Subtelomeric ACS-containing Proto-silencers Act as Antisilencers in Replication Factors Mutants in *Saccharomyces cerevisiae*

Muhammad Attiq Rehman,* Dongliang Wang,* Genevieve Fourel,[†] Eric Gilson,[†] and Krassimir Yankulov*

*Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada; and [†]Laboratoire de Biologie Moléculaire de la Cellule de l'Ecole Normale Supérieure de Lyon, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5161, F-69364, Lyon, France

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Subtelomeric genes are either fully active or completely repressed and can switch their state about once per 20 generations. This meta-stable telomeric position effect is mediated by strong repression signals emitted by the telomere and relayed/enhanced by weaker repressor elements called proto-silencers. In addition, subtelomeric regions contain sequences with chromatin partitioning and antisilencing activities referred to as subtelomeric antisilencing regions. Using extensive mutational analysis of subtelomeric elements, we show that ARS consensus sequence (ACS)-containing proto-silencers convert to antisilencers in several replication factor mutants. We point out the significance of the *B1* auxiliary sequence next to ACS in mediating these effects. In contrast, an origin-derived ACS does not convert to antisilencer in mutants and its *B1* element has little bearing on silencing. These results are specific for the analyzed ACS and in addition to the effects of each mutation (relative to wild type) on global silencing. Another line of experiments shows that Mcm5p possesses antisilencing activity and is recruited to telomeres in an ACS-dependent manner. Mcm5p persists at this location at the late stages of S phase. We propose that telomeric ACS are not static proto-silencers but conduct finely tuned silencing and antisilencing activities mediated by ACS-bound factors.

INTRODUCTION

Genes positioned close to telomeres are either fully active or completely repressed. They switch their state of expression once per 20 generations on average. This meta-stable telomeric position effect (TPE) is governed by strong repression signals emitted by the telomeres (Fourel *et al.*, 2002a; Tham and Zakian, 2002; Rusche *et al.*, 2003). Weaker repressor elements in the *core X*- and *Y'*-subtelomeric elements, called proto-silencers, can relay and enhance repression signals away from telomeres (Fourel *et al.*, 2002a). To date, proto-silencer activity has been demonstrated for ARS consensus sites (ACS) and for the binding sites for Rap1p and Abf1p (Boscheron *et al.*, 1996; Fourel *et al.*, 1999; Pryde and Louis, 1999; Lebrun *et al.*, 2001). In addition, subtelomeric *core X*- and *Y'*-elements contain sequences, which display chromatin partitioning or antisilencing activities and are referred to as subtelomeric antisilencing regions (STARS) (Fourel *et al.*, 1999, 2004; Pryde and Louis, 1999). The state of gene expression at individual telomeres is determined by the complex interplay

between the telomere, proto-silencers, and antisilencers (Fourel *et al.*, 1999; Pryde and Louis, 1999; Lebrun *et al.*, 2001; Fourel *et al.*, 2002a, 2004; Rehman *et al.*, 2006); however, it is not clear whether and how these elements contribute to the variegated nature of TPE.

ACS is a conserved 11-base pair sequence found in all yeast origins of replication, in subtelomeric *core X*- and *Y'*-elements and in the silencers of the mating type loci. ACS binds origin recognition complex (ORC) and is essential for replication initiation at origins and for silencing at the mating type loci (Bell and Stillman, 1992; Diffley and Cocker, 1992; Rusche *et al.*, 2003). A less conserved *B1* element upstream of ACS serves as an additional docking site for ORC and contributes to, but is not essential for replication initiation (Marahrens and Stillman, 1992; Rao and Stillman, 1995; Lee and Bell, 1997). The *B1* element of the HMR-E silencer is dissimilar to the *B1* elements found in origins (Palacios DeBeer and Fox, 1999; Palacios DeBeer *et al.*, 2003). It confers high affinity to ORC and contributes to robust silencing of HMR (Palacios DeBeer and Fox, 1999; Palacios DeBeer *et al.*, 2003). The role of *B1* and ACS in subtelomeric *core X*- and *Y'*-elements has not been addressed.

TPE is modulated by a significant number of genes. For example, mutations in general regulators of silencing such as the *SIR* genes, in the ORC genes, *ABF1* and *RAP1*, reduce the silencing at telomeres, the mating type loci and at the ribosomal gene loci (Aparicio *et al.*, 1991; Pryde and Louis, 1999; Roy and Runge, 2000). In contrast, genes involved in telomere maintenance and localization (*HDF1*, *HDF2*, *MRE11*, *XRS2*, and *RAD50*) specifically affect silencing at telomeres (Boulton and Jackson, 1998; Laroche *et al.*, 1998;

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Address correspondence to: Krassimir Yankulov (yankulov@uoguelph.ca).

Abbreviations used: ACS, ARS consensus sequence; FOA, fluoroorotic acid; MCM, minichromosome maintenance; ORC, origin recognition complex; STAR, subtelomeric antisilencing region; TPE, telomeric position effect.

Figure 1. Schematic representation of the constructs used in this study. (A) Telomeric repeats are represented as solid arrowheads; the *core X* as gray rectangle; *ARS1* as dotted rectangle; *STAR*, *URA3*, *TRP1*, and the portion of *ADH4* used for targeted integration in the *VII-L* telomere as open rectangles. Arrows above *TRP1* and *URA3* indicate the orientation of the gene. *ACS* (solid diamond), *B1* (solid triangle), *B2* (inverted open triangle), and *Abf1* binding site (open circle) are depicted within *ARS1* and *core X*. The *Gal4* binding site in *GF100* and *URA-UAS_{GAL}-tel* is shown as an open rectangle. (B) The consensus *B1/ACS* sequence (solid rectangle and solid triangle indicate the positions of *B1* and *ACS*) is shown on top. The *HMR-E*, *core X* and *ARS1* sequences encompassing *B1/ACS* are shown in capital letters with the consensus bases shown in bold. The mutated sequences in the *ACS⁻* and *B1⁻* constructs are shown in small letters and are underlined.

Fisher and Zakian, 2005; Mueller *et al.*, 2006). Finally, the histone methylase COMPASS (Mueller *et al.*, 2006), the histone acetyl transferase NuA4 (Babiarz *et al.*, 2006; Clarke *et al.*, 2006), the histone chaperone CAF-1 (Mueller *et al.*, 2006; Tamburini *et al.*, 2006), cohesin (Suter *et al.*, 2004; Chang *et al.*, 2005), *SUM1* (Irlbacher *et al.*, 2005), and *SCP160* (Marsellach *et al.*, 2006) were also implicated in telomeric silencing.

There is a puzzling link between DNA replication and silencing. The establishment of silent chromatin requires the passage through S-phase (Miller and Nasmyth, 1984), but does not require DNA replication per se (Dubey *et al.*, 1991; Kirchmaier and Rine, 2001; Li *et al.*, 2001). Even so, many genes that affect silencing encode DNA replication factors. These include *ORC2*, *ORC5*, *MCM5*, *MCM10*, *CDC44*, *CDC45*, *POL30* (Ehrenhofer-Murray *et al.*, 1995; Dillin and Rine, 1997; Fox *et al.*, 1997; Ehrenhofer-Murray *et al.*, 1999; Dziak *et al.*, 2003; Suter *et al.*, 2004; Liachko and Tye, 2005). Paradoxically, an earlier comprehensive study on telomeric silencing found that mutations in *orc2* and *orc5* have little effect on gene expression in the vicinity of *ACS* (Pryde and Louis, 1999). Similarly, the deletion of *ACS* in the *HMR-E* silencer actually increased *HMR* repression in *orc2* mutants (Palacios DeBeer and Fox, 1999). On the other hand,

we have demonstrated that mutations in *orc2*, *orc5* and *mcm5* exert their effects on subtelomeric silencing mostly via *ACS* (Rehman *et al.*, 2006). In an attempt to resolve this apparent inconsistency on the role of *ACS* in silencing, we further explored the *ACS*-mediated effects in different mutants. Intriguingly, we noticed that in certain mutants the subtelomeric *ACS* alter their behavior and act as antisilencers.

MATERIALS AND METHODS

Yeast Strains and Reporter Constructs

The yeast strains used are shown in Supplemental Table 1. All experiments were performed at the permissive temperature of 23°C. The constructs used are shown in Figure 1A. *GF6* and *GF44* were described in Fourrel *et al.* (1999); *GF100* in (Fourrel *et al.*, 2002b); *URA3-tel* and *URA3-UAS_{GAL}-tel* in (Jacobson and Pillus, 2004), *ARS1-URA3-tel*, and *URA3-ARS1-tel* in (Rehman *et al.*, 2006). *GF6(ACS⁻)*, *GF44(ACS⁻)*, *GF6(B1⁻)*, *GF44(B1⁻)*, *ARS1(ACS⁻)-URA3-tel*, *ARS1(B1⁻)-URA3-tel*, and *URA3-ARS1(B1⁻)-tel* were generated by site-directed mutagenesis of *GF6*, *GF44*, *ARS1-URA3-tel*, and *URA3-ARS1-tel* as depicted in Figure 1B; *GF6(ACS⁻ΔSTAR)* and *GF6(B1⁻ΔSTAR)* were produced by removing *STAR* elements from *GF6(ACS⁻)* and *GF6(B1⁻)*. All reporter constructs include a portion of *ADH4* and a telomeric *TG_{(1-3)n}* repeat to direct their insertion between *ADH4* and the telomere of the *VII-L* arm. After transformation with the constructs cells were selected on SC-ura (and SC-ura-trp in the case *GF100*) plates and variegated reporter expression was

confirmed by restreaking positive clones on SC-ura and SC/FOA plates (see below). Three colonies from each transformation were then subjected to analysis by the reporter activity assays described below.

GAL4DBD-Fusion Protein Expression Cassettes

The coding sequence of *ORC1*, *ORC2*, and *MCM3* were produced by polymerase chain reaction (PCR) of genomic DNA from W303. *MCM5* was produced by PCR from pIP122CDC46 (Gauthier *et al.*, 2002). The *mcm5-461* and *mcm5-1* alleles were produced by PCR with genomic DNA from the corresponding strains in Supplemental Table 1. All fragments were cloned into pGBKT7 to produce GAL4DBD fusion cassettes and then subcloned in pRS315 (*CEN4*, *ARS1*, *LEU2*). pGAL4DBD-GCN5, pGAL4DBD-SIR1 and pGAL4DBD-MCM10 have been described in Jacobson and Pillus (2004) and Liachko and Tye (2005), respectively. The expression plasmids were transformed into cells with previously integrated *URA3*-tel, *URA3*-UAS-*GAL4*⁺-tel, or GF100 constructs.

Reporter Activity Assays

URA3 expression renders the cells sensitive to fluoroorotic acid (FOA). Hence, the proportion of cells with repressed *URA3* was assessed as percentage of FOA resistant cells (%FOA^R). The proportion of cells expressing *TRP1* in the GF100 construct was assessed by growth on SC-trp plates. For the selection of the GAL4DBD-fusion protein expression plasmids, additional nutrients (leucine or histidine) were omitted as appropriate.

For each measurement, three colonies were grown in nonselective (YPD) medium for 20–30 generations. Serial 1:10 dilutions from each individual culture were spotted on SC, SC/FOA, or SC-trp plates as appropriate. The %FOA^R or %TRP⁺ was calculated as the average number of colonies on SC/FOA or SC-trp plates divided by the average number of colonies on SC plates. The average values $\pm$ SE (calculated in Excel; Microsoft, Redmond, WA) of *n* triplicate measurements were then calculated and are shown in Supplemental Tables 2–6.

Chromatin Immunoprecipitation (ChIP)

Cells were arrested in mitosis by 12 μ g/ml nocodazole and released from the block as described in Rehman *et al.* (2006). Samples of 50 ml were cross-linked at the time points indicated in text. Cross-linking was as in Kurdistani and Grunstein (2003) with modifications. Briefly, 50-ml cultures at OD₆₀₀ = 1.2 were suspended in 20 ml of PBS/10 mM dimethyl adipimidate for 15 min at 25°C, and then 30 ml of PBS/1.6% formaldehyde was added for 25 min. After quenching with glycine cells were suspended in 2.5 ml of LIP (Lysis/IP) buffer (50 mM Tris-HCl, 140 mM NaCl, 3 mM EDTA, 1 $\times$ protease inhibitor cocktail (P8215; Sigma-Aldrich, St. Louis, MO) and broken with glass beads. Chromatin and cell debris were collected by spinning, suspended in LIP and sonicated for 12 min in Bioruptor (Diagenode, Liege, Belgium) at maximum output. Debris was removed by spinning, and an aliquot was uncross-linked to confirm an average size of DNA of 500 base pairs. The samples received Triton X-100 and Na-deoxycholic acid to 0.5 and 0.05%, and then they were immunoprecipitated with 10 μ g of anti-MCM5 (Sc-6680; Santa Cruz Biotechnology, Santa Cruz, CA) antibody for 3 h. Load, wash and eluate samples were uncross-linked and analyzed by PCR with primers specific for *ARS1*, *ARS305*, *ARS501*, and *core X*. *Core X* signals were also confirmed by slot-blot. The primers for the *core X* element in GF44 and GF44(ACS[−]) anneal to the plasmid backbone of the integrating constructs and do not amplify genomic *core X*. Primer sequences are available upon request.

RESULTS

Experimental Strategy

Recently, we correlated the telomeric antisilencing defects of several mutants (*mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1*) to the presence of ACS (Rehman *et al.*, 2006). These mutations significantly weaken telomeric silencing (see Supplemental Tables 2 and 3), whereas the deletion of ACS diminished these effects (Rehman *et al.*, 2006). A separate study has shown that deletion of *sas2* also requires intact ACS for its effect on silencing (Ehrenhofer-Murray *et al.*, 1997). *cdc7-1* displays both ACS-dependent and ACS-independent effects (Rehman *et al.*, 2006). *cdc6-1* and *Δ sir2* reduced silencing independently of ACS (Ramachandran *et al.*, 2006; Rehman *et al.*, 2006).

In the current study, we present extensive mutational analyses of ACS and the auxiliary *B1* element in these mutant strains. Two well characterized constructs, GF6 and GF44 (Fourel *et al.*, 1999; Rehman *et al.*, 2006), contain a conserved subtelomeric *core X* elements plus a *STAR* ele-

ment and a *URA3* reporter (Figure 1A). Two other constructs, *ARS1*-*URA3*-tel and *URA3*-*ARS1*-tel (Rehman *et al.*, 2006) contain the 150-base pair genomic sequence encompassing the *ARS1* origin of DNA replication (Figure 1A).

The ACS- and *B1*-mutations in *core X* and in *ARS1* are depicted in Figure 1B. ACS is highly conserved and plays key roles in ORC binding and in origin and silencer function. It has been shown that single A $\rightarrow$ C mutations in it abolishes both ORC binding and the stability of *ARS1*-harboring minichromosomes (Marahrens and Stillman, 1992; Rao and Stillman, 1995). In our constructs, we have substituted AAA with CCC or CGC so we expect these substitutions to preclude the binding to ORC and any effects associated with ACS. The neighboring *B1* element is loosely conserved (Palacios DeBeer *et al.*, 2003) with A_WN_Y forming its core (Figure 1B). It acts as a secondary docking site for ORC (Rao and Stillman, 1995; Lee and Bell, 1997). In *ARS1*, single mutations in AW (A₈₃₉A₈₄₀) (Rao and Stillman, 1995) reduced but did not abolish binding to ORC and also decreased the stability of *ARS1*-harboring minichromosomes. Total replacement of *B1* by a linker did not produce stronger effects. In *HMR-E*, substitutions in *B1* reduced its affinity to ORC and converted it to a poor silencer but a good origin of replication (Palacios DeBeer *et al.*, 2003). We have mutated the AW doublets in *ARS1* and *core X* to CC. We expect these mutations to decrease, but not abolish, the binding to ORC. In terms of function, we expect the mutations to preclude any effects mediated by *B1*.

All mutant and wild-type constructs were integrated at the same position in *VII-L* telomere of *mcm5-461*, *orc2-1*, *orc5-1*, *cdc45-1*, *Δ sas2* *cdc6-1*, *cdc7-1*, and *Δ sir2* or the corresponding wild-type strains *MCM5* and *W303*. Three colonies (per experiment) were streaked on SC-ura and SC/FOA plates to confirm variegated expression and then grown for 30 generations in YPD medium to allow for telomeric expression equilibrium to be reached under nonselective conditions. The %FOA^R for the three colonies was assessed by the FOA^R reporter activity assay and then average %FOA^R and errors for (*n*) independent triplicate experiments were calculated (Supplemental Tables 2 and 4).

Finally, we divided the average %FOA^R value in the (ACS[−]) and (*B1*[−]) constructs where the effects of ACS are abolished, by the average %FOA^R value in the constructs with intact *B1*/ACS elements (Figures 2–4). Hence, the calculated values specifically assess the net silencing contribution of ACS and *B1* at these loci independently and above the effects on global silencing of the gene mutations. Because *mcm5-461*, *orc2-1*, *orc5-1*, *cdc46-1*, *cdc6-1*, and *cdc7-1* affect essential genes, we assume that these mutations are still capable of mediating effects through the essential ACS element. In these graphs, values below 1 signify antisilencing effect of the destroyed element, whereas values above 1 signify silencing effects of the destroyed element.

Silencing Effects of ACS

We used the ACS[−]/ACS ratios for the *ARS1*-*URA*-tel, GF44, and GF6 constructs to specifically assess the silencing effects of ACS in each individual mutant. As expected, in all three constructs the ablation of ACS decreased the silencing of *URA3* in the *MCM5* and *W303* strains by 2- to 4-fold, thus reiterating that in wild-type context ACS acts as a protosilencer (Figure 2A). Unexpectedly, however, the destruction of ACS had entirely different effect in the *mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1* mutants. In *ARS1*-*URA*-tel the elimination of ACS in *ARS1* caused reduction of repression, but to a significantly larger extent as compared with wild-type cells (Figure 2A). In contrast, the elimination of ACS in

Figure 2. ACS-mediated silencing effects in *ARS1* and *core X*. ARS1-URA-tel, GF6, GF44, ARS1(ACS⁻)-URA3-tel, GF6(ACS⁻), GF44(ACS⁻) (diagrams shown on top) were inserted in the VII-L telomere of wild-type (MCM5, W303) and mutant (*mcm5-461*, *orc2-1*, *orc5-1*, *cdc45-1*, Δ *sas2*, *cdc7-1*, *cdc6-1*, and Δ *sir2*) strains, and %FOA^R was determined for each construct/strain combination. Raw data are shown in Supplemental Table 2. (A) The ratio %FOA^R(ACS⁻)/%FOA^RACS as indicated above each graph represents the effect of destruction of ACS in the construct shown on top in the strains shown on the left. In these exponential graphs, values below 1 depict derepression and values above 1 depict increased repression. (B) The ratio %FOA^RGF6(ACS⁻ Δ STAR)/%FOA^RGF6(Δ STAR) shows the effect of destruction of ACS in the absence of STAR element.

the *core X* elements in GF44 and GF6 consistently increased repression. We also noticed a great position variation in the magnitude of increase in *URA3* repression in *orc2-1* (Figure 2A, compare GF6 and GF44) but not in other mutants (Figure 3A). Although we do not completely understand these effects of *orc2-1*, they suggest that this particular allele may alter the directionality of silencing driven by ACS as reported earlier for the mating type loci (Zou *et al.*, 2006a,b). The other three mutants had similar effects on GF44 and GF6. We concluded that in *mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1* the *core X*-ACS displayed properties typical for an antisilencer (Figure 2A).

We considered that this surprising behavior of ACS in the mutants could be a nonspecific effect of overall reduction in silencing. For this reason, we performed similar assays in four other mutants. As mentioned, Δ *sas2* requires intact ACS for its effect (Ehrenhofer-Murray *et al.*, 1997), *cdc7-1* has both ACS-dependent and ACS-independent effects (Rehman *et al.*, 2006), whereas *cdc6-1* and Δ *sir2* act independently of ACS (Ramachandran *et al.*, 2006; Rehman *et al.*, 2006). As expected, in Δ *sas2* cells the destruction of ACS exacerbated the loss of silencing in all three constructs albeit to a different level compared with wild-type cells (Figure 2A). The *cdc7-1* mutation showed little difference compared with the wild-type cells. The *cdc6-1* and the Δ *sir2* mutations showed mild increase in repression in ARS1-URA-tel and GF44 and derepression in GF6. In these two mutants, we see weak correlation to the position of *URA3* relative to the telomere and the proto-silencer rather than to the nature of the proto-

silencer. In summary, these four mutants did not show any unexpected effects. Importantly, they indicated that the conversion of ACS to antisilencer in *core X* (GF44 and GF6) is specific for *mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1* and not general consequence of deregulation of silencing.

Finally, we tested whether the observed effects of the *core X*-ACS element could somehow be driven by the neighboring STAR element. In Figure 2B we show that the deletion of STAR in GF6(ACS⁻) does not alter the pattern of repression in *mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1*.

Silencing Effects of B1

At least two studies have reported functional dissimilarities between different B1 elements that relate to the replicator or silencer efficiency (Palacios DeBeer and Fox, 1999; Palacios DeBeer *et al.*, 2003; Chang *et al.*, 2008). We therefore asked whether the apparent differences of ACS in ARS1 and *core X* could be extended to their B1 elements. We mutated the B1 elements in the ARS1-URA-tel, URA-ARS1-tel, GF44, and GF6 and used the B1⁻/B1 ratios to evaluate the contribution of B1 to telomeric silencing. The destruction of B1 in ARS1 only marginally affected silencing in the mutants and actually slightly increased repression in the wild-type strains (Figure 3A). Switching of the position of ARS1 and URA3 relative to the telomere did not make difference in the lack of effect of the destruction of B1 (Figure 3A, compare ARS1-URA-tel and URA-ARS1-tel). We concluded that, independently of position or orientation, the B1 element of ARS1

Figure 3. B1-mediated silencing effects in ARS1 and core X. ARS1-URA-tel, URA-ARS1-tel, GF6, GF44, ARS1(B1⁻)-URA-tel, URA-ARS1(B1⁻)-tel, GF6(B1⁻), and GF44(B1⁻) were inserted in the VII-L telomere of wild type (MCM5, W303) and mutant (*mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1*) strains and %FOA^R was determined for each construct/strain combination. Raw data are shown in Supplemental Table 3. (A) The ratio %FOA^R(B1⁻)/%FOA^R(B1⁺) for ARS1-URA-tel and URA-ARS1-tel is plotted to show the effect of destruction of B1 in the strains shown on the left. (B) The ratio %FOA^R(B1⁻)/%FOA^R(B1⁺) for GF44 and GF6 is plotted to show the effect of destruction of B1 in the strains shown on the left. (C) The ratio %FOA^RGF6(B1⁻ΔSTAR)/%FOA^RGF6 shows the effect of destruction of B1 in the absence of STAR element.

has no bearing on silencing at telomeres. In contrast, the destruction of the *coreX-B1* in GF6 and GF44 produced very similar gain of repression in *mcm5-461*, *orc2-1*, *orc5-1*, and *cdc45-1* compared with the elimination of *core X-ACS* (compare Figure 2A and Figure 3B). Again, the level of repression in *orc2-1* was dependent on the position of the ACS/B1 element (Figure 3B). Finally, we tested whether the observed effects of mutations in the B1 element could somehow be driven by the neighboring STAR element. In Figure 3C, we show that the deletion of STAR in GF6(B1⁻) does not alter the pattern of repression in any of the mutants (compare Figure 3, A and B, with C).

The significance of the data presented in Figures 2 and 3 is twofold. First, we show that, unlike ARS1-derived B1/ACS, subtelomeric *core X*-derived B1/ACS convert from proto-silencers in wild-type cells to antisilencers in four replication/silencing factor mutants. Second, we demonstrate that, unlike ARS1-B1, the *core X-B1* element has a profound effect on silencing.

Mcm5p Has Antisilencing Activity

The conversion of *coreX-B1/ACS* from proto-silencer to anti-silencer suggests that a hypothetical ACS-binding antisilencing factor could be more active in the analyzed mutants. This idea prompted us to look for possible antisilencing activities in transactors, which are known to associate with ACS. We exploited an assay, which has been extensively used to identify general antisilencing activities out of the context of ACS or other silencers (Fourrel *et al.*, 1999, 2002b, 2004; Jacobson and Pillus, 2004; Liachko and Tye, 2005). It measures the expression of reporters inserted in the VII-L telomere upon the tethering of recombinant GAL4-fusion proteins via a nearby UAS_{GAL4-10} site (Figure 4). Using this assay, we tested the silencing activities in an array of proteins, which are known to associate with ACS. The open reading frames of the ORC1, ORC2, MCM3, MCM5, *mcm5-461*, *mcm5-1* (Dziak *et al.*, 2003) (see Supplemental Figure 1 for details on these mutations), *mcm5(bob1-1)* (Hardy *et al.*, 1997), MCM10, *mcm10-1* (Liachko and Tye, 2005), and SIR1 alleles, respectively, were fused to a GAL4DBD expression cassette and subcloned in low copy plasmids (pRS315). The produced plasmids were then transformed into a wild-type (W303) strain containing the URA3-UAS_{GAL}-tel reporter inserted in the VII-L telomere (Jacobson and Pillus, 2004). The expression of the recombinant proteins was confirmed by Western blot with anti-GAL4 antibodies (data not shown). Next, using the FOA^R assay, we evaluated how these proteins influenced the repression of URA3 (Figure 4 and Supplemental Table 5). The specific effect of each protein was calculated as the ratio of FOA^R cells expressing GAL4DBD-fusion proteins divided by FOA^R cells expressing GAL4DBD alone (Figure 4A). Again, values below 1 signify derepression and values above 1 signify repression.

In tune with several previous reports (Rusche *et al.*, 2003; Liachko and Tye, 2005), the tethering of Orc1p, Orc2p, Sir1p, Mcm10p, and the mutant Mcm10-1p increased the repression (albeit at different levels) at the VII-L telomere relative to GAL4DBD alone. However, Mcm5p or the mutant Mcm5-461p and Mcm5-1p proteins displayed distinct antisilencing activity. The mutations in Mcm5-461p and Mcm5-1p only moderately reduced the antisilencing effect of the wild-type protein (Figure 4A); however, the Mcm5(*bob1-1*)p protein showed no antisilencing activity. Because the *mcm5(bob1-1)* mutation by-passes the requirement for CDC7 (Hardy *et al.*, 1997), we speculate that the antisilencing activity of Mcm5p is at least partially regulated by CDC7. Another member of the MCM family, Mcm3p, showed silencing rather than antisilencing properties (Figure 4A). This important control indicates that antisilencing is specific for Mcm5p and not a general feature of the MCM proteins.

We tested whether the antisilencing activity of the Mcm5p proteins was due to side effects of overexpression not mediated by the UAS_{GAL4} site. In Figure 4B, we show that the expression of wild-type GAL4-Mcm5p causes threefold derepression on a UAS_{GAL4}-less cassette (LPY1029; (Jacobson and Pillus, 2004) relative to GAL4DBD alone, whereas the mutant proteins had weaker effects. GAL4-Orc1p also reduced repression by approximately threefold. However, the ratio %FOA^R(URA3-UAS_{GAL}-tel)/FOA^R(URA-tel) indicated that the antisilencing effect of the Mcm5 proteins is 80–90 times stronger on the URA3-UAS_{GAL}-tel cassette (LPY1030; Jacobson and Pillus, 2004), whereas Orc1p increased silencing by approximately twofold (Figure 4B). We conclude that it is the tethering of the Mcm5 proteins that is mostly responsible for the derepression of URA3-UAS_{GAL}-tel. Consequently, we concluded that most of the tested proteins, which build up

Figure 4. Mcm5p has antisilencing activity. (A) Wild-type cells (W303) harboring URA3-UAS_{GAL}-tel (shown on the top) were transformed with plasmids carrying expression cassettes for the GBD-fusion proteins shown on the left. Percentage of FOA^R for each recombinant protein is plotted in the top graph. Raw data are shown in Supplemental Table 5. The ratio %FOA^RGAL4-fusion/%FOA^RGAL4DBD is shown in the bottom graph and indicates the effect of each recombinant protein. Values below 1 depict antirepression activity, whereas values above 1 depict repression activity. (B) *LPY1030* cells harboring URA-UAS_{GAL}-tel (top) and *LPY1029* cells harboring URA3-tel (middle) were transformed with plasmids expressing the proteins shown on the left. Percentage of

FOA^R was measured for each recombinant protein in each strain and was plotted. Raw data are shown in Supplemental Table 5. The ratio %FOA^RURA3-UAS-tel/%FOA^RURA3-tel (bottom) shows how the effect of these proteins depends on the UAS_{GAL} site. (C) Wild-type cells (BY4742) and Δ hat1, Δ gcn5, Δ sas2, and Δ sas3 isogenic deletion mutants harboring URA3-UAS_{GAL}-tel (shown on the top) were transformed with plasmids expressing GBD-Mcm5p or GAL4DBD, respectively. Percentage of FOA^R for each recombinant protein was measured. Raw data are shown in Supplemental Table 5. The ratio %FOA^RGAL4-Mcm5p/%FOA^RGAL4DBD indicates the effect of each gene deletion on the antisilencing activity of Mcm5p.

prereplicative complexes and presumably also associate with subtelomeric ACS, possess silencing activities. Among them, Mcm5p clearly displayed antisilencing activity.

Next, we asked what could be the mechanism, which is engaged in this antisilencing activity of Mcm5p. We performed similar tethering assays in strains with deletions in four nonessential histone-acetyl-transferase genes: Δ hat1, Δ gcn5, Δ sas2, and Δ sas3 (Figure 4C). It is noteworthy that Δ sas2 and Δ sas3 dramatically derepress truncated telomeres, but it had significantly weaker effect on the more “complex” constructs including GF6, GF44, and URA-UAS-tel (Supplemental Figure 3). Whereas the deletion of *SAS2* and *SAS3* only slightly reduced the antisilencing effect of GAL4DBD-Mcm5p, the deletion of *HAT1* and *GCN5* essentially abol-

ished it (Figure 4C). We concluded that the antisilencing effect of Mcm5p is mediated by the histone-acetyl-transferases *HAT1* and *GCN5* and thus conforms with a general expectation of an antisilencing factor.

Mcm5 Is a Poor Chromatin Insulator

Certain proteins or DNA elements impede the spreading of chromatin modifications into adjacent genomic domains thus acting as chromatin insulators or “partitioners” (Fourel *et al.*, 2002b, 2004; Ishii *et al.*, 2002). We considered the possibility that the observed effects of the Mcm5 proteins are a consequence of chromatin partitioning rather than of genuine antisilencing. We addressed this issue in the *GF100* strain (Figure 5; Fourel *et al.*, 2002b), which harbors a double

Figure 5. MCM5 has poor insulating activity. W303 cells (A) and W303 Δ *rif1* (B) cells harboring the GF100 reporter (shown on the top) in the VII-L telomere were transformed with plasmids carrying expression cassettes for the GDB-fusion proteins shown on the left. %*FOA^R*, %*TRP⁺*, and %*FOA^R&TRP⁺* cells for each recombinant protein were measured and plotted in the top, middle, and bottom graphs, respectively. Raw data are shown in Supplemental Table 6.

reporter cassette (*URA3* and *TRP1*) and a *STAR* element inserted in the VII-L telomere. These two reporters are separated by *UAS_{GAL}* so that *TRP1* is proximal to the telomeric repeats. If a protein tethered to *UAS_{GAL}* equally increases or decreases the levels of expression of *TRP1* and *URA3*, then this protein has antisilencing or silencing activities only. However, if the tethered protein increases the levels of expression of *TRP1* and *URA3* remains repressed, it acts as a chromatin insulator (Fourel *et al.*, 2002b, 2004). In this assay, the level of repression of *URA3* is assessed as %*FOA^R* cells, the level of repression of *TRP1* is assessed as %*TRP⁺* cells, whereas partitioning activity is assessed as %*FOA^R&TRP⁺* cells.

Using the GF100 strain, the three GAL4-Mcm5 proteins were compared with GAL4DBD and to well characterized silencing (GAL4-*Orc2p*) (Rusche *et al.*, 2003) or antisilencing (GAL4-*Gcn5p*) (Jacobson and Pillus, 2004) proteins. As expected, GAL4-*Orc2p* increased the %*FOA^R* cells and decreased the %*TRP⁺* cells, whereas GAL4-*Gcn5p* had the opposite effect relative to GAL4DBD alone (Figure 5A and Supplemental Table 6). The MCM5 proteins behaved as antisilencers similarly to GAL4-*Gcn5p* (Figure 5A). Importantly, GAL4DBD and all fusion proteins produced <0.01% *FOA^R&TRP⁺* cells thus indicating poor partitioning activity (Figure 5A).

Because MCM5p and Gcn5p induce significant levels of derepression at the VII-L telomere and as a result low % *FOA^R* cells, it may be difficult to detect partitioning activity under

these experimental conditions. We addressed this concern by deleting *RIF1* in the GF100 strain (Fourel *et al.*, 2002b). *RIF1* counteracts the repression signals emitted by the telomere via Rap1p (Fourel *et al.*, 2002b). Indeed, the deletion of *RIF1* increased ~10 times the repression of both *URA3* and *TRP1* (Figure 5B). Correspondingly, the %*FOA^R&TRP⁺* cells increased from ~0.01 to ~1% for all proteins tested (Figure 5B), but again no substantial difference between GAL4DBD and the fusion proteins was observed. These experiments argue that MCM5p does not have partitioning properties under the conditions of either high or low levels of local repression.

Mcm5p Remains Associated with Core X in Late S Phase

Prior studies have shown abundant association of MCM proteins with subtelomeric regions (Wyrick *et al.*, 2001), but it is not known whether this abundance is dependent on subtelomeric ACS. We tested whether this is the case. We conducted ChIP with anti-Mcm5p antibodies in W303 strains harboring the GF44 or GF44(ACS⁻) constructs in the VII-L telomere. After determining the linear range for PCR in input samples (data not shown), the input and immunoenriched DNA was quantified by PCR with primers for *ACT1*, *ARS305*, *ARS501*, total genomic core X, and the core X element in the VII-L telomere in GF44. The signals from the eluates relative to input in the W303 and W303/GF44 strains are plotted in Figure 6A. No specific association of MCM proteins with the *ACT1* gene was detected. In contrast, comparable strong signals in both strains were observed for two origins of replication, *ARS305* and *ARS501*, and for total genomic core X DNA. Importantly, the elimination of ACS from the core X-element in GF44 precluded the binding of MCM5p at this particular position (Figure 6A). Finally, the anti-Mcm5p antibody did not work in the *mcm5-461* strain, supporting the notion that all signals are specific for MCM5p. We concluded that MCM5p associates with subtelomeric core X elements in an ACS-dependent manner.

Next, we analyzed the subtelomeric occupancy by MCM5p in two mutants, which display strong ACS-dependent anti-silencing activity, *orc2-1* and *orc5-1*. Wild-type (W303) and mutant cells were arrested in mitosis by nocodazole for 3 h and then washed and suspended in YPD. Samples were collected at 10, 35, 60, and 75 min after release from the point of mitotic arrest. These time points correspond to early G1, late G1/S, S, and late S/G2 phases of the cell cycle, respectively (Rehman *et al.*, 2006). The cells were cross-linked, lysed, and chromatin was immunoprecipitated with anti-Mcm5p antibodies. The association of MCM5p with a very early (*ARS305*), an early (*ARS1*), and a late (*ARS501*) origins as well as with core X DNA was measured by PCR and is plotted in Figure 6B. At all four genomic loci a high levels of association were observed in early G1 phase, which corresponds to the time of loading of MCM proteins on chromatin (Lei and Tye, 2001). The MCM5p association markedly diminishes in the subsequent time points at the two early origins (Figure 6B, top two rows) and seems to persist longer for the late *ARS501* origin (Figure 6B, third row). This pattern of occupancy is in good agreement with the expected time of firing and MCM displacement from origins (Lei and Tye, 2001). Remarkably, ChIP signals with core X-DNA were quite dissimilar compared with the pattern displayed by the origins (Figure 6B, bottom row). At core X, we observed the typical peak of ChIP signal in early G1 but also a subsequent gradual increase in mid- and late S phase in both wild-type and mutant cells. It is possible that this increase is produced by enhanced exposure of epitope on already loaded MCM proteins, for example as a result of phosphorylation. This conclusion would be in tune with most current concepts on

Figure 6. (A) Mcm5p association with telomeres depends on ACS. ChIP with anti-Mcm5p antibodies (Santa Cruz Biotechnology) was performed in the W303/GF44, W303/GF44(ACS⁻), and *mcm5-461* strains. Each experiment was performed with parallel controls where cross-linking or primary antibody incubation was omitted. The abundance of different DNAs (shown on the left) was quantified by PCR after confirming linear PCR range for the input samples. The primers for the *core X* element in GF44 and GF44(ACS⁻) anneal to the

plasmid backbone of the integrating constructs and do not amplify genomic *core X*. *core X* signals were also confirmed by slot-blot. The signals in the eluates were subtracted by the signals in the final washes and then divided by the signals in the input fractions to produce "net signals." Finally, all net signals were normalized so that the corresponding values in GF44 are 100% and then compared with the values in W303/GF44(ACS⁻) and *mcm5-461*. The figure is representative of two independent ChIP experiments. (B) Wild-type (W303), *orc2-1* and *orc5-1* cells (shown at the bottom top) were released from mitotic arrest and samples were collected after 10, 35, 60, and 75 min (early G1, late G1/S, S, and G2 phases of the cell cycle, respectively) as shown at the bottom. ChIP with anti-Mcm5p antibodies was conducted as described above. The signals in the eluates were subtracted by the signals in the final washes and then divided by the signals in the input fractions and plotted as % enrichment relative to input. The analyzed genomic loci are indicated in the top left corner of each graph. The figure is representative of two independent ChIP experiments.

the turnover of MCM proteins throughout the cell cycle (Lei and Tye, 2001). Although we do not favor the idea, our data do not exclude a continuous loading of MCMs at telomeres through S-phase or enrichment of MCMs via arriving replication forks. In any case we can make the minimal conclusion that considerable amounts of Mcm5p are present at telomeres through the late stages of S phase. Regardless of the mechanism, this pattern is in stark contrast to the loading and displacement of MCM proteins at origins (Figure 6A). It is notable that the two mutants have extended S phase (Rehman *et al.*, 2006), providing opportunity for longer action of the antisilencing activity of Mcm5p.

DISCUSSION

Subtelomeric ACS Convert to Antisilencers in Replication Factors Mutants

ACS represents the essential core of all origins of DNA replication (Breier *et al.*, 2004) and of the silencers at the

maturing type loci (Rusche *et al.*, 2003). At telomeres, ACS operate as weaker proto-silencers, which relay/enhance the repression signals emitted by the telomere, whereas their destruction causes modest decrease in silencing (Fourel *et al.*, 2002a). At telomeres, we also find other regulatory units such as antisilencers and chromatin insulators (Fourel *et al.*, 2004). A peculiar feature of gene expression at telomeres is its meta-stable character, meaning that subtelomeric genes switch at low frequency from complete repression to full expression (Rusche *et al.*, 2003). It is not clear how this meta-stability persists and why the combination of a strong silencer (the telomere) and proto-silencers (ACS, Abf1p-, and Rap1p-binding sites) can successfully establish, but then fail to maintain the repressed state. One can speculate that some of these repressor elements temporarily reduce their strength or alternatively, antisilencer elements temporary increase their activity to allow for the reversal.

In this article, we analyze the role of ACS in telomeric silencing and show that their destruction increases repres-

sion in *orc2-1*, *orc5-1*, *mcm5-461*, and *cdc45-1* mutants (Figure 2), whereas in the corresponding wild-type strains it leads to modest derepression (Figure 2). Similar increase in repression was observed when the adjacent *B1* element was eliminated (Figure 3). A straightforward interpretation of these data are that within these mutants, the subtelomeric *B1/ACS* elements have antisilencing activity. These effects are concurrent with the overall decrease in silencing in the mutants.

Are the reported effects indeed mediated by ACS? First, we point out that the observed conversion cannot be a mere consequence of global derepression in the mutants. For example, the *cdc6-1*, *cdc7-1*, and Δ *sir2* mutations caused significant reduction in telomeric silencing (Ramachandran *et al.*, 2006; Rehman *et al.*, 2006) but did not alter the nature of ACS (Figure 2). Second, we can reasonably assume that the *orc2-1* and *orc5-1* mutations as well as the other mutations do not preclude association of ORC with ACS, otherwise they would be lethal. Therefore, we favor the interpretation that in each specific genetic context our data represents the roles of ACS and *B1* as occupied by ORC. Third, although it is conceivable that some of the observed effects in Figures 2 and 3 are influenced by the different genomic environment in *ARS1* and in *core X*, our calculations (ACS⁻/ACS; *B1*⁻/*B1*) are heavily geared toward detecting the effects mediated by ACS and *B1*. Finally, in *orc2-1* and *orc5-1*, we (Figure 2) and others (Palacios DeBeer and Fox, 1999; Pryde and Louis, 1999; Lebrun *et al.*, 2001; Fourel *et al.*, 2002b) have observed very strong de-repression uncharacteristic for other mutants. Hence, we do not exclude the possibility that the strong effects in Figure 2 are consequence of the combination of the gene mutations and the ablation of ACS and do not represent ACS-driven effects only.

A very recent article (Chang *et al.*, 2008) mapped the essential replication activity of *HMR-E* to two overlapping 9/11 ACS matches, but not to the 11/11 ACS match of the silencer (*ARS317*). Is it then possible that the antisilencing activity at telomeres is mediated by near-ACS matches? We see no such overlapping near-ACS matches at the telomere whereas the individual near-ACS matches are eight of 11 at best. At the same time, mutation of the *B1* site next to the 11/11 ACS match did not have strong effect on the replication activity of *HMR-E* (*ARS317*) and the subtelomeric *ARS319* (Chang *et al.*, 2008) but have profound effect on silencing at *HMR-E* (Palacios DeBeer *et al.*, 2003) and at the telomere (our findings). These observations provide circumstantial support to the notion that the *B1/ACS* element rather than near-ACS matches are responsible for the silencing effects at these positions.

The conclusion that subtelomeric ACS act as an antisilencers in certain replication factor mutants challenges many earlier studies, which consistently portray ACS as silencers or proto-silencers (Fourel *et al.*, 2002a; Rusche *et al.*, 2003). Yet, our observations do not stand alone. A systematic study on the silencing of a reporter along the *VII-L* subtelomere has paradoxically shown that *orc2-1* and *orc5-1* cause significant derepression at most subtelomeric positions but have almost no effect in the vicinity of ACS (Pryde and Louis, 1999). In addition, similarly to the data presented here, the destruction of ACS in the *HMR-E* silencer in *orc2-1* actually increased repression (Palacios DeBeer and Fox, 1999). We extend these observations and propose that subtelomeric ACS have weak and/or short-lived antisilencing activity, which is somehow revealed in replication factor mutants.

Functional Differences between Subtelomeric and Origin *B1/ACS*

In Figures 2 and 3, we show that *core X*-derived *B1/ACS*, but not origin-derived *B1/ACS* convert to antisilencers in the tested mutants. At the same time, the destruction of *B1* does not affect the proto-silencing properties of *ARS1*, whereas its counterpart in *core X* is as important as ACS (Figures 2 and 3). It should be noted that the *B1* element in *ARS1* has a significant albeit not essential contribution to the origin activity of *ARS1* (Marahrens and Stillman, 1992; Rao and Stillman, 1995). In contrast, mutations in *B1* of *HMR-E* make this element a poor silencer but a good origin (Palacios DeBeer and Fox, 1999; Palacios DeBeer *et al.*, 2003). Furthermore, the silencing, but not the replication strength of several *B1/ACS* combinations was correlated to their high affinity to ORC (Palacios DeBeer *et al.*, 2003).

It is important that the *B1* elements of *core X* (Figure 3) and *B1* in *HMR-E* (Palacios DeBeer *et al.*, 2003) but not *B1* in *ARS1* (Figure 2) were identified as contributors to silencing. Even more, the sequence of the *core X-B1* (Figure 1) is related to *B1* of *HMR-E* and synthetic silencers rather than to that of several origins of replication (figure 1 in Palacios DeBeer *et al.*, 2003). We anticipate the *coreX-B1/ACS* element to have high-affinity to ORC. Notwithstanding the affinity to ORC, it seems that *B1* elements can distinguish between replicator and silencer types of ACS. There are ~10,000 ACS matches or near matches in the yeast genome (Breier *et al.*, 2004), but only a few hundred of them have been functionally assigned as origins and only several as silencers/proto-silencers. We propose that the adjacent *B1* elements can predict the functions of ACS, which are not experimentally accessible at the threshold of genome-wide studies.

How Can ACS Be Proto-Silencer and Antisilencer?

Many of the proteins, which work in both replication and silencing, bind ACS or regulate the action of ACS-bound proteins (Rusche *et al.*, 2003). In DNA replication, ACS is a focal point of a rigorously regulated protein complex, which controls origin licensing and firing (Rusche *et al.*, 2003). It would be surprising if the same ACS-bound proteins in *core X* are spared from similar control and do not undergo modifications during G1 and S phases. At this point, it is not clear how *B1* elements (and affinity to ORC) affect these modifications. Still, it is possible that such modifications temporarily unleash antirepression activity to balance the repression signals emitted by other ACS-bound factors. In tune with this idea, we have observed that mutations in *CDC7* (a protein kinase) modulate the silencing properties of proteins tethered to the telomere (Supplemental Figure 2). If alterations in ACS-bound proteins can temporarily convert these complexes to antisilencers, then mutations in them can unduly unleash antisilencing or, being also mutations in replication factor, can simply extend S phase thus prolonging the time of the ACS-driven antirepression. The net effect will lead to the observations in Figure 3.

Is *Mcm5p* an Antisilencing Module on ACS-bound Complexes?

We show that the *Mcm5p* is as strong antisilencer as the histone acetyl transferase *Gcn5p*, whereas other ACS associating proteins (*Orc1p*, *Orc2p*, *Mcm3p*, and *Mcm10p*) are silencers (Figures 4 and 5). Although we consider the artificial nature of our tethering assay, we stress that it recapitulated well documented effects of *Gcn5p*, *Orc1p*, *Orc2p*, and *Mcm10p* detected by similar or other assays (Rusche *et al.*, 2003; Jacobson and Pillus, 2004; Liachko and Tye, 2005). It is

noteworthy that the antisilencing activity of Mcm5p is dependent on GCN5 and HAT1 (Figure 4C). At this point, it is not clear how these two histone-acetyl-transferases communicate with Mcm5p and whether their communication is modulated by modification of the MCM complex for example by phosphorylation. Regardless, the current observations support the idea that Mcm5p could indeed be involved in antisilencing.

Equally importantly, we show that unlike origins of replication, *core X* associates with Mcm5p later in S phase (Figure 6B). Although we do not observe any dramatic differences in this occupation in two of the analyzed mutants, we still believe that this peculiar dissimilarity to origins provides clues on how Mcm5p may exhibit stronger antisilencing in replication factor mutants. In addition, the Mcm5p antisilencing activity is not affected by the amino acid substitutions in *mcm5-461* and *mcm5-1*, whereas these two mutations lead to telomeric derepression (Dziak *et al.*, 2003). We postulate that the *mcm5-461*, *mcm5-1*, and other mutations probably boost antisilencing by prolonging S phase (Rehman *et al.*, 2006) rather than by affecting the Mcm5p antisilencing activity itself.

In conclusion, we propose that there is a dynamic interplay of weak antirepression (Mcm5p) and stronger repression (Orc1p, Orc2p, Mcm3p, and Mcm10p) signals on telomeric ACS. Mutations, which prolong the time of manifestation or boost these antisilencing activities by other means can in turn convert ACS from proto-silencers to antisilencers.

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