DOI: 10.1534/genetics.106.063446

# Differential Requirement of DNA Replication Factors for Subtelomeric *ARS* Consensus Sequence Protosilencers in *Saccharomyces cerevisiae*

**Muhammad Attiq Rehman,* Genevieve Fourel,† Amit Mathews,* Danielle Ramdin,* Maria Espinosa,* Eric Gilson† and Krassimir Yankulov*,[1]**

**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, CNRS UMR5161, IFR 128, F-69364, Lyon, France*

Manuscript received July 19, 2006
Accepted for publication September 8, 2006

ABSTRACT

The establishment of silent chromatin requires passage through S-phase, but not DNA replication *per se*. Nevertheless, many proteins that affect silencing are *bona fide* DNA replication factors. It is not clear if mutations in these replication factors affect silencing directly or indirectly via deregulation of S-phase or DNA replication. Consequently, the relationship between DNA replication and silencing remains an issue of debate. Here we analyze the effect of mutations in DNA replication factors (*mcm5-461*, *mcm5-1*, *orc2-1*, *orc5-1*, *cdc45-1*, *cdc6-1*, and *cdc7-1*) on the silencing of a group of reporter constructs, which contain different combinations of "natural" subtelomeric elements. We show that the *mcm5-461*, *mcm5-1*, and *orc2-1* mutations affect silencing through subtelomeric ***A**RS* **c**onsensus **s**equences (*ACS*), while *cdc6-1* affects silencing independently of *ACS*. *orc5-1*, *cdc45-1*, and *cdc7-1* affect silencing through *ACS*, but also show *ACS*-independent effects. We also demonstrate that isolated nontelomeric *ACS* do not recapitulate the same effects when inserted in the telomere. We propose a model that defines the modes of action of *MCM5* and *CDC6* in silencing.

THE position-dependent repression of genes via distal nonpromoter regulatory elements is referred to as "silencing." In *Saccharomyces cerevisiae*, the mating-type (*HM*) loci *HML* and *HMR*, the subtelomeric portion of the chromosomes, and the *rRNA* genes are the main genome regions where silencing has been observed and extensively studied (reviewed in Rusche *et al.* 2003).

At *HML* and *HMR* silencing is established through two specialized silencer elements that flank each of these loci (Rusche *et al.* 2003). These elements contain binding sites for Rap1p and Abf1p, which act as transcriptional activators/repressors in other contexts (Eisenberg *et al.* 1988; Biswas and Biswas 1990; Planta *et al.* 1995), as well ***A**RS* **c**onsensus **s**ites (*ACS*), which bind the origin recognition complex (ORC) (Labib and Diffley 2001; Lei and Tye 2001). It is believed that the juxtaposition of Rap1p, Abf1p, and ORC creates a high local concentration of the main components of silenced chromatin, the SIR proteins, which eventually leads to the nucleation and spreading of the silent chromatin state (Rusche *et al.* 2003).

At telomeres, gene expression is reversibly silenced to produce either active or completely repressed chromatin, a phenomenon referred to as *t*elomeric *p*osition *e*ffect (TPE) (Tham and Zakian 2002; Rusche *et al.* 2003). Mutations in the regulators of silencing at *HM*, the *SIR* genes, the *ORC* genes, *ABF1*, and *RAP1*, also have an effect on TPE (Aparicio *et al.* 1991; Pryde and Louis 1999; Roy and Runge 2000). On the other hand, genes involved in nonhomologous end joining, telomere maintenance, or histone methylation (*HDF1* and *HDF2*, *MRE11*, *XRS2*, *RAD50*, COMPASS) as well as localization at the nuclear periphery affect silencing at telomeres, but not at the *HM* loci (Boulton and Jackson 1998; Laroche *et al.* 1998; Fisher and Zakian 2005; Mueller *et al.* 2006). Hence, silencing of the telomeres is mediated by similar, but not identical mechanisms to these employed at the mating loci. The growing list of factors engaged in silencing also includes the histone acetyl transferase NuA4 (Babiarz *et al.* 2006; Clarke *et al.* 2006), the histone chaperone CAF-1 (Tamburini *et al.* 2006), cohesin (Suter *et al.* 2004; Chang *et al.* 2005), *SUM1* (Irlbacher *et al.* 2005), and *SCP160* (Marsellach *et al.* 2006).

Unlike the *HM* loci, subtelomeric regions do not contain apparent strong silencers. At these positions the telomeric ($TG_{1-3}$) repeats, which contain binding sites for Rap1p (Gilson 1989; Marcand *et al.* 1996; Grunstein 1997), cooperate with Abf1p-binding sites (*ABF1-BS*) and *ACS*, which are found in the *core X*- and *Y′*-subtelomeric elements. *ABF1-BS* and *ACS* do not act as silencers on their own and hence they are referred to as protosilencers (Boscheron *et al.* 1996; Fourel *et al.*

[1]*Corresponding author:* Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON N1G 2W1, Canada.
E-mail: yankulov@uoguelph.ca

1999; Pryde and Louis 1999; Lebrun *et al.* 2001). In the *core X*- and *Y′*-elements, protosilencers coreside with sequences that have chromatin partitioning or antisilencing activities that are referred to as *s*ub*t*elomeric *a*ntisilencing *r*egions (STARs) (Fourel *et al.* 1999, 2004; Pryde and Louis 1999). Thus, silencing at telomeres seems to be regulated by a complex assembly of proto- and antisilencers that is not so well understood.

A most puzzling issue regarding gene silencing is its link to DNA replication. The establishment of silent chromatin requires passage through S-phase (Miller and Nasmyth 1984), but does not require DNA replication (Dubey *et al.* 1991; Kirchmaier and Rine 2001; Li *et al.* 2001). Furthermore, robust silencing at *HMR* does not take place until M-phase (Lau *et al.* 2002). Nevertheless, many of the genes that affect silencing both at telomeres and at *HM* loci encode for *bona fide* DNA replication factors such as *ORC2*, *ORC5*, minichromosome maintenance (*MCM*) *5*, *MCM10*, *CDC44*(RF-C), *CDC45*, and *POL30*(PCNA) (Ehrenhofer-Murray *et al.* 1995, 1999; Dillin and Rine 1997; Fox *et al.* 1997; Dziak *et al.* 2003; Suter *et al.* 2004; Liachko and Tye 2005).

We have shown that a mutation in *MCM5* (*mcm5-461*) derepresses multiple natural subtelomeric genes as well as reporter cassettes inserted next to telomeres (Dziak *et al.* 2003). To clarify the mechanism by which the *mcm5-461* allele disturbs silencing, we attempted to identify natural subtelomeric sequences that confer its effect. We also compared the effect of *mcm5-461* to that of mutations in several other replication genes. We show that replication factor mutants differentially affect silencing at telomeres. Interestingly, we show that nontelomeric *ACS* does not recapitulate the effects observed with natural telomeric *ACS*.

## MATERIALS AND METHODS

**Yeast strains and plasmids:** The strains used in this study are shown in supplemental Table 4 at http://www.genetics.org/supplemental/. All experiments were performed at the permissive temperatures as stated in the original studies in supplemental Table 4.

The GF2, GF3, GF6, GF9, GF10, GF44, GF46, and GF61 constructs are described in Fourel *et al.* (1999). URA3-tel (pURTEL) was described in Gottschling *et al.* (1990). *ARS1-URA3-tel* and *URA3-ARS1-tel* were produced by inserting the *Bam*HI/*Eco*RI fragment of pARS1wt (Marahrens and Stillman 1992) in the indicated positions in pURTEL. *ARS1ΔACS-URA3-tel*, *ARS1ΔABF1-URA3-tel*, GF6ΔACS, and GF44ΔACS were generated by site-directed mutagenesis of *ACS* and *ABF1-BS* in *ARS1-URA3-tel*, GF6, and GF44, respectively.

**Yeast transformation and fluoroorotic acid resistance assays:** Integrating constructs were produced by restriction digestion of the GF plasmids as described in Fourel *et al.* (1999) or by PCR. Cells were transformed and colonies were selected on SC/−Ura plates. For each measurement of percentage of fluoroorotic acid resistance (%FOA$^R$) three colonies were streaked on SC/FOA plates to confirm the variegated expression and then inoculated in 3 ml SC medium and diluted 1000 times into a fresh SC medium for 3 consecutive days. Serial 1:10 dilutions were prepared for each culture and 5-μl aliquots were spotted on SC and SC/FOA plates. The %FOA$^R$ cells was acquired as the average number of cells on SC/FOA plates divided by the average number of cells on SC plates. The numbers presented in supplemental Tables 1–3 at http://www.genetics.org/supplemental/ are average values ± standard error of *n* triplicate measurements with each strain/construct combination. Average values, error measurement, and ratios between %FOA$^R$ in different strains were calculated in Microsoft Excel.

Replication timing was estimated by the transient hemimethylation assay (Friedman *et al.* 1995). *Dam*I *methylase*-expressing yeast strains were generated by transforming cells with *Xho*I-linearized pDP6-Dam1 (Hoekstra and Malone 1985). Transformants expressing *Dam*I *methylase* and their genomic DNAs were tested for sensitivity to *Dpn*I. Cells at $OD_{600}$ = 0.3–0.4 were arrested at G2/M in YPD medium containing 15 μg/ml nocodazole [Sigma (St. Louis) M1404] for 3 hr. An aliquot of arrested cells was collected and the extent of arrest was determined by FACS and by microscope (>90% large budded, not shown). The cells were released from the arrest by resuspension in fresh media after washing twice with $H_2O$. Samples were harvested at 0, 25, 35, 45, 55, 65, 75, 85, 95, 105, and 125 min. Genomic DNA was isolated, digested with *Eco*RI or *Xho*I and *Dpn*I, respectively, separated on 0.8% agarose gels, and transferred to Nylon membranes. The membranes were probed with a PCR-generated probe to *ARS305* or with a 1.3-kb *Xho*I fragment *Y′*-probe (Brevet *et al.* 2003), respectively. Signals were acquired by a Typhoon phosphorimager (Amersham Biosciences, Arlington Heights, IL) and analyzed with Kodak ID image analysis software.

## RESULTS

**Experimental strategy:** The evaluation of the expression of a reporter gene (most frequently *URA3*) inserted in the subtelomeric region of a chromosome (most frequently in the *VII-L* telomere) is a routine assay for telomere position effect (TPE). At these positions *URA3* shows variegated expression—it is either fully transcribed or completely repressed. The proportion of cells that do not express *URA3* is used as an indirect measure of the extent of subtelomeric silencing. Because *URA3* expression renders the cells sensitive to FOA, the proportion of nonexpressing cells is assessed as % FOA$^R$.

To identify sequences that confer the effect of *mcm5-461* and other alleles on TPE, we selected nine previously published *URA3* reporter cassettes (Fourel *et al.* 1999) (Figure 1). These cassettes contain a telomere repeat [$TG_{(1–3)n}$] and different combinations of natural *Y′*- or *core X*- or STAR elements. For cross-reference, we kept the names of the constructs (GF2, GF3, etc.) as in the original article (Fourel *et al.* 1999). In Figure 1 we show the positions of *ACS* and the binding sites of Reb1p, Tbf1p, and Abf1p in the building blocks of these constructs.

All these constructs were integrated in the *VII-L* telomere of *mcm5-461*, *mcm5-1*, *orc2-1*, *orc5-1*, *cdc45-1*, *cdc7-1*, and *cdc6-1* and the corresponding isogenic wild-type strains. Variegated expression was confirmed by

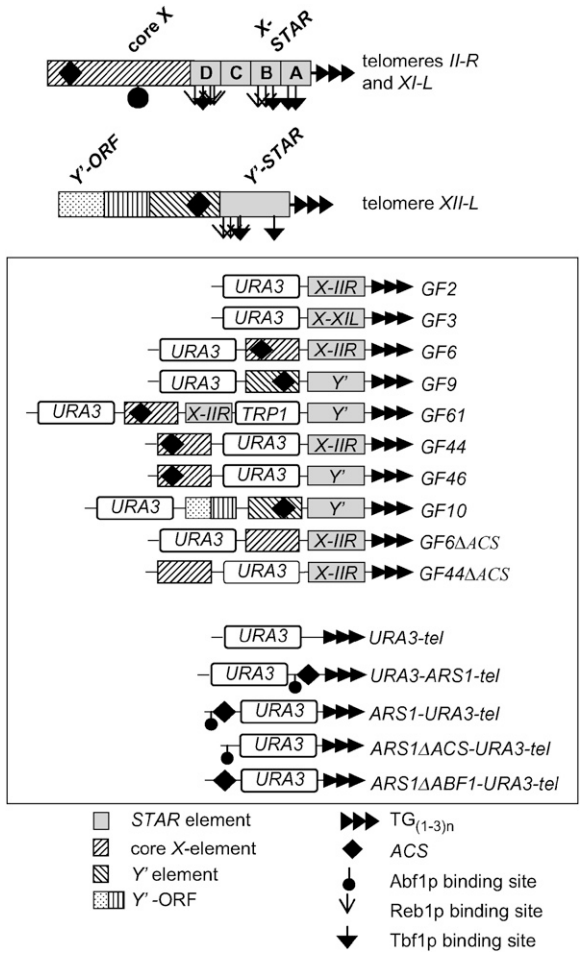


FIGURE 1.—Diagrams of the constructs used in this study. Maps (not to scale) of the *core X*-element at the *II-R* and *XI-L* telomeres and the *Y′*-element at the *XII-L* telomere are shown at the top. The positions of *STAR*, the *core X*- and *Y′*-elements, the telomeric $TG_{1-3}$ repeats, *ACS*, and the binding sites for Abf1p, Reb1p, and Tbf1p are indicated by different patterns and the symbols shown below. The combinations of these elements and the position of the *URA3* reporter in the different constructs are shown in the middle.

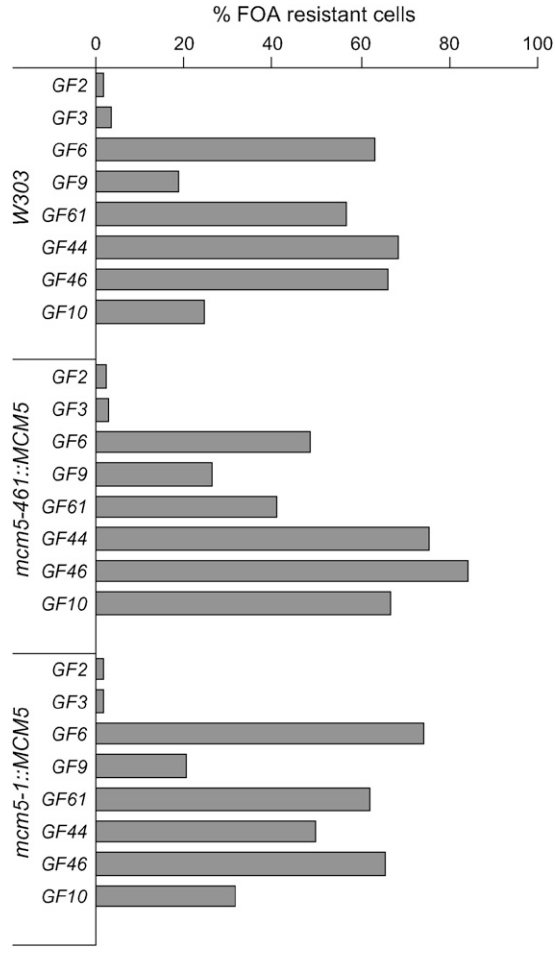


FIGURE 2.—Reporter constructs display similar repression in different wild-type strains. The GF series of constructs in Figure 1 were integrated in the wild-type *W303* and the merodipolid *mcm5-461::MCM5* and *mcm5-1::MCM5* strains and $\%FOA^R$ was measured in ($n$) triplicate measurements. Average $\%FOA^R$ cells for the different strain/construct combinations are plotted. Data are from supplemental Table 1 at http://www.genetics.org/supplemental/.

streaking the cells on SC/−Ura and SC/FOA plates. Subsequently, three colonies (per experiment) were picked and grown for >30 generations in nonselective medium to reach an equilibrium of variegated expression and to allow for the complete loss of any nonintegrated cassettes. Serial dilutions of these cultures were then spotted on SC and SC/FOA plates and the average $\%FOA^R$ was measured. Initial experiments showed that the three wild-type strains (*W303*, *mcm5-461::MCM5*, and *mcm5-1::MCM5*) impose similar levels of *URA3* repression in identical constructs (Figure 2). GF10 showed about two times higher repression in *mcm5-461::MCM5* (Figure 2). While we do not understand the reason for this dissimilarity, it appears on a background of comparable patterns of repression between all other constructs in the three strains (Figure 2). Hence, our reporter cassettes are subject to only minor strain-specific variations. We postulate that any significant variation in the repression of these constructs in mutant strains should be attributed to the mutant alleles.

Next, we calculated the average $\%FOA^R$ for each strain/construct combination in several ($n$) independent experiments (supplemental Table 1 at http://www.genetics.org/supplemental/). Finally, the ratio of $\%FOA^R$ in the mutant strain *vs.* the $\%FOA^R$ in the isogenic wild-type strain was calculated and plotted to represent the effect of different mutations on the silencing of *URA3* in different constructs (Figure 3, A–G). In these graphs, values below one signify derepression of *URA3* in the mutant strain, while values above one signify increased repression.

***orc2-1*, *mcm5-461*, and *mcm5-1* perturb silencing predominantly via *ACS*-containing *core X*- and *Y′*-elements:** We noted significant similarities in the effects of *mcm5-461*, *mcm5-1*, and *orc2-1* (Figure 3, A–C). These mutations

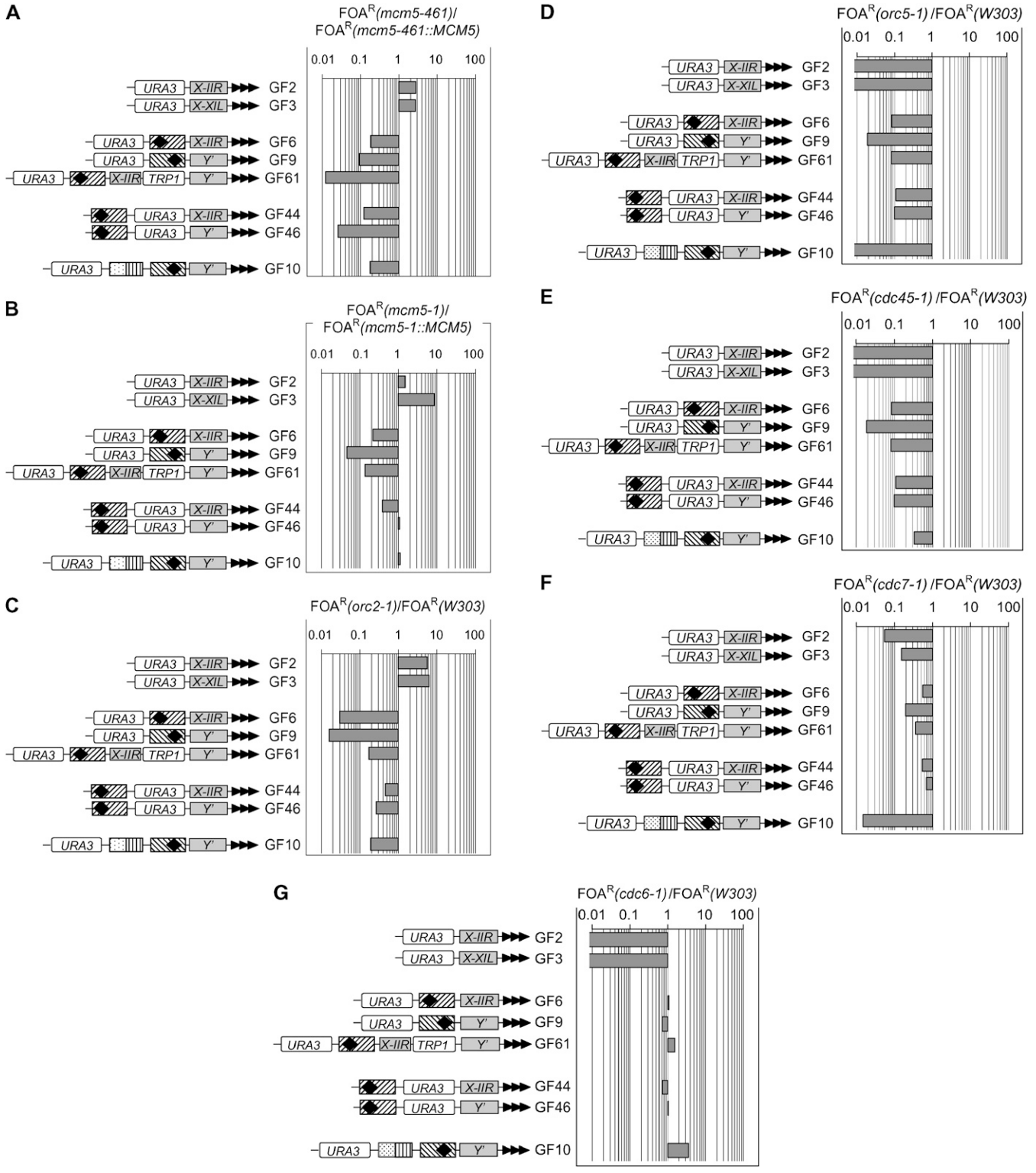


FIGURE 3.—Differential silencing of *URA3* in mutant strains. The GF constructs in Figure 1 were inserted in the *VII-L* telomere of *mcm5-461*, *mcm5-461::MCM5*, *mcm5-1*, *mcm5-1::MCM5*, *W303*, *orc2-1*, *orc5-1*, *cdc45-1*, *cdc7-1*, and *cdc6-1* strains and %FOA$^R$ was determined for each construct/strain combination. The graphs show the ratio of %FOA$^R$ in the mutant *vs.* %FOA$^R$ in the isogenic wild-type strain. Data are from supplemental Table 1 at http://www.genetics.org/supplemental/. The diagrams from Figure 1 are shown on the left. Values above one indicate increased repression of *URA3* in this construct in the mutant relative to the isogenic wild-type strain. Values below one indicate derepression. The following ratios are shown: (A) %FOA$^R$ *mcm5-461 vs.* %FOA$^R$ *mcm5-461::MCM5*; (B) %FOA$^R$ *mcm5-1 vs.* %FOA$^R$ *mcm5-1::MCM5*; (C) %FOA$^R$ *orc2-1 vs.* %FOA$^R$ *W303*; (D) %FOA$^R$ *orc5-1 vs.* %FOA$^R$ *W303*; (E) %FOA$^R$ *cdc45-1 vs.* %FOA$^R$ *W303*; (F) %FOA$^R$ *cdc7-1 vs.* %FOA$^R$ *W303*; (G) %FOA$^R$ *cdc6-1 vs.* %FOA$^R$ *W303*.

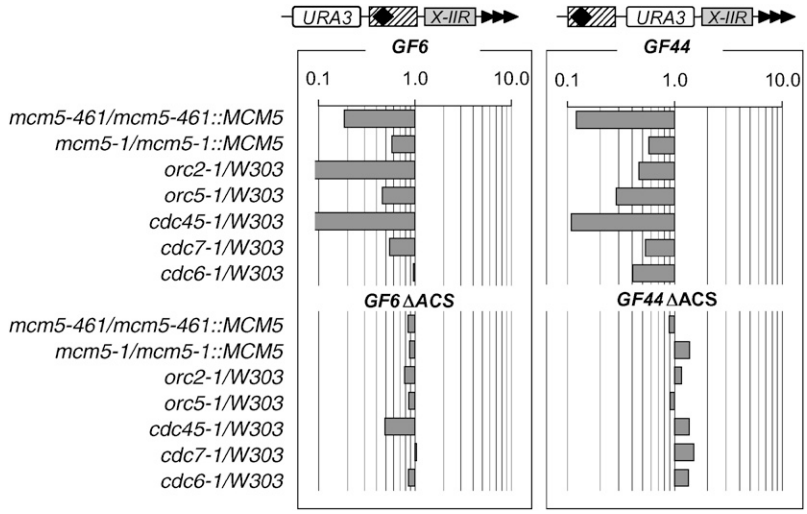


FIGURE 4.—Deletion of *ACS* in GF6 and GF44 abolishes the effect of mutations. The ratio of %FOA$^R$ in mutant *vs.* %FOA$^R$ in isogenic wild-type strains as indicated on the left was calculated from the numbers in supplemental Table 2 at http://www.genetics.org/supplemental/ and plotted. Each series of bars represents the calculations for the constructs shown at the top.

caused derepression of *URA3* in the constructs, which harbor the *ACS*-containing *core X*- and *Y′*-elements (GF6, GF9, GF61, GF44, GF46, and GF10). This effect was stronger in GF6, GF9, and GF61, in which the *ACS*-containing element was juxtaposed to *URA3* toward the telomere. The constructs, in which the *ACS*-containing element was positioned next to *URA3* toward the centromere (GF44, GF46) or was separated from *URA3* by additional *Y′*-sequences (GF10) showed a weaker effect in *mcm5-461* and *orc2-1* and almost no effect in *mcm5-1* (Figure 3, A–C). The results indicate that in these constructs *URA3* is exposed to *ACS*-emitted silencing signals, which are compromised in *orc2-1*, *mcm5-461*, and *mcm5-1*.

The two constructs that contained no *ACS* plus a STAR between the telomere and *URA3* (GF2, GF3) showed a moderate increase in silencing in *mcm5-461*, *mcm5-1*, and *orc2-1* (Figure 3, A–C). These data are consistent with the idea that a general genomewide decrease in *ACS*-mediated silencing could release factors that can engage in telomere-mediated silencing. The alternative interpretation is that *mcm5-461*, *mcm5-1*, and *orc2-1* have a direct positive effect on the STARs in constructs that are missing *ACS*. We favor the former idea because the MCM and ORC complexes associate with subtelomeric *ACS* (WYRICK *et al.* 2001) (M. A. REHMAN, unpublished data) and because we have no reasonable model to explain how they could work on *ACS*-less chromatin.

***orc5-1*, *cdc45-1*, and *cdc7-1* show *ACS*-independent effects:** *orc5-1*, *cdc45-1*, and *cdc7-1* caused significant derepression of *URA3* in all constructs (Figure 3, D–F). Remarkably, strongest derepression was observed in the *ACS*-less constructs (GF2, GF3) and the construct with the greatest distance between *ACS* and *URA3* (GF10). These data do not exclude a role of these alleles via *ACS*; however, they strongly imply that *orc5-1*, *cdc45-1*, and *cdc7-1* profoundly affect another step in silencing that is *ACS* independent.

***cdc6-1* does not affect silencing via *ACS*:** *cdc6-1* showed strong derepression of *URA3* in the *ACS*-less constructs (GF2, GF3) while the *ACS*-containing constructs were unaffected (Figure 3G). The results suggest that the *cdc6-1* allele does influence silencing, but this effect is independent of *ACS*.

*cdc6-1*, *orc5-1*, *cdc45-1*, and *cdc7-1* strongly to moderately decreased the repression of *URA3* in the GF2 and GF3 constructs, which contain only a STAR element next to the telomere (Figure 1). Considered separately, these data point to the possibility that the four wild-type genes counter the effect of STARs; however, the mechanism of this action remains completely unknown.

**The deletion of *ACS* precludes the effect of the mutations:** To evaluate the dependence of the observed effects on *ACS*, we destroyed *ACS* in two of the tested constructs, GF6 and GF44. They differ in the position of the *X*-element and *URA3* relative to the telomere (Figure 1). We inserted these constructs in the *VII-L* telomere and performed FOA$^R$ assays (supplemental Table 2 at http://www.genetics.org/supplemental/). We estimated the effect of each mutation by dividing the %FOA$^R$ cells in the mutants by the %FOA$^R$ cells in the corresponding wild-type strains (Figure 4). Consistent derepression (although at varying magnitude) of *URA3* in GF6 and GF44 in all mutants relative to wild-type strains was observed except for GF6 in *cdc6-1* (Figure 4, top graphs). The destruction of *ACS* strongly reduced (GF6ΔACS) or eliminated (GF44ΔACS) these effects with residual derepression still notable for GF6ΔACS in *cdc45-1* and *cdc7-1* (Figure 4, bottom graphs). These results clearly show that the effects of all mutants (with the exception of *cdc6-1*) are mediated by *ACS*. In conjunction with Figure 3, the data in Figure 4 provide the conclusion that *orc5-1*, *cdc45-1*, and *cdc7-1* affect silencing through the *ACS*, but also though another unknown mechanism.

**Nontelomeric *ACS* does not recapitulate the effects of *core X*- and *Y′-ACS*:** The *ACS* sequences within the *X*- and

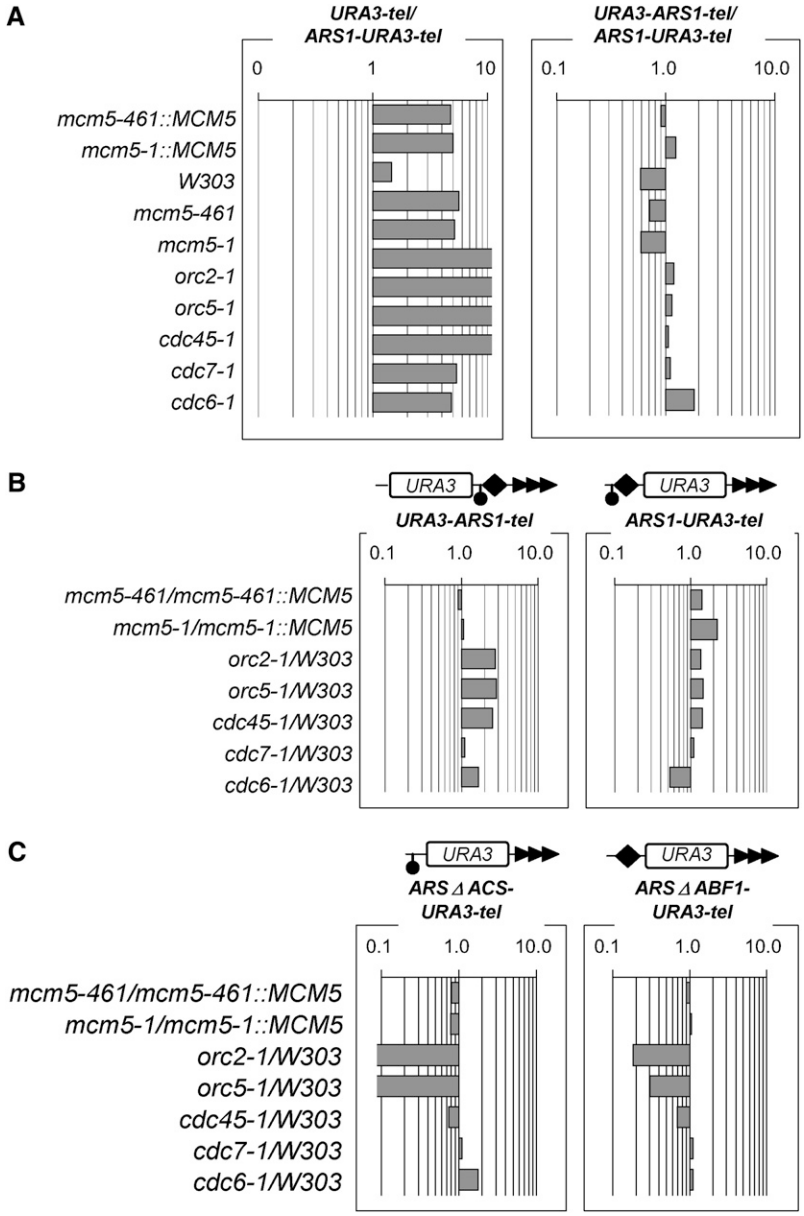


FIGURE 5.—Isolated nontelomeric *ACS* does not recapitulate the effects of mutations in the GF constructs. (A) The ratio of %FOA$^R$ in *URA-tel vs. ARS1-URA3-tel* and in *URA3-ARS1-tel vs. ARS1-URA3-tel* in the strains shown on the left was calculated and plotted. Data are from supplemental Table 3 at http://www.genetics.org/supplemental/. (B and C) The ratio of %FOA$^R$ in mutant *vs.* %FOA$^R$ in wild-type strains as indicated on the left was calculated and plotted from the numbers in supplemental Table 3. Each series of bars represents the calculations for the constructs shown at the top.

the *Y′*-elements are the obvious candidates that convey the effect of mutations in DNA replication factors on TPE. We therefore tested if isolated nontelomeric *ACS* could recapitulate these effects. We constructed five reporter cassettes containing *URA3*, a telomeric repeat, and the genomic *ARS1* (encompassing *ACS* through *ABF1-BS*) (MARAHRENS and STILLMAN 1992) (Figure 1). We must note that the consensus *ACS* and *ABF1-BS* in the *X*-element and in *ARS1* are not identical. *URA3-ARS1-tel* and *ARS1-URA3-tel* differ in the position of *ARS1* and *URA3* relative to the telomere (Figure 1). In *ARS1ΔABF1-URA3-tel* and *ARS1ΔACS-URA3-tel* the *ABF1-BS* and *ACS* were destroyed by site-directed mutagenesis. *URA3-tel* does not contain *ARS1* (Figure 1). These five constructs were integrated in the *VII-L* telomere of all mutant and isogenic wild-type strains and TPE assays were performed (supplemental Table 3 at http://www.genetics.org/supplemental/). As expected, *ARS1* acted as a protosilencer in all strains as exemplified by the moderate to strong increase in repression in *ARS1-URA3-tel* relative to *URA3-tel* (Figure 5A). We also show that the position of *ARS1* has little effect on *URA3* in all mutants tested (Figure 5B).

The impact of mutations in replication factors on the repression of these constructs was assessed by dividing the %FOA$^R$ in the mutant strains by %FOA$^R$ in the isogenic wild-type strains (Figure 5B). Surprisingly, despite the fact that the net effect of *ACS* in *URA3-ARS1-tel* and *ARS1-URA3-tel* is increased silencing (supplemental Table 3 at http://www.genetics.org/supplemental/), the mutations had very little or even a weak derepression effect in these constructs (Figure 5B). Even more, the destruction of *ACS* in *ARS1-URA3-tel* strongly decreased repression in *orc2-1* and *orc5-1* cells, while the

other mutants were indifferent to the omission of this element (Figure 5C). Corresponding, but weaker derepression was observed upon deletion of *ABF1-BS* (Figure 5C), arguing that the effect of these mutations was only partially mediated by *ACS*. For comparison, *orc2-1* and *orc5-1* did not affect repression in the GF6ΔACS and GF44ΔACS constructs (Figure 4) and most mutations reduced repression in GF6 and GF44 (Figure 4). The overall impression from these experiments was that isolated nontelomeric *ACS* responds quite differently to mutations in replication factors as compared to "natural" telomeric *ACS*.

**Repression in the GF constructs does not correlate with deregulation of the cell cycle or the replication timing of telomeres:** In an attempt to correlate the results obtained so far to a foreseeable deregulation of cell cycle or DNA replication, we directly compared the cell cycle distribution, minichromosome stability, and the replication timing of telomeres in the mutant strains.

Cell cycle distribution was evaluated in exponentially growing cells in rich medium at the permissive temperatures. We noted a moderate increase in the fraction of G2/M cells in *cdc6-1* and *mcm5-461* and a more substantial increase in *cdc45-1* (supplemental Figure 1 at http://www.genetics.org/supplemental/). There was also a moderate increase in the fraction of S-cells in *cdc7-1*. Apart from these differences, we did not note a correlation between the effect of these mutations on the cell cycle (supplemental Figure 1) and that on telomeric silencing (Figure 3). All strains demonstrated significant instability of minichromosomes (data not shown), but again failed to draw a parallel to the pattern of silencing.

Replication timing of telomeres in the different mutants was estimated by the transient hemimethylation assay (Friedman *et al.* 1995) in nocodazole-synchronized cultures. Briefly, the *Dam*I *methylase* gene was introduced in all strains and the cells were arrested in mitosis by exposure to 15 μg/ml nocodazole. The cells were then released and aliquots were taken at different time points. The genomic DNA from these samples was isolated and digested with *Dpn*I. Because *Dpn*I digests yeast DNA only if it is methylated by *Dam*I and because newly replicated DNA is not immediately methylated by *Dam*I, the detection of *Dpn*I-resistant genomic DNA indicates the time when this DNA is replicated (Friedman *et al.* 1995). We evaluated the timing of replication of two genomic loci, the early firing *ARS305* and telomeres (Figure 6). In all strains *ARS305* replicated between 45 and 55 min after the release from mitotic arrest. In most strains subtelomeric *Y′*-DNA was replicated ~90 min after the release from mitotic arrest. In *mcm5-461* the *Y′*-DNA was replicated 100 min after the release. Notably, *cdc45-1* did not display a distinct peak of hemimethylated DNA during the cell cycle, thus suggesting untimed replication of subtelomeric DNA (Figure 6).

Taken together, these data revealed no correlation between the effect of the mutations on silencing on the one hand (Figure 3) and that on cell cycle, minichromosome stability, or replication timing of telomeres on the other hand.

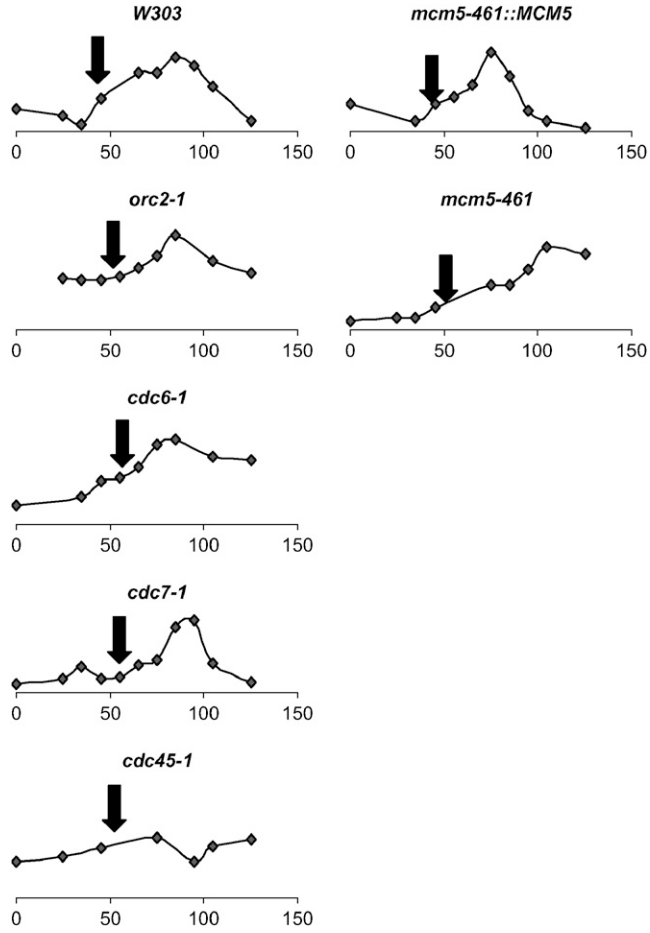


Figure 6.—Replication timing of telomeric DNA in different mutants. Each graph represents the relative abundance of *Dpn*I-resistant telomeric DNA in nocodazole synchronized cells that express *Dam*I methylase. The name of each strain is shown at the top of each graph. Time (minutes) after release of mitotic block is shown at the bottom. The arrow indicates the peak of abundance of *Dpn*I-resistant *ARS305* DNA. Details are provided in materials and methods.

## DISCUSSION

**DNA replication factors differentially regulate subtelomeric protosilencers:** The understanding of the relationship between silencing and DNA replication is a challenge. The establishment of silent chromatin requires a passage through S-phase (Miller and Nasmyth 1984), but the requirement for DNA replication *per se* has been ruled out (Dubey *et al.* 1991; Kirchmaier and Rine 2001; Li *et al.* 2001; Sharma *et al.* 2001). Nevertheless, the list of DNA replication factors that affect silencing has been steadily growing (Ehrenhofer-Murray *et al.* 1995, 1999; Dillin and Rine 1997; Fox *et al.* 1997; Dziak *et al.* 2003; Suter *et al.* 2004; Liachko

and Tye 2005). In many instances it is not clear if mutations in these factors affect silencing directly or indirectly through effects on DNA replication or cell cycle. Here we provide conclusive evidence regarding the mode of action of two of these genes and details on the roles of others.

First, we show that the *mcm5-461* and *mcm5-1* alleles share a high level of similarity with *orc2-1*. Previously, disruption of silencing at multiple genomic loci by the *orc2-1* allele was conclusively demonstrated (Rusche *et al.* 2003; Ramachandran *et al.* 2006). *mcm5-461* and *mcm5-1* as well as *orc2-1* reduced repression in the constructs, which contain *URA3* next to *ACS*-containing *core X*- or *Y′*-elements (Figure 3, A–C). In support of *ACS*-dependent function, we show that in *mcm5-461*, *mcm5-1*, and *orc2-1* the deletion of *ACS* in GF6 and GF44 abolished the effect of these mutations (Figure 4). Altogether, we present evidence that, similar to *ORC2*, *MCM5* is directly involved in *ACS*-mediated silencing at telomeres. Most, if not all of the effects of these two genes on silencing are via *ACS*. In constructs lacking *ACS* (GF2 and GF3) these mutations produced a moderate increase in silencing (Figure 3, A–C). These results are consistent with a genomewide deficiency in the *ACS*-mediated silencing, which in turn releases and redirects silencing factors to the telomere. However, the proposed scenario may not be universal. For example, a telomeric *URA3* reporter without a STAR element (*URA-tel*) was actually derepressed in *orc2-1* and *orc5-1*, but not in *mcm5-461* mutants (supplemental Table 3 at http://www.genetics.org/supplemental/). Hence, the response of repressed genes at ACS-independent locations may vary in different mutants.

Our second finding is that *CDC6* (or at least the *cdc6-1* allele) regulates silencing independently of *ACS*. Cdc6p is essential for the loading of the MCM complex on ORC-bound *ACS* at origins of replication (Blow and Dutta 2005). The *cdc6-1* allele impairs DNA replication (Weinreich *et al.* 2001; Tsuyama *et al.* 2005). However, it selectively caused derepression only in the *ACS*-less GF2 and GF3 constructs, while having negligible effects on the *ACS*-containing constructs (Figure 3G). In agreement with our results, derepression of genes in the nontelomeric *DAL* gene cluster on chromosome *IX* in the *cdc6-1* mutant occurs independently of several nearby *ACS* (W. Burhans, personal communication). This behavior of *cdc6-1* contrasts with the derepression of the same genes by the *orc2-1* mutation, which is *ACS* dependent (Ramachandran *et al.* 2006). The experiments in Figure 3G and this unrelated set of data reinforce the conclusion that *cdc6-1* affects silencing, however, in an *ACS*-independent fashion.

Our data do not allow unambiguous conclusions about the modes of action of *ORC5*, *CDC45*, and *CDC7*. *orc5-1*, *cdc45-1*, and *cdc7-1* decreased silencing in all constructs regardless of the presence of *ACS* (Figure 3, D–F). The trivial interpretation of these data is that, like *cdc6-1*, the three alleles do not affect silencing via *ACS*. However, the deletion of *ACS* in GF6 and GF44 reduced repression in these mutants (Figure 4), arguing in favor of *ACS*-dependent function. The most likely scenario is that *orc5-1*, *cdc45-1*, and *cdc7-1* act via *ACS*, but the specificity of this action is masked by effects on other *ACS*-independent process(es). We can only speculate on the nature of these *ACS*-independent processes. For example, a recent article shows extensive crosstalk between DNA replication/silencing factors and genes, which regulate sister chromatid cohesion or histone acetylation (Suter *et al.* 2004). It is possible that *orc5-1*, *cdc45-1*, and *cdc7-1* disturb the links to cohesins or some unknown feature of the maintenance of the silent chromatin state in addition to decreasing the efficiency of silencing emitted by ORC/MCM complexes on *ACS*.

***ACS* are not created equal:** We attempted to recapitulate the effects of mutations in replication factors in a more defined experimental system. We used isolated *ACS* and *ABF1-BS* derived from *ARS1* as opposed to the *ACS* and *ABF1-BS* in the subtelomeric *core X*- and *Y′*-elements. We stress that the consensus *ACS* and *ABF1-BS* in *ARS1* differ from the ones in *core X*. The *ARS1* containing constructs (Figure 5) and the *core X* containing GF6 and GF44 (Figure 4) provide a basis for comparison between these two types of *ACS*. We noted that the telomere- and *ARS1*-derived *ACS* display extensive dissimilarities in terms of telomeric silencing (Figures 4 and 5). The results suggest that subtelomeric *ACS* are not functional replicas of origin *ACS* and their action as protosilencers is not subject to the same regulatory mechanisms. This idea is in agreement with Palacios DeBeer *et al.* (2003), who demonstrate that origin and *HM* silencer *ACS* differ in their affinity for the ORC and that this difference reflects the preferential function of the corresponding *ACS* in silencing or origin firing. We propose that an additional functional dissimilarity of these two types of *ACS* is their dependence on the *cdc6-1* allele (Figure 3) (Weinreich *et al.* 2001; Tsuyama *et al.* 2005). It is possible that the surrounding sequence of *core X*- and *Y′*-elements influences the *ACS* silencing specificity. More focused work is needed to address in detail how origin and subtelomeric *ACS* differ.

**Concluding remarks:** There is an extensive overlap of proteins, which work in both replication and silencing. Many of these bind *ACS* or regulate the function of *ACS*-bound proteins (Rusche *et al.* 2003). In DNA replication, these proteins control origin licensing and firing (Rusche *et al.* 2003). It remains unclear how the same proteins work in silencing. Here we show that *ACS*-related factors require different subtelomeric elements for their silencing effects and are not uniform *ACS*-dependent repressors. We support the idea that at least some of these factors work directly on the silenced domains rather than affecting them indirectly via DNA replication or the cell cycle.

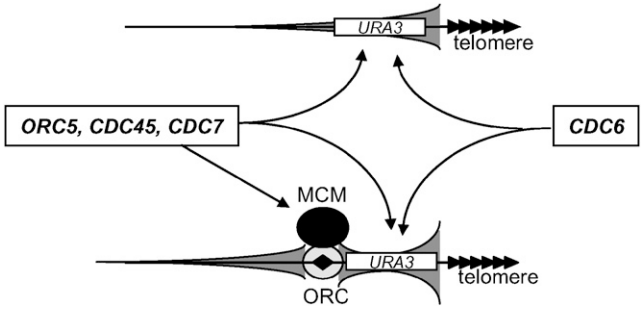


FIGURE 7.—A model for *ACS*-mediated silencing at yeast telomeres. The array of solid triangles represents the telomeric repeat. The solid diamond represents *ACS*. The oval with light shading over *ACS* represents the ORC. The solid oval represents MCM proteins. *URA3* is shown as a rectangle. The elongated cones with dark shading represent areas and levels of repression. In the absence of protosilencers the only silencing element is the telomere. Its activity is independent of ORC and MCM proteins. Subtelomeric *ACS* protosilencers require the binding of ORC and MCM proteins.

We propose that the MCM proteins (or at least Mcm5p) are recruited to subtelomeric *ACS*, where together with the ORC they build up a protosilencer (Figure 7). The protosilencer activity is directly influenced by Orc5p, Cdc45p, and Cdc7p. However, these three proteins also modulate silencing independently of *ACS* via other processes (Figure 7). Finally, Cdc6p does not participate in the regulation of protosilencers. Still, Cdc6p can control silencing via some unknown *ACS*-independent mechanism. Future studies should address how these genes are involved in telomeric silencing.

We thank J. Rine, B. Stillman, P. Pasero, B. Duncker, and S. Jacobson for the generous donation of strains and plasmids. This study was supported by a grant to K.Y. from National Science and Engineering Research Council (Canada) and to E.G. from the Ligue Nationale Contre le Cancer (France).

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Communicating editor: B. J. Andrews