P* mating type region.

to Longtime *et al* (24). Each loss rate value is based on at least five measurements.

Northern analysis

Isolation of *S.pombe* RNA and Northern blotting were perfomed as described previously (13). The plasmid pDW232 was linearized with EcoRV (Amersham), and SP6 polymerase (Pharmacia-LKB) was used to create an RNA probe against *ura4*$^+$ mRNA. The level of trancription was measured by scanning several exposures using a BioRad model 620 video densitometer combined with a 1-D Analyst program for Macintosh (BioRad/Apple computer). Nonspecific hybridisation to ribosomal RNA was used as an internal standard.

RESULTS

High transformation efficiency with fragments both to the left and to the right of the silent mating type promoter

We have previously described a 3.9 kb BglII/HindIII fragment from the *mat2-P* locus, which contains the silent *mat2-P* cassette and four sequences necessary for its transcriptional repression (13, figure 2). In order to investigate the possibility of *ars* activity in this unexpressed region we chose to insert subfragments from that locus into pDW228 (figure 1), a non-replicating bacterial vector with a fission yeast *ura4*$^+$ marker. Transformations with the vector without an insert resulted in the production of microcolonies. Cells from these microcolonies were not able to grow further when transferred to a new selective plate. When fragments from the silent mating type locus were inserted into pDW228 we found that several of the resulting plasmids gave large colonies with high transformation frequencies (figure 2). The colonies grew equally well after transfer to another selective plate. The frequencies reached several thousand transformants per μg of plasmid DNA and were comparable to those obtained with an *ars1* plasmid (pDW232, data not shown). The smallest active fragments to the left (*ars2PL*), and to the right (*ars2PR*) of the promoter region were defined and were further tested with Southern analysis for rearrangements (see below). None of the elements previously defined as necessary for repression of plasmid-borne *P* genes (13) could alone function as an *ars* element (pTO21, pTO10, pTO11 and pTO3)(figure 2). Deletion of element 2, the strongest repressive element, from pTO19, gave no effect on the transformation frequency (not shown).

Ars2PL and *ars2PR* containing plasmids are maintained as extrachromosomal polymers

In order to determine whether the plasmids were integrated or maintained extrachromosomally we analyzed several transformants by Southern analysis. Undigested total DNA was compared to DNA digested with an enzyme that does not cut plasmid DNA, and another enzyme that cuts the plasmid once. As a DNA probe we used the bacterial vector pGEM3, that does not hybridize with fission yeast sequences under the applied conditions. Plasmids harbouring only *ars2PL* (pTO26) or *ars2PR* (pTO4) are maintained mainly as polymers that migrate extremely slowly in an agarose gel. This is also true for the vector pDW232, which contains the previously isolated *ars1*, and for pTO15, a plasmid containing both *ars2PL* and *ars2PR* (figure 3). When the plasmids are digested with an enzyme that linearizes them, they all appear as one band, with the expected size of a monomer.

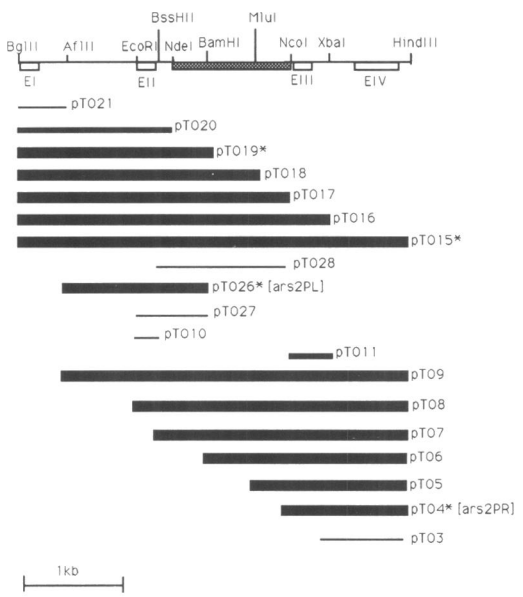

Figure 2. Transformation efficiency supported by different inserts from the *mat2-P* locus. After 3.5 days of growth at 30°C, colonies with a diameter of 1 mm or larger were scored. Thin bar: < 50 colonies/0.1 μg plasmid DNA, intermediate bar: 50-200 colonies/0.1 μg DNA and thick bar: > 1000 colonies/0.1 μg plasmid DNA. Constructs indicated with asterisk were further analyzed by Southern analysis. The plasmids pTO26 (*ars2PL*) and pTO4 (*ars2PR*) represent the smallest fragments found with *ars* activity. The long filled box shows the position of the *P* mating type cassette and the open boxes labelled EI-EIV show the positions of elements involved in transcriptional repression of the *P* cassette.

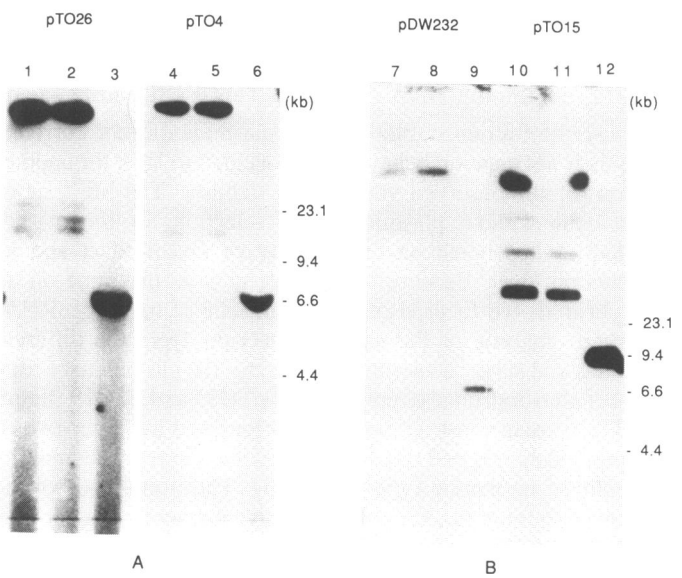

Figure 3. Hybridisation of radioactive pGEM3 to DNA from *S.pombe* cells transformed with pTO26 (*ars2PL*), pTO4 (*ars2PR*), pDW232 (*ars1*) and pTO15 (*ars2PL* and *ars2PR*). Lanes 1,4,7 and 10 contain undigested DNA. DNA in lanes 2, 5, 8 and 11 is cut with BglII (no sites in the plasmids). Lane 3, DNA cut with BssHII (one site in pTO26). Lane 6, DNA cut with HindIII (one site in pTO4). Lanes 9 and 12, DNA cut with BamHI (one site in pDW232 and one site in pTO15). Since pGEM3 has little homology to the chromosomal DNA, all bands correspond to plasmid DNA. A and B indicate two different gels.

Table 1. Properties of the two new ars elements compared to *ars1*

Plasmid	ars	Generation time (h)[a]	Total copy number[a]	Mitotic stability[b]	Loss rate[b] per generation
pDW232	*ars1*	4.37±0.11	7.8±3.7	28%±12	0.07±0.04
pTO26	*2PL*	3.76±0.16	3.0±0.6	78%±10	0.08±0.03
pTO19	*2PL*	3.63±0.05	8.5±3.2	82%±8	0.05±0.05
pTO4	*2PR*	4.34±0.03	0.5±0.2	45%±18	0.24±0.00
pTO15	*2PL/2PR*	3.42±0.13	7.7±2.9	93%±5	0.03±0.01

[a]For generation time and total copy number three measurements were made.
[b]For loss rate and mitotic stability five measurements were made.

This indicates that the structure of the plasmid monomers has not changed apart from the polymerization.

Different mitotic stability does not reflect different segregation properties

To characterize the new *ars* sequences we measured how many cells contained a plasmid with these sequences during selection for ura prototrophy. This number, mitotic stability, depends directly on the fidelity of plasmid segregation and inversely on the ability of cells to survive after the loss of the plasmid. The survival in its turn is dependent on the presence of a sufficient amount of the selected product, here ura4 protein. The mitotic stability of pTO19 (*ars2PL*) is high compared to pDW232 (*ars1*) and pTO4 (*ars2PR*) (figure 4A). This difference is not due to improved segregation function in pTO19, since plasmid loss rates in the absence of selection (figure 4B) are not significantly different between pDW232, pTO26, pTO19 and pTO15. It is notable that the *ars2PR* containing plasmid pTO4 has an exceptionally high tendency to segregate assymetrically during mitotic growth. This plasmid also has a much lower total copy number compared to all the other tested plasmids (table 1).

Ars2PL containing DNA fragments repress the *ura4*$^+$ gene

Because the mitotic stability of our plasmids did not correlate with their segregation behaviour we decided to look for another reason for the observed variation in stability. The life time of yeast cells without plasmids under selection for the marker product is dependent on the stability of this product and its production level. Because the marker gene is the same on all our plasmids we focused on the abundance of marker mRNA. Northern analysis of the *ura4*$^+$ transcripts from the different plasmids and subsequent normalization for the plasmid total copy number shows strongest repression in pTO15 and pTO19 (figure 5). The former construction contains four and the latter two of the previously mapped *cis* elements involved in repression of the plasmid borne mating type genes (13). The importance of the region containing the leftmost repressive element (element 1) is underlined by the relative behaviour of pTO19 containing this region and pTO26, where this region is missing. The copy number of pTO26 is lower than that of pTO19 and most significantly the *ura4*$^+$ gene on pTO26 is repressed tenfold less than on pTO19 (figure 5).

The influence of marker gene expression on plasmid stability should be a general phenomenon. To test this generality we chose four plasmids containing *ars1* (pDW232, pKE10, pKE12 and pKE15) (13) and inserts from the silent *mat2-P* locus (figure 1). The mitotic loss rates of these plasmids in the absence of marker selection do not differ significantly from each other (table 2).

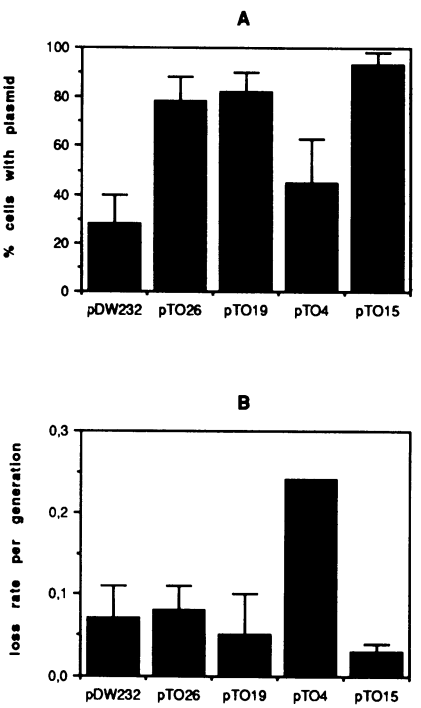

Figure 4. A. Mitotic stability of *ars2PL* and *ars2PR* containing plasmids compared to pDW232, which contains the previously isolated *ars1*. See fig. 1 for details of the constructs. **B.** Loss rates of *ars2PL* and *ars2PR* containing plasmids compared to pDW232.

The negative correlation between *ura4*$^+$ expression and plasmid stability under selection could be clearly seen with pKE12 and pDW232 (table 2).

Repression of the *ura4*$^+$ transcription is dependent on the *ars* function

The dependence of *ura4*$^+$ expression on the identity of the *ars* containing DNA fragments from the silent mating type locus could be interpreted such that replication influences *ura4*$^+$ expression. In order to test this hypothesis we compared the marker gene expression in the *ars2PL* containing plasmids pTO15 and pTO19 to that in two plasmids, pKE10 and pKE15, containing both *ars2PL* and *ars1*. The inserts in pTO15 and pKE10 are identical and in pTO19 and pKE15 equal with respect to the earlier defined repressive elements (figures 1 and 6). The effect of the *ars2PL* was substantially diminished through the presence of the additional replication sequence. When compared

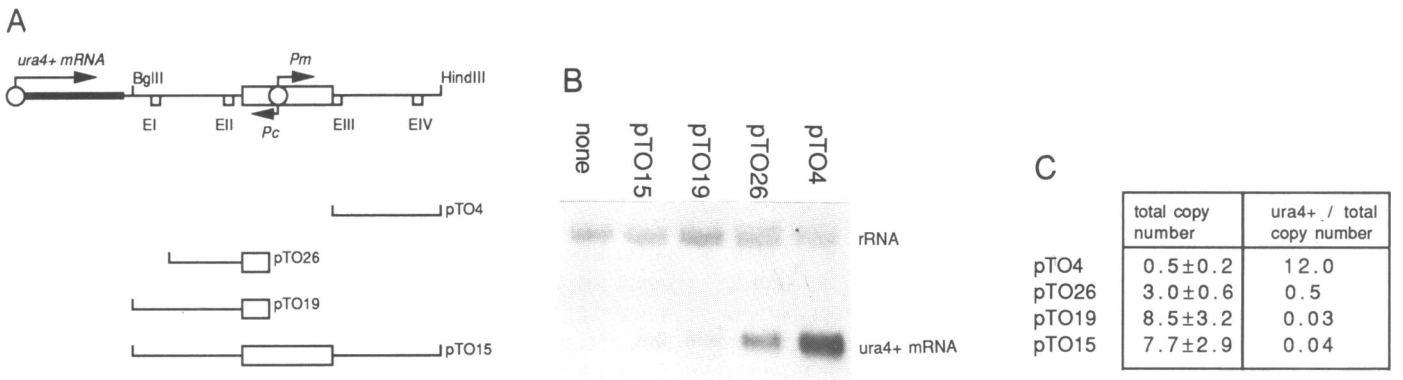

Figure 5. A. A schematic figure (not drawn to scale) of the different inserts in the four tested plasmids. The small white boxes: EI-EIV represent *cis* elements important for repression of the plasmid-borne mating type genes (13). **B.** Northern analysis of the constructs. Nonspecific hybridisation of the probe to ribosomal RNA was used as an internal standard. **C.** Ura4$^+$ expression based on Northern analysis and total copy numbers based on Southern analysis.

Table 2. Mitotic stability, loss rate and *ura4*$^+$ expression of four *ars1* based plasmids

Plasmid	Mitotic stability[a]	Loss rate[b] per generation	ura4$^+$/total copy[c]
pDW232	42% ± 4.1	0.08 ± 0.04	14
pKE10	91% ± 9.5	0.06 ± 0.00	1.5
pKE12	12% ± 9.5	0.09 ± 0.04	17
pKE15	87% ± 17.3	0.08 ± 0.02	0.8

[a]Mitotic stability is an average of three independent measurements of one representative isolate.
[b]Loss rates are based on at least three independent measurements.
[c]The values for *ura4*$^+$ expression are directly comparable to the values in figure 5.

with the plasmid where only *ars1* is present (pDW232) *ura4*$^+$ expression was, however, repressed by more than an order of magnitude (figure 6).

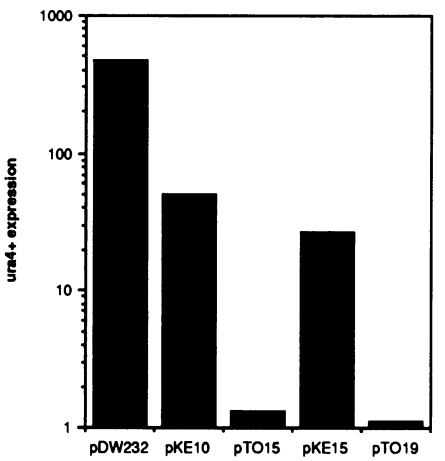

Figure 6. A comparison of the *ura4*$^+$ gene expression in plasmids containing *ars1* (pDW232, pKE10 and pKE15) to similar plasmids with inserts from the *mat2P* locus as *ars* elements (pTO15 and pTO19). The *ura4*$^+$ expression is normalized to the lowest value (pTO19). For details of the plasmid structures, see figure 1.

DISCUSSION

Ars elements overlap with DNA elements important for repression of plasmid borne mating type genes

Four cis elements important for repression of plasmid borne *mat2-P* cassette have previously been defined (13). Two of them are located to the left and two to the right of the cassette. We find two independent *ars* elements, *ars2PL* and *ars2PR*, that overlap with the elements involved in repression. They can support replication of fission yeast plasmids in a multimeric form very much like the well characterized *ars10* (25) from the *ura1* region and the most common ars element, *ars1* (26), do. The *ars2PL* includes *cis* repression element II and the *ars2PR* both elements III and IV (figure 4). Elements I and II are necessary for complete transcriptional repression of plasmid borne mating type genes, while only one of the elements III and IV is required (13). Here we find that *ura4*$^+$ gene expression is repressed much more efficiently by *ars2PL*, than by *ars2PR*. Deletion of a fragment containing element I from pTO19 to make pTO26 causes significant derepression of the marker gene. This deletion leads also to significant reduction of plasmid copy number without a change in segregation properties. We believe that the altered copy number depends on reduced frequency of replication initiation. The remaining insert containing element II can still repress the marker gene more than tenfold. Therefore elements I and II are the most important elements not only for mating type gene repression but also for repression of an unrelated gene. This shows that the same DNA elements and most likely the same mechanism cause major part of the repression of the *ura4*$^+$ gene and the mating typegenes. The function of elements III and IV remains more obscure, because they alone do not significantly affect *ura4*$^+$ expression. It might be that these elements and their replication capability are used to block competing replication from outside into the silent mating type region (see below).

The repression is promoter independent, but requires a specific *ars* element

We have previously reported transcriptional repression of the bacterial kanamycin resistance gene by sequences close to the *mat2-P* cassette (14). This taken together with the fact that an *ars2PL* containing DNA fragment is able to repress the *ura4*$^+$ gene three hundred fold, supports the idea that these sequences

cause promoter independent repression. Another important aspect of this effect concerns the role of replication. If the repression was a general consequence of replication, then any two vectors having their *ars* sequences in a similar position with respect to the marker gene should show this repression. In pDW232 the positions of *ars1* and *ura4*$^+$ are very similar to the positions of *ars2PL* and *ura4*$^+$ in pTO15 and pTO19 (figure 1). However, the expressional level of *ura4*$^+$ is more than two orders of magnitude higher in pDW232 than in pTO15 or pTO19. Moreover, pTO4 contains *ars2PR* and *ura4*$^+$ at similar locations and the marker gene is expressed at a level comparable to that in pDW232. We conclude that transcriptional repression is not an effect of replication per se, but rather a specific property of *ars2PL*.

Does *ars2PL* contain a silencer?

A silencer was defined as a DNA element capable of repressing transcription independent of its orientation, distance and position with respect to the silenced promoter (27). In our case we see repression of mating type genes and the *ura4*$^+$ gene simultaneously at two different sides of the *ars2PL* element. This satifies the third requirement. The distance of the *ura4*$^+$ gene is ca. 2 kb from the edge of the *ars2PL* element, which appears to satisfy the second requirement. However, insertion of a kanamycin resistance gene (1.3 kb) causes derepression of the mating type genes when inserted at either side of the mating type cassette (14). We have also shown that there are several positions inside the *mat2-P* region where the resistance gene is unexpressed in either orientation. In that case it is, however, not clear if this is due to one single negative element, although the resistance gene is most strongly affected by element II (14). Finally the kanamycin resistance gene in a position similar to the *ura4*$^+$ gene in the plasmid pTO15, is not repressed (14). We conclude that although there are similarities between the *ars2PL* element and silencers, this element does not satisfy all of the requirements for a silencer sequence.

Plasmid segregation is not affected by the silent *mat2-P* region

In the budding yeast, *HMR* silencer sequences increase the mitotic stability of plasmids by improving their fidelity of segregation (28). In fission yeast results from mitotic stability measurements of plasmids harbouring fragments from the silent *mat2-P* locus were originally interpreted in the same way (13). The mitotic stability of the vector pDW232 for example, was increased from 25% to 85%, when the 3.9 kb BglII/HindIII fragment of the *mat2-P* locus was cloned into its polylinker. The increased mitotic stability is also seen with the *ars1* lacking plasmids reported in this paper. However the stability of plasmids is not only dependent on segregation during mitosis but also on the number of generations that the cell can survive after losing the plasmid (29). In the cases studied here the rate of plasmid loss in the absence of selection does not correlate with the mitotic stabilities and is relatively independent of the insert in the plasmids. The only construct that segregates significantly more assymmetrically than the others is pTO4 that contains the *ars2PR*. This plasmid has a copy number below one per cell, which alone can explain the increased loss rate.

Ura4$^+$ expression influences mitotic stability of plasmids

The constructs pKE12 and pDW232, which show low stability during selection, had *ura4*$^+$ expression levels at least ten times higher than the other plasmids. This elevated marker expression should increase the number of post plasmid generations and thereby decrease the fraction of plasmid containing cells in the selected population. The measured frequency of plasmid loss in the absence of selection was around 5% per cell division. This means that if no division is possible after dissappearance of the plasmid, then the fraction of cells with the plasmid should be close to 95%. This will be the case when the marker gene expression is barely enough to keep the plasmid containing cells alive. In those cells where several fold more marker product is made the cessation of growth after plasmid loss is caused by dilution of the marker protein under the critical limit for growth. Our results agree with the interpretation that very little *ura4*$^+$ protein is present in cells containing pTO15 and pTO19 and that pKE12 and pDW232 can support the production of much larger amounts of this protein. Therefore, the *ura4*$^+$ expression level rather than defective segregation is the best explanation for the low stability of pKE12 and pDW232. However, the results for cells with intermediate levels of *ura4*$^+$ expression (pKE10, pTO26 and pKE15), reveal that the mitotic stability of these plasmids is not significantly affected by their marker expression which is at least tenfold above that of pTO15 and pTO19. We do not have an explanation for this insensitivity apart from the possibility that the standard deviation of our results might hide small changes in mitotic stability.

Are the *ars* elements necessary components of the silencer mechanism?

All four silencers of the mating type region of *S.cerevisiae* can function as *ARS* elements. *Ars* elements are found approximately once every 20 kb in the *S.pombe* genome. We found two such sequences within 4 kb coinciding with the transcriptionally silent P mating type region. Since the fission yeast and the budding yeast are only remotely related to each other, the *ars* activities in the silent mating type loci of both organisms suggest that replication might play a part in long range repression in eukaryotes. Another example that supports this reasoning comes from recent studies on *Dictyostelium discoideum.* This organism has many of its genes on plasmids. One of these plasmids has an orientation-independent negative transcriptional control region, which co-localizes with the origin of plasmid replication (11).

Are all silencers dependent on replication? So far only the budding yeast silencers have been analyzed for origin function on the chromosome. While *HMRE* is a chromosomal origin (9), there is no detectable chromosomal origin function at the *HMLE* and *HMLI* silencers (30). Since the *HMRE ars* is used as an origin only in some cell cycles, the origin function appears to be important for the establishment rather than the inheritance of the repressed state. In the *HML* case perhaps the inheritance of the transcriptional repressed state is very efficient so that the origin is seldom needed. Such a function would be difficult to detect by the current gel techniques.

How can replication affect transcription?

We have presented data in support of a strong link between replication and transcription. The most important results are that changing the *ars* sequence at a given position as well as inclusion of both *ars2PL* and *ars1* onto the same plasmid lead to drastically different levels of transcription. Plasmids containing these two *ars* sequences give results that are intermediate between those for the individual *ars* sequences. This might mean that replication sometimes begins at one of the sites and sometimes at the other. This would lead to fully active and fully repressed marker genes

and the observed value would only reflect the proportions of each type in the mixture. This phenomenon of replication sequences influencing each other's effect on transcription is not seen when *ars2PL* and *ars2PR* are on the same plasmid. It is important to notice that this cannot be just an effect of distance because the relative positions of *ura4*$^+$ gene and the left side flanking sequence are very similar in pKE15 (*ars1* and *ars2PL*) and pTO15 (*ars2PL* and *ars2PR*). In summary, replication changes the behaviour of a promoter at a distance from its origin. This kind of effect would be impossible on naked DNA, but transcription of eukaryotic genes can be repressed by chromatin structures (31). What might be different between the different origins? It is known from studies in budding yeast that the timing of replication differs between origins (32). It is also known that *HM* silencers are dependent on several trans acting factors, which are necessary and specific for the transcriptional silencing at these sites and at the telomeres (33). We can speculate that origins may differ by offering a different set of factors for the assembly of chromatin through a different timing of replication or by having a way to engage additional factors in the replication reaction. Finally it is possible that replication has to pass through DNA sequences that modify the process. It will be interesting to determine where the replication originates on our plasmids and subsequently how the chromosomal mating type region is replicated.

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