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The requirement of yeast replication origins for pre-replication complex proteins is modulated by transcription

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ABSTRACT

The mini-chromosome maintenance proteins Mcm2–7 are essential for DNA replication. They are loaded onto replication origins during G1 phase of the cell cycle to form a pre-replication complex (pre-RC) that licenses each origin for subsequent initiation. We have investigated the DNA elements that determine the dependence of yeast replication origins on Mcm2–7 activity, i.e. the sensitivity of an origin to *mcm* mutations. Using chimaeric constructs from *mcm* sensitive and *mcm* insensitive origins, we have identified two main elements affecting the requirement for Mcm2–7 function. First, transcription into an origin increases its dependence on Mcm2–7 function, revealing a conflict between pre-RC assembly and transcription. Second, sequence elements within the minimal origin influence its *mcm* sensitivity. Replication origins show similar differences in sensitivity to mutations in other pre-RC proteins (such as Origin Recognition Complex and Cdc6), but not to mutations in initiation and elongation factors, demonstrating that the *mcm* sensitivity of an origin is determined by its ability to establish a pre-RC. We propose that there is a hierarchy of replication origins with respect to the range of pre-RC protein concentrations under which they will function. This hierarchy is both 'hard-wired' by the minimal origin sequences and 'soft-wired' by local transcriptional context.

INTRODUCTION

Genomic stability during cell proliferation demands accurate DNA replication and segregation, with the result that tight controls over these processes have evolved. For DNA replication, the primary control is at the level of initiation. On each chromosome, bi-directional replication forks initiate at multiple sites called replication origins. Replication origins are 'licensed' for replication during G1 phase of the cell cycle. Licensing involves the stepwise assembly at the origin of a series of proteins: the origin recognition complex (ORC), Cdc6, Cdt1 and the Mcm2–7 complex, which together form the pre-replication complex (pre-RC). When the cell enters S phase, the combination of two kinase activities, Cdk1 and Cdc7, initiates DNA replication. In yeast, Cdk activity inhibits pre-RC assembly, preventing re-licensing of origins until cells exit metaphase, thus ensuring only one round of DNA replication per cell cycle (1).

The protein factors involved in licensing origins are conserved throughout eukaryotes, but the DNA sequence elements with which they interact are not. Replication origins are best understood in the budding yeast *Saccharomyces cerevisiae* where specific origin sequences (called ARS elements) have been isolated. Recently, all the origins in the yeast genome have been mapped using microarray-based methods, revealing that the 16 chromosomes of the *S.cerevisiae* genome are replicated from ~400 origins (2–4). These origins differ in a number of characteristics. First, origins initiate at specific and reproducible times, with some activated early in the S phase and others later (2). Second, 'efficient' origins initiate replication in the majority (>60%) of cell cycles while 'inefficient' origins may initiate in <20% (5,6). Third, origins differ in their requirement for the Mcm2–7 proteins, with some origins being severely affected by limiting Mcm2–7 levels whereas others are relatively resistant (7). The interplay between these three effects determines which origins will fire in any one cell cycle. The molecular basis for these differences is however incompletely understood (8).

Several origins have been dissected and found to have a modular structure with a number of discrete sequence elements. All have an essential ARS consensus sequence (known as the ACS or A element) flanked by less well-conserved B

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elements that are required for efficient origin activity. The A element consensus sequence has been determined by the alignment of known essential elements to give an 11 bp motif, (A/T)AAA(C/T)ATAAA(A/T) (9), or an extended 17 bp motif based on a larger number of origins (10). Most origins have multiple matches to this motif with the best match not necessarily corresponding to the essential A element. The presence of an A element does not imply that a sequence has origin function, and so the identification of this consensus has not permitted the precise genome-wide mapping of origins.

Two particularly well-characterized origins are *ARS1* (ChrIV: coordinates 462487–462603) and *ARS121* (ChrX: coordinates 683549–684037) (11,12). In addition to the essential A element, *ARS1* has three B elements lying 5′ of the A-rich strand of the ACS: B1 participates with the A element in recruiting ORC (13,14); B2 is a second match to the ACS and is required for efficient loading of Mcm2–7 proteins (15,16); and B3 is a binding site for the transcription factor Abf1 (11,17). The essential A element of *ARS121* is a relatively poor match to the ACS motif. It is flanked to the 5′ on the A-rich strand by an AT-rich (ATR) sequence and to the 3′ by transcription factor binding sites—two Abf1 sites (18) and four Mcm1 sites (19). The precise relationship between transcription factor binding and origin function is uncertain. In the case of *ARS121*, it has been shown that transcriptionally incompetent Abf1 protein fragments still stimulate replication, suggesting that DNA binding rather than transcription factor function is important for replication (20). Abf1 has been shown to contribute to *ARS1* activity by positioning nucleosomes in a conformation that favours ORC recruitment (15).

ARS1 and *ARS121* are both efficient origins that replicate early in the S phase. They have, however, been reported to differ in their response to mutations in *MCM* genes, and this difference formed the basis for the mini-chromosome maintenance (*mcm*) genetic screen that originally identified the *MCM* genes (21). Following origin licensing, the Mcm2–7 complex is phosphorylated concomitantly with initiation, after which the complex moves with the replication machinery away from the origin (22–25). Although it is not known whether Mcm2–7 protein phosphorylation is an essential initiation step, Mcm2–7 proteins are required for continuing replication elongation (26), possibly functioning as the replicative helicase (27,28). Determining why otherwise similar origins respond differently to mutations in *MCM* genes is key to our understanding of Mcm2–7 protein function in DNA replication. Here, we show that the difference between these origins is in functional pre-RC assembly, and is determined by the core sequence elements and modulated by transcription towards the origin.

MATERIALS AND METHODS

Strains and plasmids

The *ARS1* and *ARS121* plasmids (Yp-CN1 and Yp-CN2) were isolated from a yeast genomic library (see Supplementary Table S1). The chimaeric plasmids derived from Yp-CN1 and Yp-CN2 were made via a two-step homologous recombination approach. In the first step, the 11 bp ARS consensus sequences of *ARS1* and *ARS121* were deleted from Yp-CN1 and Yp-CN2, respectively, using a cassette encoding *LEU2–ARSH4* flanked by NotI and PacI restriction sites. This cassette was amplified by PCR from pRS315 using primers to add the restriction sites and 40 bp of homology to sequences flanking each ACS. The yeast strain AY925 was transformed with either Yp-CN1 or Yp-CN2 together with the appropriate cassette and recombinants were selected on plates lacking leucine. The correct plasmid products were identified by diagnostic PCR, recovered in *Escherichia coli* and verified by sequencing.

The NotI and PacI restriction sites are unique in both resultant plasmids and after double digestion give versions of Yp-CN1 and Yp-CN2 linearized at the site of the deleted ACS. Chimaeras were generated by PCR using primers to define the required junction between *ARS1* and *ARS121* derived sequences. The resulting PCR products were transformed into AY925 with the appropriate linearized parent vector. Recombinants were then screened by diagnostic PCR, recovered in *E.coli* and verified by sequencing.

To make Yp-*ARS1* and Yp-*ARS121*, the core (373 bp) sequences of *ARS1* and *ARS121* were amplified by PCR using primers to add BamHI sites and cloned into the vector YIp5-5. Mutagenesis of Mcm1 binding sites (Yp-CN13) and B2 elements (Yp-CN14) was performed using standard PCR-based techniques in minimal constructs, verified by sequencing and then transferred to large constructs as described above. Full details of all plasmids are given in Supplementary Table S1.

For strains used in this study, see Supplementary Table S2. Cells were grown in standard synthetic complete medium or selective medium as appropriate. Oligonucleotide sequences are available on request.

Plasmid loss assay

Plasmids were transformed into the required strains, and two independent tranformants were selected for measuring plasmid loss rates. Cells were grown under selection for the plasmid at 23°C to a cell density between 5×10^6 and 1×10^7 cells/ml (Casy Counter, Schärfe Systems, Germany). A zero time point sample was taken, after which cells were diluted in synthetic complete medium and transferred to the appropriate temperature. At least three further samples were taken at succeeding time points, and the number of intervening generations was determined from the cell number. All samples were equivalent to 1×10^8 cells. DNA was then prepared from the samples as described previously (29). To determine plasmid levels, DNA samples were digested with EcoRI (NEB, UK), separated by agarose gel electrophoresis and analysed by Southern blotting with a radiolabelled probe against *CENV*. The plasmid *CENV* band was 1143 bp as compared with a genomic band of 2527 bp. Signal intensities were quantified on a phosphorimager to determine the ratio of plasmid to genomic DNA. Loss rates were determined according to the formula: $L = 1 - e^P$, where L is the loss rate (% per generation) and P is the gradient of the best fit line from a logarithmic-linear plot of plasmid level versus the number of generations. The maximum theoretical loss rate is 50% per generation. The results presented are the average loss rate determined for the two independent transformants.

2D gel electrophoresis

DNA was prepared from asynchronously growing cells (30°C) of the appropriate strains as described previously (30,31).

The 2D gels were run as described previously (32). First dimension gels were 0.4% agarose and second dimension gels were 1.1% agarose. Fragments probed were *ARS1*, NcoI/SacI, 3459 bp; *ARS121*, NcoI/KpnI, 3429 bp.

Phylogenetic analysis

The corresponding intergenic spaces from *Saccharomyces sensu stricto* species (*S.paradoxus*, *S.mikatae*, *S.kudriavzevii* and *S.bayanus*) were downloaded from the Saccharomyces Genome Database (33). Sequences were aligned using ClustalW (34) and identity at each position of the alignment was determined using Plotcon (35).

RESULTS

Chromosomal origin efficiency is differentially affected by *mcm* mutations

It has been reported that plasmids replicated by different origins are maintained to differing extents in *mcm* mutants, suggesting that origins may vary in their requirement for Mcm2–7 function. For example, plasmids replicated from *ARS1*, the *HO ARS* and the telomeric origin *ARS131* are lost more rapidly in an *mcm* mutant than those replicated from *ARS121*, *ARSH2B* and the telomeric origin *ARS120* (7). To determine whether this reported difference in plasmid stabilities reflects the effect on origins in their native chromosomal context, we used 2D gel analysis to determine the efficiency of chromosomal *ARS1* and *ARS121* in wild-type and *mcm* strains. These origins were selected because they are among the best-characterized *S.cerevisiae* replication origins and because the difference in *mcm* sensitivity is their principal reported functional difference.

The 2D gel analysis confirmed that both *ARS1* and *ARS121* origins fire efficiently in wild-type cells (Figure 1A) as demonstrated by the presence of a clear bubble arc for both origins. In the *mcm2-1* temperature-sensitive mutant strain, there is a marked reduction in the bubble arc visible at *ARS1* and a corresponding increase in the proportion of small Y-shaped structures. These changes indicate that *ARS1* activation efficiency is significantly reduced in the *mcm2* strain. In contrast, *ARS121* shows little reduction in origin firing efficiency in the *mcm2* strain (Figure 1B). Therefore, these origins differ in their efficiency of forming bubble structures when Mcm2 is limiting—an effect that could result from the reduced efficiency of pre-RC assembly at *ARS1*, differences in the efficiency with which replication initiates at pre-RCs or an increased rate of failure in the early steps of elongation at *ARS1*. Mcm2–7 proteins are implicated in each of these steps of replication.

Plasmids vary in their requirements for MCM function

Measurement of the stability (during mitotic growth) of yeast autonomously replicating plasmids offers a convenient means of quantitatively determining the efficiency of DNA replication. To establish a plasmid-based model for *mcm* sensitivity, we tested the stability of various *ARS1*- and *ARS121*-replicated plasmids. We measured the efficiency of plasmid maintenance using a Southern blotting method that compares plasmid DNA levels with genomic DNA as cells are grown without selection for the plasmid (Figure 2A) (36,37). In our hands, this method

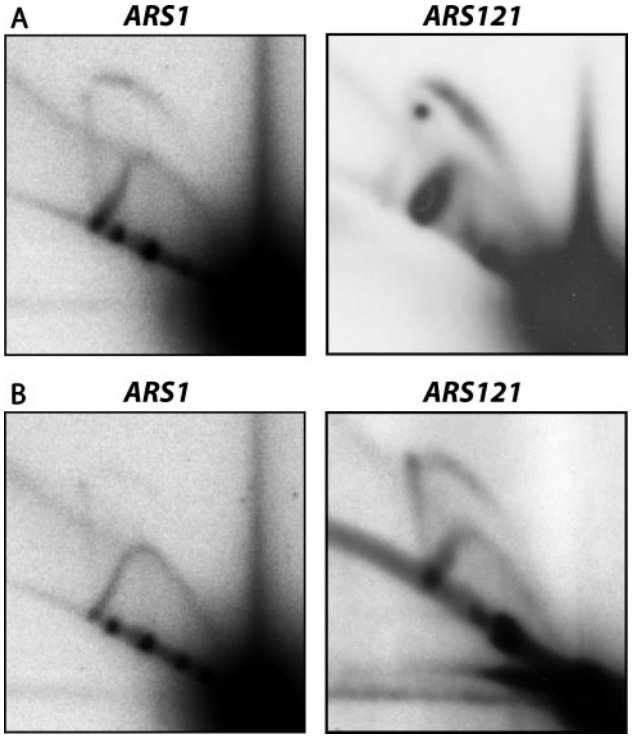

Figure 1. *ARS1* is more severely affected by *mcm* mutations than *ARS121*. The 2D gel analysis of replicating structures at origins *ARS1* (left) and *ARS121* (right) in (**A**) wild-type cells and (**B**) *mcm2-1* mutant cells.

gives results identical to, but more reproducible than, those obtained with colony-plating methods (38). We first tested two of the plasmids used in the original *mcm* screens, YCp101 (*ARS1*) and YCp121 (*ARS121*), which contain the ARS sequences on 0.8 and 6.7 kb chromosomal DNA inserts, respectively (21). We found that both plasmids were stable in wild-type cells, but in *mcm2-1* cells at 23°C YCp101 was lost at 18% per generation and YCp121 at 1% per generation (Table 1), consistent with *ARS1* being more sensitive than *ARS121* to the *mcm* mutation. To test whether the determinants of *mcm* sensitivity were completely contained within the replication origins, we constructed a second plasmid pair (Yp-*ARS1* and Yp-*ARS121*), which both have 373 bp origin inserts within the same centromeric *URA3* plasmid backbone. We chose 373 bp as the smallest possible insert size that contains all the previously identified sequence elements important for *ARS121* activity, and indeed both Yp-*ARS1* and Yp-*ARS121* were stably maintained in wild-type cells (loss rate <1.0% per generation at 23°C). In the *mcm2-1* mutant at 23°C, Yp-*ARS1* was lost at 34% per generation and Yp-*ARS121* at 14% per generation (Table 1). The fact that these 'minimal origin' plasmids had much higher loss rates than plasmids YCp101 and YCp121 raised concerns that not all the chromosomal determinants of plasmid *mcm* sensitivity are contained within these minimal origin inserts, and suggested moreover that the plasmid backbone might affect *mcm* sensitivity. To mimic as closely as possible the native chromosome contexts of the two origins, we used a third plasmid pair, Yp-CN1 and Yp-CN2, containing large (>10 kb) chromosome-derived fragments centred on *ARS1* and *ARS121*, respectively. In the wild-type strain, both of these

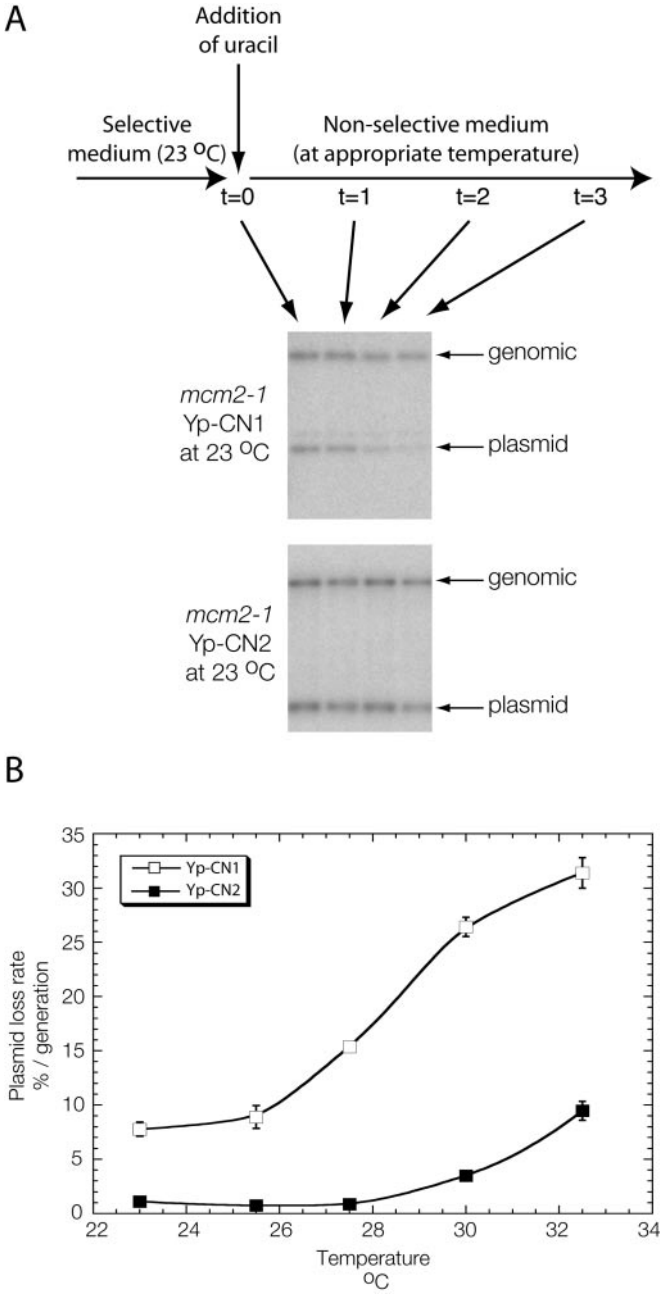

Figure 2. Measurement of plasmid loss by Southern blotting shows that an *ARS1* plasmid is less stable than an *ARS121* plasmid in the *mcm2-1* mutant. (**A**) Yeast strains transformed with the relevant plasmid were grown at 23°C under selective conditions and plasmid maintenance levels determined (time point, t = 0). Selection was relieved (by the addition of uracil) and the culture was shifted to the appropriate temperature. Further samples were taken to determine the rate of plasmid loss (t = 1, 2 and 3). Southern blotting was used to determine the proportion of plasmid relative to genomic DNA. Representative blots are shown for the loss of Yp-CN1 and Yp-CN2 at 23°C in *mcm2-1*. (**B**) The rate of plasmid loss in the *mcm2-1* strain was determined for plasmids replicated from *ARS1* (Yp-CN1) and *ARS121* (Yp-CN2) at a range of temperatures.

plasmids were completely stable, whereas in the *mcm2-1* strain at 23°C Yp-CN1 was lost at 8% per generation and Yp-CN2 plasmid at 1% per generation (Table 1). Therefore, plasmids with large *ARS1* and *ARS121* inserts show differences in *mcm* sensitivity similar to smaller insert plasmids used in previous studies, albeit with a reduced difference in plasmid loss rate. To avoid the risk of failing to identify important determinants of *mcm* sensitivity, we decided to base further analysis on the large insert plasmids Yp-CN1 and Yp-CN2.

Table 1. Plasmid loss rates at 23°C in *mcm2-1* for various *ARS1* and *ARS121* plasmids

Plasmid	Insert size[a]	Loss rate[b]
ARS1 plasmids		
YCp101	0.8	18
Yp-*ARS1*	0.4	34
Yp-CN1	16.1	8
ARS121 plasmids		
YCp121	6.7	1
Yp-*ARS121*	0.4	14
Yp-CN2	11.8	1

[a]Kilobases of chromosomal origin DNA.
[b]Percentage of plasmid loss per generation at 23°C.

We measured the stabilities of the two plasmids in the *mcm2-1* strain at a range of permissive and semi-permissive temperatures. Yp-CN1 was lost more rapidly than Yp-CN2 at all temperatures tested between 23 and 32.5°C (Figure 2B). Similar results were obtained in an *mcm5* temperature-sensitive strain, whereas in wild-type strains both plasmids were stably maintained at all temperatures (see Supplementary Table S3). We selected 30°C as suitable temperature for further experiments, since this temperature produced the largest absolute difference in plasmid loss rate between Yp-CN1 and Yp-CN2.

Our results indicate that plasmids replicated by *ARS1* are more sensitive to *mcm* mutations than plasmids replicated by *ARS121*. Measuring the stabilities of plasmids that contain origin inserts of various sizes showed that large chromosomal inserts may be required to accurately reflect the behaviour of chromosomal origins. These large origin insert plasmids (Yp-CN1 and Yp-CN2) establish a convenient system to study what determines the *mcm* sensitivity of an origin.

One interpretation of these data is that initiation at *ARS1* requires more Mcm2–7 activity than does *ARS121*. Alternatively, the differing effects on the two origins could be due to the specific nature of the mutations in the *mcm2-1* (E391K) and *mcm5-461* (C183Y) alleles (39,40). Consistent with the first interpretation, we found that an *ARS121* (Yp-*ARS121*) plasmid was significantly more stable than an *ARS1* (Yp-*ARS1*) plasmid in certain *mcm* heterozygote strains (data not shown) where the concentration of the relevant Mcm2–7 proteins is effectively halved (41).

Sequence determinants of plasmid *mcm* sensitivity

We wished to investigate the extent to which *mcm* sensitivity is determined by the origin itself or its surrounding sequence. To this end, we exchanged 2 kb of sequence (centred on the origin) between plasmids Yp-CN1 and Yp-CN2. The sequences were exchanged precisely using a multi-step homologous recombination approach (see Materials and Methods) such that no sequence was lost or introduced and the orientation of the essential ACS (A element) was maintained. Both of the new chimaeric plasmids were efficiently maintained in a wild-type strain, demonstrating that the origins remained functional. As shown in Figure 3A, exchanging 2 kb fragments

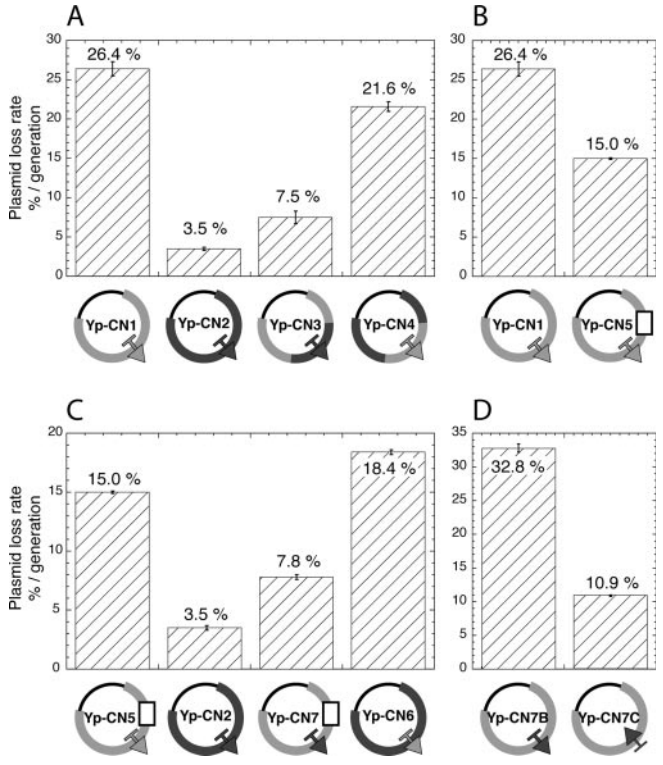

Figure 3. The minimal origin sequence is the primary determinant of *mcm*-sensitivity, but *mcm*-sensitivity is exacerbated by transcription into the origin sequence. Plasmid loss rates of Yp-CN1, Yp-CN2 and derived chimaeric constructs in the *mcm2-1* strain at 30°C. In the plasmid cartoons, light grey indicates *ARS1* chromosomal flanking sequences and dark grey represents *ARS121* chromosomal flanking sequences. Thin black lines represent the YIp5-5 vector backbone. The fused T and ▼ symbols represent the origin, with the orientation corresponding to the orientation of the T-rich strand of the ACS. (**A**) Exchanging 2 kb regions largely exchanges the *mcm* sensitivity phenotype. (**B**) The *TRP1* promoter contributes to *mcm* sensitivity. Deletion of the *TRP1* promoter is shown by the open rectangle. (**C**) In the absence of the *TRP1* promoter, the minimal origin sequences (373 bp) are the primary determinant of *mcm* sensitivity. (**D**) Orientation of the *ARS121* minimal sequences affects origin sensitivity to *TRP1* transcription. In all cases, the plasmids were stable in wild-type cells (loss rates ⩽2.5% per generation, Supplementary Table S4).

exchanged the majority of the *mcm* sensitivity effect between the two plasmids (Figure 3A: compare loss rates of chimaeric plasmids Yp-CN3 and Yp-CN4 with parent plasmids Yp-CN1 and Yp-CN2), indicating that the sequence determinants of the plasmid requirement for Mcm2–7 function are predominantly within the exchanged region. Therefore, the *mcm* sensitivity phenotype is linked to the replication origin and its immediately flanking sequences.

Like almost all origins, *ARS1* and *ARS121* lie in intergenic regions. However, they differ in chromosomal context with regard to surrounding open reading frames: *ARS121* lies in a large divergent intergenic space (1.3 kb), whereas *ARS1* lies immediately downstream of the *TRP1* gene. *ARS1* is unusual in that one of its sequence elements (the B3 box, an Abf1 binding site) lies within the *TRP1* coding sequence (see Figure 4A). The chimaeric plasmid Yp-CN4 includes the entire *TRP1* gene and 292 bp of its upstream sequence. To test whether *TRP1* transcription into *ARS1* contributes to its *mcm* sensitivity, we deleted the *TRP1* promoter from Yp-CN1 to create Yp-CN5. The *TRP1* promoter has been extensively characterized and our deletion removes the region previously shown to be required for gene expression (42–44). The *TRP1* gene of Yp-CN1 is functional and confers a Trp^+ phenotype; after deletion of the promoter Yp-CN5 is unable to confer a Trp^+ phenotype (data not shown), confirming that the majority of *TRP1* expression has been ablated. Removing *TRP1* transcription did not affect the stability of the plasmid in a wild-type strain but substantially improved its stability in an *mcm* mutant, reducing the loss rate from 26.4% per generation to 15.0% per generation (Figure 3B). Therefore, transcription from the *TRP1* promoter, which extends towards the origin, causes about half of the *mcm* sensitivity difference between Yp-CN1 and Yp-CN2.

To narrow down the elements responsible for the remaining *mcm* sensitivity, 373 bp fragments were exchanged between Yp-CN5 and Yp-CN2 to create plasmids Yp-CN7 and Yp-CN6 (Figure 3C), 373 bp being chosen as the smallest fragment containing all elements required for *ARS121* activity (see Figure 4A). Both Yp-CN7 and Yp-CN6 were efficiently maintained in wild-type cells, whereas in the *mcm2-1* strain Yp-CN7 was lost at 7.8% per generation and Yp-CN6 at 18.4% per generation (Figure 3B). The level of *mcm* sensitivity observed for Yp-CN7 is essentially the same as for Yp-CN3, and only somewhat higher than for *ARS121* parent plasmid Yp-CN2. Taken together, these data indicate that the majority of the sequence elements that allow *ARS121* to fire efficiently under conditions of reduced Mcm2–7 function are contained within the 373 bp core origin. Conversely, the *ARS1* minimal origin retained most of its *mcm* sensitivity when surrounded by *ARS121* flanking sequences (Yp-CN6). Therefore, two different regions contribute to the greater requirements of *ARS1* for Mcm2–7 protein function: the *TRP1* promoter (probably acting via transcription into the core origin sequences) and the core origin itself.

The relatively low loss rate of Yp-CN7 in the *mcm2* strain contrasts with a much higher loss rate (24%) measured at 30°C in *mcm2-1* for the minimal *ARS121* plasmid (Yp-*ARS121*) described above. Since Yp-*ARS121* and Yp-CN7 contain exactly the same 373 bp core origin inserts, this difference illustrates the importance of origin context in determining the efficiency of replication, and raises the possibility that in Yp-*ARS121* the vector backbone may contain destabilizing elements analogous in effect to the *TRP1* promoter.

To further investigate such context effects, we tested two chimaeric plasmids similar to Yp-CN7, but with the *TRP1* promoter intact (Figure 3D). In the first of these constructs, Yp-CN7B, the orientation of the *ARS121* sequence is as in Yp-CN7, whereas in the second, Yp-CN7C, the *ARS121* sequence and therefore the A element orientation are reversed. In the *mcm2-1* strain, Yp-CN7B had a very high loss rate (32.8%). Reversing the orientation of the *ARS121* origin (Yp-CN7C) gave a striking stabilization of the plasmid (10.9% loss rate), making Yp-CN7C the most stable of the constructs retaining *TRP1* transcription. The orientation of the origin relative to the neighbouring transcription unit, therefore, has an important effect on the levels of MCM function required to permit origin firing. Furthermore, the destabilizing effect of *TRP1* transcription appears to be dominant over the stabilizing effect of the minimal *ARS121* sequence in the 'unreversed' construct (compare Yp-CN7B and Yp-CN7).

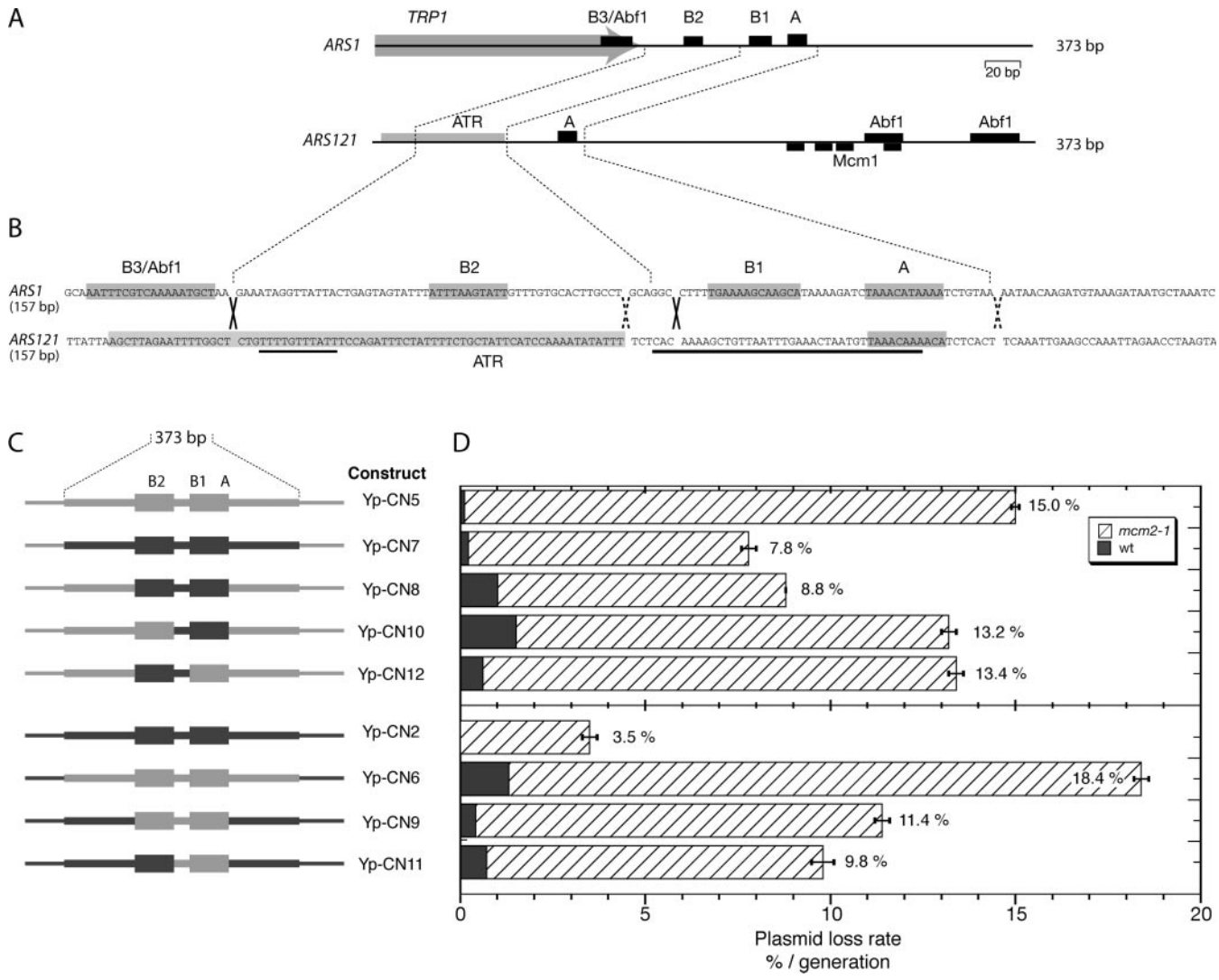

Figure 4. Analysis of origin substructure. (**A**) Schematic diagram of the regions exchanged in the 373 bp chimaeric constructs (Figure 3, Yp-CN6 and Yp-CN7). The A, B and ATR elements, transcription factor binding sites (Abf1 and Mcm1) and 3′ end of the *TRP1* ORF are marked. (**B**) Sequences of the core elements exchanged in Yp-CN8 through Yp-CN12. The regions exchanged in the A and B1 element chimaeras are indicated by dashed crossovers, and in the B2 element chimaeras are indicated by solid crossovers; note that the regions overlap by 6 bp. For the A, B1 and B2 element chimaeras the regions exchanged are from the left-most to the right-most crossovers. Underlined *ARS121* sequence indicates the regions that are most highly conserved across *sensu stricto Saccharomyces* species. (**C**) Schematic diagrams of the origin chimaeric contructs, Yp-CN6 through Yp-CN12 (not to scale). Light grey indicates *ARS1* sequence and dark grey *ARS121* sequence. In all relevant constructs (Yp-CN5, 7, 8, 10 and 12), the *TRP1* promoter was deleted. (**D**) Plasmid loss rates in the wild-type strain are indicated by grey bars and in the *mcm2-1* mutant by hatched bars. Error bars are omitted from the wild-type data for clarity; values shown in Supplementary Table S4. The loss rate of Yp-CN2 in wild type was 0%. The small differences in wild-type loss rates between these chimaeric plasmids have low statistical significance.

As mentioned previously, in a wild-type strain all of the constructs discussed had low loss rates of <2.5% (Supplementary Table S4). Although these loss rates differed between plasmids, the differences generally fall within the limits of experimental error, and do not mirror those in the *mcm2* mutant (see also Figure 4D).

Evolutionarily conserved modular replication origin structure

We have identified a 373 bp region of *ARS121* that is more resilient to reduced Mcm2–7 protein function than the equivalent 373 bp of *ARS1*. To look for potentially important sequence elements within these regions, we took advantage of the recently available genome sequences from related *Saccharomyces* species (45,46). Corresponding sequences from four closely related *sensu stricto* family members were aligned with the *S.cerevisiae* origins and the level of identity at each position was calculated (see Materials and methods). This method, called comparative genomics or phylogenetic footprinting, has previously been used to determine the locations of other important sequence elements within non-coding regions, e.g. transcription factor binding sites (47,48).

In the case of *ARS1*, the known ARS elements are insufficiently conserved in other *Saccharomyces* species to allow the identification of additional elements that might be involved in determining *mcm* sensitivity. The *ARS121* alignment

highlighted three regions of significant sequence identity among *sensu stricto* species: one overlapping with the Mcm1 transcription factor binding sites (19); a second (underlined in Figure 4B) that overlaps the essential A element and encompasses 5′ sequences that may correspond to the B1 element of *ARS1*; and a third (also underlined in Figure 4B) that lies within the ATR element and intriguingly overlaps with an exact match for the A element of *ARS121*. This conserved additional A-like element lies in the opposite orientation to the essential *ARS121* ACS, and as such resembles the B2 element of *ARS1* (16).

While initial inspection might conclude that *ARS1* and *ARS121* have rather different structures, the comparative genomic analysis indicates more similarity. To determine whether these origins do in fact have the same modular structure, we asked whether these elements are functionally equivalent. We made a series of chimaeric plasmids based on Yp-CN5 and Yp-CN2 in which: the A, B1 and B2 elements are exchanged (Yp-CN8 and Yp-CN9); the ORC binding sites (A and B1 elements) are exchanged (Yp-CN10 and Yp-CN11); or the putative B2 element from *ARS121* replaces the B2 element of *ARS1* (Yp-CN12). The precise sequences exchanged are shown in Figure 4B and the constructs are summarized in the schematics of Figure 4C.

We first tested the stability of these constructs in wild-type strains, to establish whether origin function remained intact. All the plasmids were stable (loss rates <2% per generation; black bars in Figure 4D; Supplementary Table S4) showing that all these chimaeric origins are fully functional. The portion of the ATR that we used to replace the B2 element of *ARS1* (Yp-CN12) must, therefore, provide B2 function, since loss of B2 function has previously been shown to dramatically increase *ARS1* plasmid loss rates (11,16). We conclude from these results that *ARS1* and *ARS121* do indeed have similar modular origin structures.

We also tested directly whether the conserved elements identified by phylogenetic footprinting are required for origin function. Precise deletion of the A element rendered the resulting plasmid non-transformable, confirming its essential role (data not shown). We disrupted the conserved B2 element of *ARS121* on Yp-CN2 (to give Yp-CN14). The resultant plasmid is stable in wild-type cells, indicating that this sequence element is not essential for origin activity (data not shown), although it should be noted that the ATR of *ARS121* contains multiple good matches to the ACS, any one of which could potentially fulfil the role of a B2 element. Scrambling three previously described Mcm1 binding sites (19) also resulted in a stable plasmid (Yp-CN13), demonstrating that these sites are not required for origin activity (data not shown).

Multiple elements within the core origin contribute to *mcm* sensitivity

The identification of a functional B2 element within *ARS121* had facilitated the design of a further series of chimaeric constructs with full origin activity. We tested these chimaeric constructs in the *mcm2-1* strain to see whether the reduced requirement of *ARS121* for functional Mcm2–7 protein could be attributed to one of these sequence elements. Replacing the A, B1 and B2 elements of *ARS1* in Yp-CN5 with those from *ARS121* gave Yp-CN8, which has a loss rate in the *mcm2-1* strain of 8.8% per generation, compared with the 15.0% loss rate of Yp-CN5. Just 104 bp of *ARS121* is, therefore, able to confer similar levels of resilience to reduced Mcm2–7 protein function as the 373 bp in Yp-CN7 (7.8% loss rate) and the 2 kb in Yp-CN3 (7.5% loss rate). In contrast, neither the ORC binding site (A and B1 elements) nor the B2 element from *ARS121* alone was sufficient to confer this level of resistance to reduced Mcm2 protein function on *ARS1* (constructs Yp-CN10 and Yp-CN12). Disrupting the conserved B2 element of *ARS121* (Yp-CN14) did not significantly elevate the plasmid loss rate in the *mcm2-1* strain (data not shown), suggesting that even under conditions of reduced Mcm2–7 function other sequences within the ATR can provide B2 element function. Therefore, it is only in combination that the A, B1 and B2 elements from *ARS121* are sufficient to reduce the requirement for Mcm2–7 protein function. Binding sites for the Mcm1 transcription factor have been suggested to affect *mcm* sensitivity (49). We found that mutating three Mcm1 binding sites outside this region (Yp-CN13) did not significantly elevate the plasmid loss rate in *mcm2-1* cells (data not shown), consistent with the finding that the resilience of *ARS121* to reduced Mcm2–7 function is linked mainly to the A, B1 and B2 elements.

For the reciprocal series of constructs in which *ARS1* A-B1 and/or B2 elements replace those of *ARS121*, loss rates in the *mcm2-1* strain were elevated relative to the Yp-CN2 parent, but not to the high levels seen for constructs with larger regions of *ARS1* sequence (Figure 4C). This observation suggests that while some of the determinants of *ARS1 mcm* sensitivity lie within the A, B1 and B2 elements, there are other determinants outside these elements but within the 373 bp exchanged in Yp-CN6. Nevertheless, comparing Yp-CN2, Yp-CN6 and Yp-CN9 makes it clear that we have identified the main determinants of *mcm* sensitivity for *ARS1*: the *TRP1* promoter accounting for almost half of the effect and the A, B1 and B2 elements together contributing the majority of the remaining sensitivity.

Other components of the pre-RC show an *mcm* phenotype

The Mcm2–7 proteins are involved throughout the DNA replication process. During G1 phase, they load on to the DNA to form a pre-RC and they are involved subsequently in both the origin initiation and fork elongation steps (50). Of the two main elements we have identified, the *TRP1* promoter could be envisaged to act via interference between transcription and pre-RC formation or via collisions between transcription forks and nascent replication forks. To determine whether *mcm* sensitivity differences reflect events occurring before or after replication initiation, we tested whether other mutants that affect specific stages of replication distinguish between the *ARS1* and *ARS121* plasmids Yp-CN1 and Yp-CN2. We selected alleles of ORC genes (*orc2-1* and *orc5-1*) and *cdc6-1* (51–53) as additional mutations in the pre-RC assembly pathway. We also tested the initiation factor mutant *dbf4-1* and the elongation factor mutant *cdc17-1* (53,54). The transcription factor mutant *mcm1-1* was tested to confirm previous reports that this mutant also distinguishes between these origins (21,55). The stability of Yp-CN1 and Yp-CN2 was measured

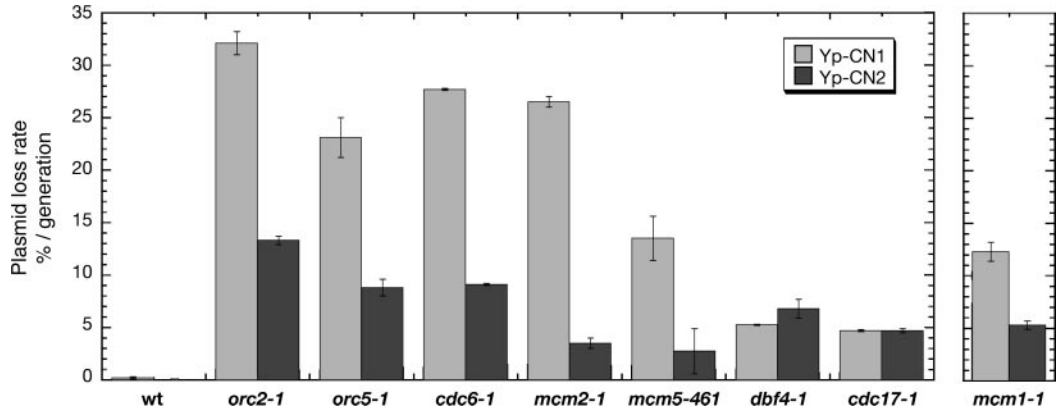

Figure 5. Mutations in other pre-RC components cause differential plasmid sensitivity. Plasmid loss rates are shown for the *ARS1* (Yp-CN1) and *ARS121* (Yp-CN2) plasmids in wild-type cells and a panel of mutants. All loss rates were measured at 30°C.

in each strain at the semi-permissive temperature of 30°C (Figure 5).

The *ARS1* plasmid (Yp-CN1) was lost much more rapidly than the *ARS121* plasmid (Yp-CN2) in all of the pre-RC component mutants. The *dbf4-1* and *cdc17-1* mutants, in contrast, affected both origins equally, with Yp-CN1 and Yp-CN2 being lost at similar rates. Another elongation mutant (*pri2-1*) behaved similarly (data not shown). We conclude that *ARS1* and *ARS121* differ in their ability to assemble a functional pre-RC when licensing components are limiting; if a pre-RC is successfully assembled, it is equally likely to be activated at either *ARS1* or *ARS121* and for the replication forks to extend successfully.

The *mcm1-1* strain behaved like the pre-RC mutants, with the *ARS1* plasmid being lost more rapidly than the *ARS121* plasmid, consistent with the previously reported results that *mcm1-1* confers an *mcm* sensitivity phenotype similar to that of the Mcm2–7 family proteins (55). This phenotype could result from the role of Mcm1 in regulating the expression of pre-RC genes, including *CDC6*, *MCM3*, *MCM5*, *MCM6* and *MCM7* (56–58), or via a direct role for Mcm1 in pre-RC assembly (49).

DISCUSSION

To understand the response of origins to *mcm* mutations, we have isolated the key sequence determinants of origin *mcm* sensitivity and identified other mutants that cause a similar phenotype. In analysing *ARS1* and *ARS121* two major sequence determinants of origin *mcm* sensitivity were identified: first, the core sequence elements (A, B1 and B2) of the *ARS121* origin are important for its relative resistance to *mcm* mutations, while second, transcription towards the *ARS1* origin from the *TRP1* promoter makes a substantial contribution to the extreme sensitivity of *ARS1* to *mcm* mutations. Furthermore, we found that these origins differ in their efficiency not just in *mcm* mutants, but in mutants of all tested pre-RC components, demonstrating that the difference is in fact more accurately termed a *pre-RC* sensitivity phenotype. Since the pre-RC is assembled on the minimal origin sequence, our results are consistent in pointing towards pre-RC assembly as the principal step where these origins differ.

Origin substructure and sensitivity to *mcm* mutations

We used phylogenetic footprinting to identify a functional B2 element within the ATR domain of *ARS121*. Improved understanding of the substructure of *ARS121* aided us in determining the sequence elements that were responsible for the resilience of *ARS121* to reduced pre-RC function. It has been suggested that the additional matches to the ACS found within *ARS121* might contribute to its relative resistance to *mcm* mutations (7). Neither *ARS1* nor *ARS121* has significantly more matches to the ACS than the other, but the *ARS121* ATR does contain several sequences that resemble the B2 element of *ARS1*. Replacement of the B2 element of *ARS1* with a region of the ATR that includes three good matches to the ACS did not, however, increase its stability in an *mcm* mutant (Yp-CN12), suggesting that neither the ATR nor additional ACS matches are sufficient to confer resistance to *mcm* mutations. Consistent with this conclusion, we found that disrupting one or two of the ACS-like sequences within the ATR of *ARS121* did not destabilize the origin in the *mcm2-1* strain. Therefore, these elements are neither sufficient nor required to confer the *mcm* resistance phenotype.

It has also been proposed that the binding of Mcm1 to a replication origin might determine its sensitivity to reduced Mcm2–7 protein function, by playing a direct role in promoting pre-RC assembly (49). Mutating three Mcm1 binding sites in *ARS121* did not significantly increase the *mcm* sensitivity of the origin (Ycp-CN13, data not shown), although there may be additional Mcm1 binding sites that remain intact in this construct. The plasmid loss rates in *mcm1* mutants could potentially be explained by the role of Mcm1 in regulating the expression of pre-RC genes, such as *CDC6* (56). However, our data do not exclude a more direct role for Mcm1, either through interacting with the assembling pre-RC or by shielding the pre-RC from the transcriptional apparatus.

To address whether the *mcm* sensitivity is determined by the ORC binding site, we exchanged A and B1 elements between *ARS1* and *ARS121*. There was no clear linkage between these elements and *mcm* sensitivity, suggesting that the ORC binding site alone is not responsible. Consistent with this observation, we did not detect any difference in the ability of these two origins to interact with Orc2 by chromatin immunoprecipitation (ChIP), even under conditions of compromised ORC

function (data not shown). However, given that the loss rates of Yp-CN1 and Yp-CN2 represent 47 and 93% origin firing efficiency and assuming that these differences quantitatively reflect differences in pre-RC assembly, such a difference (~2-fold) may be below the sensitivity of ChIP-based methods.

Finally, we tested the extent to which the core A, B1 and B2 elements together confer *mcm* sensitivity. The core elements from *ARS121* are sufficient to overcome the *mcm* sensitivity of the *ARS1* plasmid (Yp-CN8), showing that in the case of *ARS121*, a minimal 104 bp sequence containing A, B1 and B2 elements is the key determinant of resistance to reduced pre-RC function. The situation in the case of *ARS1* is slightly more complicated. The core A, B1 and B2 elements from *ARS1* confer significant *mcm* sensitivity on the *ARS121* plasmid (compare Yp-CN9 with Yp-CN2); however, sensitivity is increased by the inclusion of the sequences immediately surrounding *ARS1* along with these core elements (Yp-CN5 and Yp-CN6). Some elements influencing *ARS1* sensitivity, therefore, lie close to as well as within the origin core sequences. We refer to these effects, i.e. sensitivity elements that lie very close to or overlapping core origin activity elements, as the 'hard-wiring' of the origin with respect to pre-RC protein function.

Origin context and sensitivity to *mcm* mutations

In addition to the 'hard-wired' determinants, origin sensitivity to *mcm* mutations depends on transcriptional context. Deletion of the *TRP1* promoter leads to a Trp^- phenotype, almost certainly due to failure to transcribe the *TRP1* gene. We did not confirm directly that transcription at the locus is ablated, but since the *TRP1* open reading frame remains intact it is difficult to envisage another mechanism that could give rise to the Trp^- phenotype. The promoter deletion increased the stability of an *ARS1*-replicated plasmid in *mcm2-1* cells, indicating that under conditions of reduced Mcm2–7 protein function *ARS1* is particularly sensitive to transcription. One interpretation of this result is that there is competition between the assembling pre-RC and the transcription apparatus, such that under normal dosage of Mcm2–7 proteins the origin is successfully licensed. However, under conditions of reduced Mcm2–7 protein function, the balance tips and transcription limits licensing and reduces the efficiency of origin firing. Consistent with our findings, it has previously been reported that very high-level transcription across an origin reduces origin activity (59). Our study is the first to demonstrate that endogenous transcription is detrimental to origin function even when the origin is downstream of the transcription unit in its normal intergenic location. This finding contrasts with the positive correlation between the transcriptional activity and the origin usage observed at the yeast rDNA locus (60). However, unlike *ARS1*, the *rDNA* replication origin lies in a divergent intergenic space between the *RDN18* and the *RDN5* genes.

The susceptibility of the *ARS121* minimal sequences to *TRP1* transcription was greatly influenced by the orientation of the replication origin (Figure 3D). When *ARS121* was placed downstream of *TRP1* with its A element oriented like the normal *ARS1* A element, the plasmid (Yp-CN7B) was extremely *mcm* sensitive. Reversing the orientation of *ARS121* reduced the plasmid loss rate 3-fold. In the latter case, the Abf1 binding sites contained within *ARS121* lie between *TRP1* and the *ARS121* A element, so that one possible explanation of the orientation-dependence might be that the Abf1 binding sites influence the susceptibility of these chimaeric origins to transcription. This possibility is consistent with the fact that Abf1 binds DNA tightly but has only weak transcriptional activation function that is not required for its role at replication origins (20,61,62). Two functions have been demonstrated for Abf1 binding at *ARS1*; positioning of nucleosomes to favour ORC recruitment (15) and terminating transcription that is artificially driven through the origin (63,64). The orientation-dependent effect of transcription on *ARS121* that we have observed could potentially be mediated by Abf1 through either of these mechanisms. However, it is interesting to note that transcription also has a substantial effect on *ARS1* in its natural configuration immediately downstream of the *TRP1* gene (Figure 3B), indicating that the single intervening Abf1 site (the *ARS1* B3 element) is unable to completely protect the origin from the effects of transcription. A role in coordinating transcription with pre-RC formation could explain why the activity of many origins is influenced by flanking transcription factor binding sites.

Our discovery of an effect of transcription on origin sensitivity to pre-RC mutations has significant consequences for studies that have analysed plasmids replicated by small origin fragments, particularly in strains carrying mutations in pre-RC proteins. Minimal *ARS121* origin inserts were sufficient to confer *mcm* resistance on large plasmids with no interfering transcription (e.g. Yp-CN7), but did not stabilize our minimal origin construct (Yp-*ARS121*), in which the same fragment was inserted directly at the polylinker. Therefore, there must be dominant elements within the vector sequences that can destabilize this construct when placed close to the origin. These elements are analogous in effect to *TRP1* transcription and indeed it has been reported that flanking vector sequences can initiate transcription towards an inserted origin (44). By having the potential to dramatically affect the efficiency of the origin, proximal vector sequences may mask more subtle physiological effects under investigation.

A hierarchy of replication origins

We have found that origins differ in their response to reductions in pre-RC protein levels, with *ARS1* activity being significantly reduced, whereas *ARS121* activity is relatively resilient. We find that there are two underlying determinants of this effect: one, the minimal sequence elements, is inflexible or 'hard-wired'; the other, transcription into the origin, has the potential to be responsive and is 'soft-wired'. The pre-RC sensitivity phenotype is not limited to *ARS1*, but is shared with a number of other replication origins, including the *HO ARS* and the telomeric origin *ARS131*. Similarly, other origins are known that are relatively resistant to *mcm* mutations. Therefore, a hierarchy of origins exists with respect to requirement for pre-RC function. Under laboratory conditions, levels of pre-RC protein expression are sufficient for all these origins to fire with high efficiency, but if pre-RC levels are reduced the origins differ in their robustness such that specific origins continue to function. In this study, we artificially reduced the concentrations of pre-RC components by the use of conditional mutants, but in a population of cells there will

be stochastic variations in the concentrations of particular proteins. Differences in environment may also have an impact on protein levels. This hierarchy of origins could offer a means for the cell to react in a regulated manner to alter DNA replication in response to environmental changes. These mechanisms may not be limited to budding yeast since there are clear indications for an interplay between transcription and replication in fission yeast (65) and metazoans (66,67). Therefore, the response of origins to the level of pre-RC function may represent a key mechanism by which eukaryotes coordinate transcription and replication.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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REFERENCES

1. Diffley,J.F. (2004) Regulation of early events in chromosome replication. *Curr. Biol.*, **14**, R778–R786.
2. Raghuraman,M.K., Winzeler,E.A., Collingwood,D., Hunt,S., Wodicka,L., Conway,A., Lockhart,D.J., Davis,R.W., Brewer,B.J. and Fangman,W.L. (2001) Replication dynamics of the yeast genome. *Science*, **294**, 115–121.
3. Wyrick,J.J., Aparicio,J.G., Chen,T., Barnett,J.D., Jennings,E.G., Young,R.A., Bell,S.P. and Aparicio,O.M. (2001) Genome-wide distribution of ORC and MCM proteins in *S.cerevisiae*: high-resolution mapping of replication origins. *Science*, **294**, 2357–2360.
4. Yabuki,N., Terashima,H. and Kitada,K. (2002) Mapping of early firing origins on a replication profile of budding yeast. *Genes Cells*, **7**, 781–789.
5. Friedman,K.L., Brewer,B.J. and Fangman,W.L. (1997) Replication profile of *Saccharomyces cerevisiae* chromosome VI. *Genes Cells*, **2**, 667–678.
6. Weinreich,M., Palacios DeBeer,M.A. and Fox,C.A. (2004) The activities of eukaryotic replication origins in chromatin. *Biochim. Biophys. Acta*, **1677**, 142–157.
7. Tye,B.K. (1999) MCM proteins in DNA replication. *Annu. Rev. Biochem.*, **68**, 649–686.
8. Donaldson,A.D. and Blow,J.J. (1999) The regulation of replication origin activation. *Curr. Opin. Genet. Dev.*, **9**, 62–68.
9. Newlon,C.S. and Theis,J.F. (1993) The structure and function of yeast ARS elements. *Curr. Opin. Genet. Dev.*, **3**, 752–758.
10. Theis,J.F. and Newlon,C.S. (1997) The ARS309 chromosomal replicator of *Saccharomyces cerevisiae* depends on an exceptional ARS consensus sequence. *Proc. Natl Acad. Sci. USA*, **94**, 10786–10791.
11. Marahrens,Y. and Stillman,B. (1992) A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science*, **255**, 817–823.
12. Walker,S.S., Malik,A.K. and Eisenberg,S. (1991) Analysis of the interactions of functional domains of a nuclear origin of replication from *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **19**, 6255–6262.
13. Rao,H. and Stillman,B. (1995) The origin recognition complex interacts with a bipartite DNA binding site within yeast replicators. *Proc. Natl Acad. Sci. USA*, **92**, 2224–2228.
14. Lee,D.G. and Bell,S.P. (1997) Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol. Cell. Biol.*, **17**, 7159–7168.
15. Lipford,J.R. and Bell,S.P. (2001) Nucleosomes positioned by ORC facilitate the initiation of DNA replication. *Mol. Cell*, **7**, 21–30.
16. Wilmes,G.M. and Bell,S.P. (2002) The B2 element of the *Saccharomyces cerevisiae* ARS1 origin of replication requires specific sequences to facilitate pre-RC formation. *Proc. Natl Acad. Sci. USA*, **99**, 101–106.
17. Diffley,J.F. and Stillman,B. (1989) Similarity between the transcriptional silencer binding proteins ABF1 and RAP1. *Science*, **246**, 1034–1038.
18. Walker,S.S., Francesconi,S.C., Tye,B.K. and Eisenberg,S. (1989) The OBF1 protein and its DNA-binding site are important for the function of an autonomously replicating sequence in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **9**, 2914–2921.
19. Chang,V.K., Fitch,M.J., Donato,J.J., Christensen,T.W., Merchant,A.M. and Tye,B.K. (2003) Mcm1 binds replication origins. *J. Biol. Chem.*, **278**, 6093–6100.
20. Wiltshire,S., Raychaudhuri,S. and Eisenberg,S. (1997) An Abf1p C-terminal region lacking transcriptional activation potential stimulates a yeast origin of replication. *Nucleic Acids Res.*, **25**, 4250–4256.
21. Maine,G.T., Sinha,P. and Tye,B.K. (1984) Mutants of *S.cerevisiae* defective in the maintenance of minichromosomes. *Genetics*, **106**, 365–385.
22. Lei,M., Kawasaki,Y., Young,M.R., Kihara,M., Sugino,A. and Tye,B.K. (1997) Mcm2 is a target of regulation by Cdc7–Dbf4 during the initiation of DNA synthesis. *Genes Development*, **11**, 3365–3374.
23. Young,M.R. and Tye,B.K. (1997) Mcm2 and Mcm3 are constitutive nuclear proteins that exhibit distinct isoforms and bind chromatin during specific cell cycle stages of *Saccharomyces cerevisiae*. *Mol. Biol. Cell*, **8**, 1587–1601.
24. Tanaka,T., Knapp,D. and Nasmyth,K. (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell*, **90**, 649–660.
25. Aparicio,O.M., Weinstein,D.M. and Bell,S.P. (1997) Components and dynamics of DNA replication complexes in *S.cerevisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell*, **91**, 59–69.
26. Labib,K., Tercero,J.A. and Diffley,J.F. (2000) Uninterrupted MCM2–7 function required for DNA replication fork progression. *Science*, **288**, 1643–1647.
27. Tye,B.K. and Sawyer,S. (2000) The hexameric eukaryotic MCM helicase: building symmetry from nonidentical parts. *J. Biol. Chem.*, **275**, 34833–34836.
28. Labib,K. and Diffley,J.F. (2001) Is the MCM2–7 complex the eukaryotic DNA replication fork helicase? *Curr. Opin. Genet. Dev.*, **11**, 64–70.
29. Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. Huberman,J.A., Spotila,L.D., Nawotka,K.A., el-Assouli,S.M. and Davis,L.R. (1987) The *in vivo* replication origin of the yeast 2 micron plasmid. *Cell*, **51**, 473–481.
31. Brewer,B.J., Lockshon,D. and Fangman,W.L. (1992) The arrest of replication forks in the rDNA of yeast occurs independently of transcription. *Cell*, **71**, 267–276.
32. Friedman,K.L. and Brewer,B.J. (1995) Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. *Methods Enzymol.*, **262**, 613–627.
33. Balakrishnan,R., Christie,K.R., Costanzo,M.C., Dolinski,K., Dwight,S.S., Engel,S.R., Fisk,D.G., Hirschman,J.E., Hong,E.L., Nash,R. *et al.* (2005) Fungal BLAST and Model Organism BLASTP Best Hits: new comparison resources at the *Saccharomyces* Genome Database (SGD). *Nucleic Acids Res.*, **33**, D374–D377.
34. Thompson,J.D., Higgins,D.G. and Gibson,T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.
35. Rice,P., Longden,I. and Bleasby,A. (2000) EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet.*, **16**, 276–277.
36. Donaldson,A.D., Fangman,W.L. and Brewer,B.J. (1998) Cdc7 is required throughout the yeast S phase to activate replication origins. *Genes Dev.*, **12**, 491–501.

37. Sinha,P., Chang,V. and Tye,B.K. (1986) A mutant that affects the function of autonomously replicating sequences in yeast. *J. Mol. Biol.*, **192**, 805–814.
38. Huberman,J.A. (1999) Genetic methods for characterizing the *cis*-acting components of yeast DNA replication origins. *Methods*, **18**, 356–367.
39. Yan,H., Gibson,S. and Tye,B.K. (1991) Mcm2 and Mcm3, two proteins important for ARS activity, are related in structure and function. *Genes Dev.*, **5**, 944–957.
40. Hopwood,B. and Dalton,S. (1996) Cdc45p assembles into a complex with Cdc46p/Mcm5p, is required for minichromosome maintenance, and is essential for chromosomal DNA replication. *Proc. Natl Acad. Sci. USA*, **93**, 12309–12314.
41. Lei,M., Kawasaki,Y. and Tye,B.K. (1996) Physical interactions among Mcm proteins and effects of Mcm dosage on DNA replication in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **16**, 5081–5090.
42. Kim,S., Mellor,J., Kingsman,A.J. and Kingsman,S.M. (1986) Multiple control elements in the TRP1 promoter of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **6**, 4251–4258.
43. Kim,S.Y., Mellor,J., Kingsman,A.J. and Kingsman,S.M. (1988) An AT rich region of dyad symmetry is a promoter element in the yeast TRP1 gene. *Mol. Gen. Genet.*, **211**, 472–476.
44. Braus,G., Paravicini,G. and Hutter,R. (1988) A consensus transcription termination sequence in the promoter region is necessary for efficient gene expression of the TRP1 gene of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **212**, 495–504.
45. Kellis,M., Patterson,N., Endrizzi,M., Birren,B. and Lander,E.S. (2003) Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature*, **423**, 241–254.
46. Cliften,P., Sudarsanam,P., Desikan,A., Fulton,L., Fulton,B., Majors,J., Waterston,R., Cohen,B.A. and Johnston,M. (2003) Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting. *Science*, **301**, 71–76.
47. Cliften,P.F., Hillier,L.W., Fulton,L., Graves,T., Miner,T., Gish,W.R., Waterston,R.H. and Johnston,M. (2001) Surveying *Saccharomyces* genomes to identify functional elements by comparative DNA sequence analysis. *Genome Res.*, **11**, 1175–1186.
48. Moses,A.M., Chiang,D.Y., Kellis,M., Lander,E.S. and Eisen,M.B. (2003) Position specific variation in the rate of evolution in transcription factor binding sites. *BMC Evol. Biol.*, **3**, 19.
49. Chang,V.K., Donato,J.J., Chan,C.S. and Tye,B.K. (2004) Mcm1 promotes replication initiation by binding specific elements at replication origins. *Mol. Cell. Biol.*, **24**, 6514–6524.
50. Lei,M. and Tye,B.K. (2001) Initiating DNA synthesis: from recruiting to activating the MCM complex. *J. Cell. Sci.*, **114**, 1447–1454.
51. Foss,M., McNally,F.J., Laurenson,P. and Rine,J. (1993) Origin recognition complex (ORC) in transcriptional silencing and DNA replication in *S.cerevisiae*. *Science*, **262**, 1838–1844.
52. Loo,S., Fox,C.A., Rine,J., Kobayashi,R., Stillman,B. and Bell,S. (1995) The origin recognition complex in silencing, cell cycle progression, and DNA replication. *Mol. Biol. Cell*, **6**, 741–756.
53. Hartwell,L.H. (1973) Three additional genes required for deoxyribonucleic acid synthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.*, **115**, 966–974.
54. Johnston,L.H. and Thomas,A.P. (1982) A further two mutants defective in initiation of the S phase in the yeast *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **186**, 445–448.
55. Elble,R. and Tye,B.K. (1992) Chromosome loss, hyperrecombination, and cell cycle arrest in a yeast mcm1 mutant. *Mol. Biol. Cell*, **3**, 971–980.
56. McInerny,C.J., Partridge,J.F., Mikesell,G.E., Creemer,D.P. and Breeden,L.L. (1997) A novel Mcm1-dependent element in the SWI4, CLN3, CDC6, and CDC47 promoters activates M/G1-specific transcription. *Genes Dev.*, **11**, 1277–1288.
57. Fitch,M.J., Donato,J.J. and Tye,B.K. (2003) Mcm7, a subunit of the presumptive MCM helicase, modulates its own expression in conjunction with Mcm1. *J. Biol. Chem.*, **278**, 25408–25416.
58. Spellman,P.T., Sherlock,G., Zhang,M.Q., Iyer,V.R., Anders,K., Eisen,M.B., Brown,P.O., Botstein,D. and Futcher,B. (1998) Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol. Biol. Cell*, **9**, 3273–3297.
59. Snyder,M., Sapolsky,R.J. and Davis,R.W. (1988) Transcription interferes with elements important for chromosome maintenance in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **8**, 2184–2194.
60. Muller,M., Lucchini,R. and Sogo,J.M. (2000) Replication of yeast rDNA initiates downstream of transcriptionally active genes. *Mol. Cell*, **5**, 767–777.
61. Cho,G., Kim,J., Rho,H.M. and Jung,G. (1995) Structure-function analysis of the DNA binding domain of *Saccharomyces cerevisiae* ABF1. *Nucleic Acids Res.*, **23**, 2980–2987.
62. Goncalves,P.M., Griffioen,G., Minnee,R., Bosma,M., Kraakman,L.S., Mager,W.H. and Planta,R.J. (1995) Transcription activation of yeast ribosomal protein genes requires additional elements apart from binding sites for Abf1p or Rap1p. *Nucleic Acids Res.*, **23**, 1475–1480.
63. Tanaka,S., Halter,D., Livingstonezatchej,M., Reszel,B. and Thoma,F. (1994) Transcription through the yeast origin of replication ARS1 ends at the ABFI binding site and affects extrachromosomal maintenance of minichromosomes. *Nucleic Acids Res.*, **22**, 3904–3910.
64. Chen,S., Reger,R., Miller,C. and Hyman,L.E. (1996) Transcriptional terminators of RNA polymerase II are associated with yeast replication origins. *Nucleic Acids Res.*, **24**, 2885–2893.
65. Gomez,M. and Antequera,F. (1999) Organization of DNA replication origins in the fission yeast genome. *EMBO J.*, **18**, 5683–5690.
66. Danis,E., Brodolin,K., Menut,S., Maiorano,D., Girard-Reydet,C. and Mechali,M. (2004) Specification of a DNA replication origin by a transcription complex. *Nature Cell. Biol.*, **6**, 721–730.
67. Aladjem,M.I. and Fanning,E. (2004) The replicon revisited: an old model learns new tricks in metazoan chromosomes. *EMBO Rep.*, **5**, 686–691.