The Origin Recognition Complex, *SIR1*, and the S Phase Requirement for Silencing

Catherine A. Fox,* Ann E. Ehrenhofer-Murray, Stephen Loo, Jasper Rine†

Silencing of transcription in *Saccharomyces cerevisiae* has several links to DNA replication, including a role for the origin recognition complex (ORC), the DNA replication initiator, in both processes. In addition, the establishment of silencing at the *HML* and *HMR* loci requires cells to pass through the S phase of the cell cycle. Passage through S phase was required for silencing of *HMR* even under conditions in which ORC itself was no longer required. The requirement for ORC in silencing of *HMR* could be bypassed by tethering the Sir1 protein to the *HMR-E* silencer. However, ORC had a Sir1-independent role in transcriptional silencing at telomeres. Thus, the role of ORC in silencing was separable from its role in initiation, and the role of S phase in silencing was independent of replication initiation at the silencers.

Silencing is a form of transcriptional repression that involves the assembly of specialized, heritable structures of chromatin confined to certain domains within chromosomes. ORC, the eukaryotic replication initiator (*1*), has a role in silencing the cryptic mating-type loci *HMR* and *HML* of *Saccharomyces cerevisiae* (*2*, *3*). This finding links a protein responsible for the initiation of DNA replication at chromosomal origins with the assembly of repressive domains of chromatin.

Silencing of *HMR* and *HML* requires regulatory sites called silencers that flank both loci. Silencing also requires proteins that directly bind silencers, such as ORC, as well as the core nucleosome proteins histone H3 and H4 and the four Sir proteins (*4*). The *HMR-E* silencer, the most thoroughly characterized of the four silencers, is both necessary and sufficient for repressing gene expression at *HMR*. *HMR-E* consists of binding sites for ORC, Rap1, and Abf1 proteins, each of which contributes to silencer function (*5*). Furthermore, *HMR-E* contains two ORC binding sites (autonomous replication consensus sites, ACSs) and several near matches to the ORC binding site (*4*). A synthetic silencer consisting of a single binding site for ORC, Rap1p, and Abf1p is fully functional in silencing (*6*).

In addition to the role of ORC in replication and silencing, several other observations suggest a connection between DNA replication and transcriptional silencing in yeast. First, both *E* and *I* silencers at *HML* and *HMR* promote the replication of plasmids on which they reside (*7*), and at least two silencers, *HMR-E* and *HMR-I*, are bona fide chromosomal origins of replication (*8*). Second, passage through the S phase of the cell cycle is required to silence *HMR* and *HML* (*9*). A simple model that would unite these observations is that ORC's role in silencing is synonymous with the S phase requirement to establish silencing.

However, the simplest form of this model is inadequate because certain alleles of *ORC1* and *ORC5* can function in replication initiation but not silencing (*3*, *10*). In addition, ORC has a role in silencing outside the S phase of the cell cycle (*3*). Thus, ORC has a role in silencing beyond its role as a replication initiator, and ORC's role in replication is not sufficient for silencing. However, these observations do not address whether ORC's function as a replication initiator at *HMR-E* is necessary for silencing or whether ORC contributes the S phase requirement for silencing.

To address the role of ORC in silencing, we first determined whether tethering Sir1p to the silencer could bypass the requirement for ORC in silencing *HMR*. Second, we examined whether establishment of the silenced state with a tethered Sir1 protein bypassed the S phase requirement for silencing. Third, we determined whether ORC could function in silencing without functioning as a replication initiator. Last, we determined whether ORC had a role in silencing that was independent of Sir1p.

Fusion proteins in which a protein of interest is joined to a discrete and unrelated DNA binding domain provide a versatile way of tethering proteins to particular DNA sequences in chromosomes. Previous studies established that a fusion protein consisting of the DNA binding domain of the Gal4 protein, which has no role in silencing, joined to the complete sequence of the Sir1 protein complemented the silencing defect of a *sir1* mutant and, when tethered at *HMR-E*, could silence *HMR* (*11*). However, the interpretation of this experiment was limited with respect to the role of ORC in silencing, because the experiments were performed in Orc^+ cells and in the presence of a functional ORC binding site in the *HMR-I* silencer, which is itself an origin of replication (*8*). To extend these experiments, we constructed a new Gal4-Sir1p fusion protein (*12*) that complemented *sir1* mutations and expressed the protein in cells with synthetic derivatives of the *HMR-E* silencer that contained one, three, or five Gal4 binding sites in place of the ACS (*13*). We refer to these silencers as NxGal4-RAP-ABF. These mutant silencers, when substituted for the wild-type *HMR-E* silencer in the chromosome, completely abolished silencing in cells with a wild-type *SIR1* gene, but efficiently silenced *HMR* in cells expressing *GAL4-SIR1*, as measured by quantitative mating assays that can detect the expression of **a** genes at *HMR* in a *MATα* strain (Table 1) (*14*). As expected, this silencing required the function of the other three Sir proteins, as established previously for Gal4-Sir1p–targeted silencing (*11*). However, in contrast to previous studies, the *HMR-I* silencer was deleted in this study to avoid any potential complications, and the Gal4-Sir1 protein was still able to silence *HMR*. In control experiments with strains containing mutant silencers lacking Gal4 binding sites, Gal4-Sir1p was unable to mediate silencing (*14*). Thus, silencing that depended on a Gal4-Sir1p fusion was extremely efficient and independent of any ORC binding sites at *HMR*.

Sequence-specific DNA binding proteins can sometimes exert an effect on genes lacking a binding site for those proteins. Perhaps the best known example is the dependence of genes that lack TATA elements in their promoters on the function of the TATA binding protein for their transcription (*15*). To examine whether ORC played a role in silencing at silencers lacking an ACS, we determined whether silencing mediated by Gal4-Sir1p functioned in *orc2-1* or *orc5-1* cells. In the presence of Gal4-Sir1p, neither *orc2-1* nor *orc5-1* caused a significant defect in silencing mediated by Gal4-Sir1p at the 5xGal4-RAP-ABF synthetic silencer (Table 1). In contrast, in strains containing a synthetic silencer with ORC, Rap1, and Abf1 binding sites, either *orc2-1* or *orc5-1* caused approximately a 100-fold loss of silencing (*3*). Thus, silencing achieved by tethering a Gal4-Sir1p directly to the silencer bypassed the requirement both for ORC binding sites

Department of Molecular and Cell Biology, Division of Genetics, 401 Barker Hall, University of California, Berkeley, CA 94720, USA.

*Present address: Department of Biomolecular Chemistry, University of Wisconsin, 1300 University Avenue, Madison, WI 53706, USA.

†To whom correspondence should be addressed. E-mail: jasper@mendel.berkeley.edu

in the silencer and for ORC function.

The simplest interpretation of these data was that Gal4-Sir1p bypassed the requirement both for ORC and for replication initiation at the silencer for silencing. An alternative though unprecedented possibility was that the Gal4-Sir1p itself caused replication initiation at Gal4 binding sites. To investigate this possibility, we examined replication initiation at the 5xGal4-RAP-ABF silencer using two-dimensional origin-mapping gels (Fig. 1). As shown previously (*8*), the synthetic *HMR-E* silencer functioned as a chromosomal origin. In contrast, the synthetic silencer in which the ACS has been replaced with five Gal4 binding sites failed to function as an origin of replication in cells with Gal4-Sir1p. Taken together, these data indicated that Gal4-Sir1p–dependent silencing was independent of both ORC and of replication initiation at *HMR*.

A classic silencing study showed that establishment of silencing at *HML* and *HMR* could occur in cells that passed from G_1 to the beginning of M phase, but could not occur in cells that passed from G_1 to early S phase of the cell cycle (*9*). These observations were interpreted as evidence of an S phase requirement for silencing, although a possible G_2 role could not be excluded. One hypothesis to explain these observations is that ORC and replication initiation at silencers define the S phase dependence for establishment of silencing.

We tested this hypothesis by determining whether silencing still required passage through S phase under conditions that bypassed the requirement for ORC. For this purpose, reciprocal shift experiments analogous to those in the earlier study (*9*) were performed. The rationale of our experiments was to arrest cells that were not expressing Gal4-Sir1p in one phase of the cell cycle, induce the synthesis of Gal4-Sir1p, and then allow the cells to proceed to a block in another phase of the cell cycle. We evaluated silencing by measuring the amount of **a**1 mRNA transcribed from *HMR* at the second block. The exceedingly short half-life of the **a**1 mRNA (<3 min) (*9*) allowed us to monitor rapid changes of silencing. For these experiments, expression of *GAL4-SIR1* from the *MET3* promoter (*16*) provided a regulated source of Gal4-Sir1p, allowing silencing of the **a**1 gene at *HMR* in a conditional manner (Fig. 2A, lanes 1 and 2).

We first examined whether silencing could be achieved by passage through S phase under these conditions. Cells lacking Gal4-Sir1p were arrested in early S phase with hydroxyurea, an inhibitor of ribonucleotide reductase (*17*). Gal4-Sir1p was induced and the cells were then released from this block and rearrested in M phase with nocodazole, an inhibitor of mitotic spindle formation (*18*). This protocol resulted in silencing of *HMR* (Fig. 2A, lane 5), thus confirming the earlier observation that silencing could be established in cells that passed through S phase. This silencing was noteworthy both by its completeness and its rapidity. In the absence of Gal4-Sir1p, *HMR* failed to be silenced (Fig. 2A, lane 3), demonstrating that passage through S phase alone did not cause silencing. In addition, releasing the cells without subsequent block, either in the presence or absence of Gal4-Sir1p, revealed that the M phase block itself did not affect the outcome (Fig. 2A, lanes 4 and 6). Hence, the ORC-independent silencing mediated by Gal4-Sir1p could occur if cells passed through S phase.

We next examined whether silencing could be established in the reverse situation, when cells passed from M phase to early S phase. Cells lacking Gal4-Sir1p were arrested in M phase, Gal4-Sir1p was induced, and then cells were released and rearrested in early S phase. In contrast to the previous experiments, transit through the cell cycle from the beginning of M phase to the beginning of S phase was insufficient to silence *HMR* (Fig. 2A, lane 9). Cells that continued beyond the S phase block silenced *HMR*, presumably because they passed through S phase (Fig. 2A, lane 10). No silencing occurred in the absence of Gal4-Sir1p (Fig. 2A, lanes 7 and 8), regardless of whether the cells were blocked in S phase, confirming that passage through the cell cycle did not affect silencing. Thus, the Gal4-Sir1p–dependent silencing could not be established during passage from M phase to the beginning of S phase. Therefore, this ORC-independent silencing still required passage through S phase.

A potential limitation to interpreting these data was the presence of an ORC binding site at both the synthetic *HMR-E* silencer used in this experiment and at the *HMR-I* silencer on the opposite side of *HMR*. Therefore, an identical set of experiments was performed in a strain lacking the *HMR-I* element altogether and lacking the ORC binding site at *HMR-E*. The results from this experiment paralleled those from the previous experiment (Fig. 2B). Therefore, silencing mediated by Gal4-Sir1p was ORC-independent but still required passage beyond early S phase.

One trivial explanation for the inability of these cells to establish silencing when prevented from passing through S phase is the lack of *GAL4-SIR1* expression under these conditions. To examine this possibil-

Table 1. Tethering Sir1p, Orc2p, or Orc5p to the *HMR-E* silencer provided silencing at *HMR*. The efficiency of the tethered proteins in silencing *HMR***a** was measured by determining the mating efficiency of a *MAT*α strain with the 5xGal4-RAP-ABF silencer (JRY4981; *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 gal4*Δ*::HIS3*) and its isogenic derivatives (JRY4986 for *orc2-1*, JRY4987 for *orc5-1*, and JRY4983 for *sir2*Δ*::LEU2*) with the indicated mutations when transformed with the denoted plasmid (pJR1205 for Gal4, pCF117 for Gal4-Sir1, pJR1640 for Gal4-Orc2, and pJR1641 for Gal4-Orc5).

Plasmid	Relevant genotype	Mating efficiency
Gal4(1–147)	Wild type	$<1 \times 10^{-5}$
Gal4(1–147)-Sir1p	Wild type	0.8
Gal4(1–147)-Sir1p	*orc2-1*	0.41
Gal4(1–147)-Sir1p	*orc5-1*	0.61
Gal4(1–147)-Sir1p	*sir2*Δ*::LEU2*	$\sim 10^{-6}$
Gal4(1–147)-Orc5p	Wild type	0.12
Gal4(1–147)-Orc2p	Wild type	0.02
Gal4(1–147)-Orc5p	*sir1*Δ*::LEU2*	5×10^{-5}

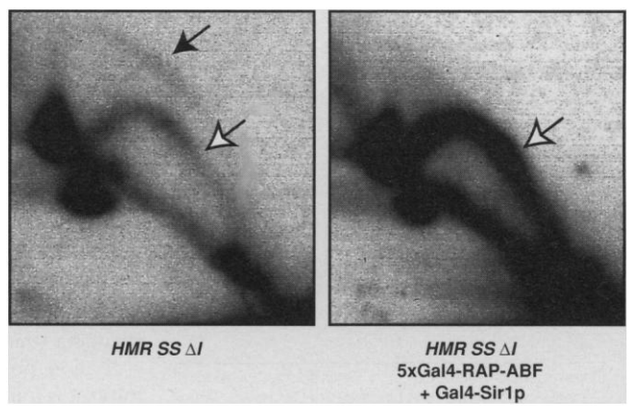

Fig. 1. Gal4-Sir1p did not cause replication initiation at a synthetic *HMR-E* silencer consisting of five tandem Gal4 binding sites, a Rap1 binding site, and an Abf1 binding site (5xGal4-RAP-ABF). A Hind III–Bgl II *HMR* fragment containing this silencer was analyzed for the presence of replication intermediates through use of two-dimensional origin-mapping gels (*32*) as described (*3*). The black arrow indicates bubble-shaped replication intermediates, which result from replication initiation on the fragment. The white arrows denote fork-shaped intermediates, which arise from replication by an origin lying outside of the fragment. The strains used were JRY4473 (*MAT*α *HMR-SS ΔI ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and JRY4806 (JRY4473, *HMR-SS ΔI*, 5xGal4-RAP-ABF) carrying pJR1815 (Gal4-Sir1p under control of the *ADH1* promoter).

ity, we quantitated mRNA levels of *GAL4-SIR1* and normalized them to those of the *SCR1* gene (*19*), whose expression is unaffected by changes in silencing or the cell cycle. This analysis showed that *GAL4-SIR1* expression could be induced regardless of the position of the cells in the cell cycle (*14*).

Because tethering Sir1p to the silencer bypassed ORC's role in silencing *HMR*, perhaps the chief role of ORC in silencing was to recruit Sir1p to the silencer, as recently suggested (*20*). There were two attractive models for how ORC might recruit Sir1p. The simplest model was through direct protein-protein interactions. A more complex model was that initiation of replication might be required to recruit Sir1p, perhaps through some interaction between Sir1p and a component of the replication machinery. These models could be tested if ORC could be bound to a silencer in some manner that prevented it from initiating replication. The ability of such an ORC to mediate Sir1p-dependent silencing would support the first model, whereas an inability to silence would support the second model.

To determine whether ORC could mediate silencing in the absence of a functional origin, we fused the entire *ORC2* or *ORC5* sequence to the Gal4p DNA binding domain (*21*). Both Gal4-Orc fusion proteins complemented the temperature sensitivity and the silencing defects of their respective *orc* mutation, establishing that both fusion proteins were functional (*14*). Both Gal4-Orc2p and Gal4-Orc5p caused silencing at *HMR* in the strain with the 5xGal4-RAP-ABF synthetic silencer (Table 1). Gal4-Orc5p was somewhat more effective at silencing in this context than was Gal4-Orc2p, and neither ORC fusion was as effective as Gal4-Sir1p. This difference in silencing capability was not a function of expression levels of the fusion proteins as judged by immunoblots with antibodies directed against the Gal4 DNA binding domain (*14*). Moreover, the efficiency of silencing was proportional to the number of Gal4 binding sites present in the silencer. Nevertheless, both Gal4-Orc fusion proteins restored a significant amount of silencing, as reflected in the 10^4 or better increase in mating efficiency. Thus, either Orc2p or Orc5p could function in silencing *HMR* in the absence of an ACS if tethered to the *HMR-E* silencer through an unrelated DNA binding domain.

At all yeast origins examined, replication initiation requires an intact ACS (*22*). Nevertheless, it was conceivable that the Gal4-Orc fusion proteins could initiate replication at Gal4 binding sites even in the absence of an ACS. However, no initiation of replication was observed at the 5xGal4-RAP-ABF synthetic silencer, whether or not the Gal4-Orc2 fusion protein was expressed (Fig. 3). Therefore, as with Gal4-Sir1p, both the Gal4-Orc2p and the Gal4-Orc5p could silence *HMR* if tethered to the silencer, and at least in the case of Gal4-Orc2p, this silencing occurred in the absence of replication initiation. Thus, under these conditions, ORC could function in silencing without acting as a replication initiator.

If ORC were to recruit Sir1p to the silencer, then silencing caused by either Gal4-Orc2p or Gal4-Orc5p would still require Sir1p. Indeed, this silencing required Sir1p because it was abolished in cells containing a mutant allele of *SIR1* (Table 1). Thus, if ORC recruits Sir1p to the silencer, then it can do so independently of replication initiation.

Telomeric silencing requires several of the same proteins required to silence *HMR* and *HML*. The most significant distinction between the two types of silencing is that telomeric silencing does not require Sir1p, whereas silencing of *HML* and *HMR* does (*23*). If the only function of ORC in silencing were to recruit Sir1p to the silencers (*20*), then mutations in *orc2-1* and *orc5-1* should not affect telomeric silencing. On the other hand, if mutations in *ORC* genes decrease telomeric silencing, then ORC

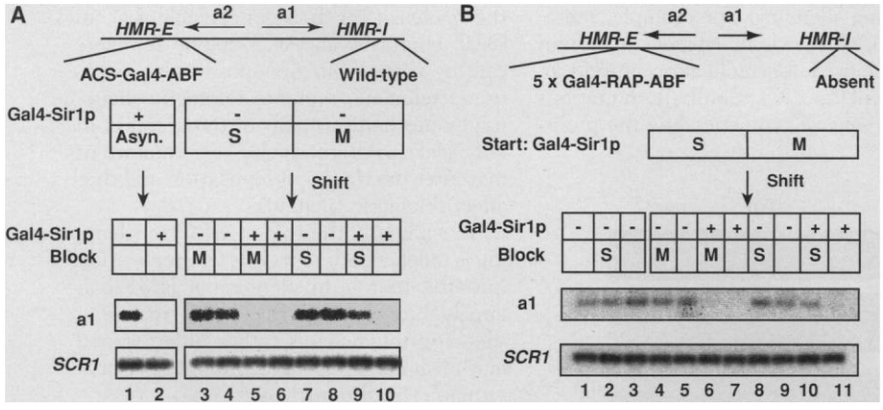

Fig. 2. Gal4-Sir1p–mediated silencing required passage through S phase. (**A**) Strain JRY5278 , which carried a synthetic silencer allele with the Rap1 binding site replaced by a Gal4 binding site, was transformed with a plasmid containing *GAL4-SIR1* under control of *MET3* (pJR1811). JRY5278 with pJR1811 was grown in selective medium to an absorbance at 600 nm (A_{600}) of 0.5 in the presence or absence of 50 μM methionine (Start). Cells were harvested and suspended at an A_{600} of 0.25 either in hydroxyurea-containing medium (0.2 M; S), in nocodazole-containing medium (10 μg/ml; M), or in drug-free medium (open boxes). Cells were allowed to arrest at 23°C until the arrest was complete (~3 hours). For induction of *GAL4-SIR1* expression, cells were harvested, washed, and incubated for 30 min in media containing the appropriate drug, but without methionine. They were then allowed to proceed to the next block by incubation for 3.5 hours in the appropriate medium. Total yeast RNA was prepared as described (*3*). RNA blot hybridization was performed with probes for **a**1 and, as a loading control, with a probe for *SCR1*. (**B**) The identical experiment as above was performed with a strain containing 5xGal4-RAP-ABF at the *HMR-E* silencer and lacking *HMR-I* (JRY4806).

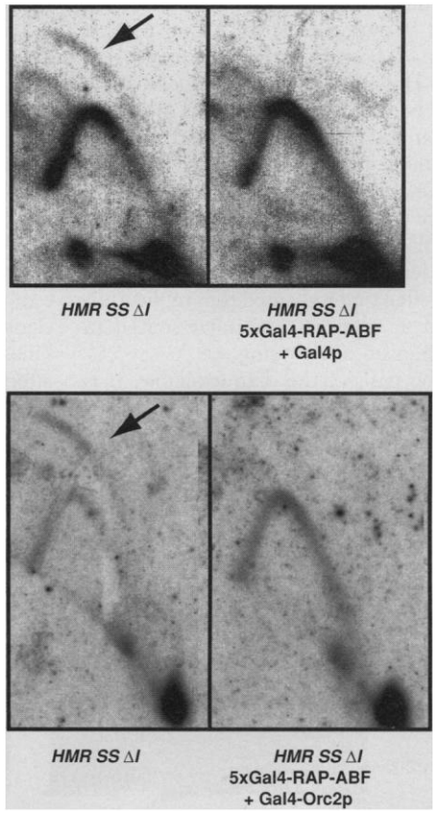

Fig. 3. Gal4-Orc2 did not cause replication initiation at the 5xGal4-RAP-ABF *HMR*-E silencer. Two-dimensional origin mapping was done as in Fig. 1. The strains were JRY4473 (*HMR SS ΔI*), JRY4981 pJR1205 (*HMR SS ΔI*, 5xGal4-RAP-ABF *gal4Δ::HIS3* + Gal4), and JRY4981 pJR1600 (*HMR SS ΔI*, 5xGal4-RAP-ABF *gal4Δ::HIS3* + Gal4-Orc2).

must have a second and *SIR1*-independent role in silencing.

Silencing of the *TRP1* gene placed near a synthetic telomere on the left end of chromosome VII (*24*) was evaluated by the ability of cells containing this gene to grow in the absence of exogenous tryptophan. Telomeric silencing of the *TRP1* gene required Sir2p but not Sir1p (Fig. 4), consistent with earlier studies (*23*). In fact, in the absence of Sir1p, telomeric silencing improved slightly in these experiments. In contrast, both *orc2-1* and *orc5-1* cells were defective in telomeric silencing (Fig. 4), although less defective than *sir2Δ* cells. Because ORC was required for telomeric silencing whereas Sir1p was not, ORC had two different roles in silencing, one that was *SIR1*-dependent and another that was *SIR1*-independent.

The experiments presented here critically examined possible mechanisms of how ORC, the replication initiator, contributes to silencing. The most salient feature of ORC's contribution was its independence of replication initiation at the silencer. This result was surprising both because of ORC's well-documented role in replication initiation and because the *HMR-E* and *HMR-I* silencers are both bona fide origins of replication. However, these data resolved the disparity between the replication initiation evident at the *HMR* silencers and the lack of detectable initiation at *HML* silencers (*25*). The ability of the Gal4-Orc2p fusion to silence when tethered to the 5xGal4-RAP-ABF synthetic silencer, and the ORC-independence of tethered Sir1-dependent silencing indicated that replication initiation at silencers was not essential to the mechanism of silencing.

On the basis of the data available, it is conceivable that the only role of ORC at *HMR-E* is the recruitment of Sir1p to the silencer. For example, the silencing mediated by a tethered ORC still required Sir1p function, but the silencing mediated by tethered Sir1p did not require ORC function. These data provide functional significance to the interactions between ORC and Sir1p detected in a two-hybrid interaction (*20*). However, it is unlikely that ORC alone recruits Sir1p to the silencer, because every origin binds ORC, but few if any other origins repress expression of adjacent genes.

With tethered Sir1p at the *HMR-E* silencer and no need for ORC in silencing, repression of *HMR* still required passage through S phase. This aspect of silencing distinguishes it from many other types of gene regulation that display no cell cycle dependence. In fact, silencing could be established only in cells that progressed beyond the hydroxyurea block in early S phase. This result confirmed the earlier studies (*9*) and extended them by the finding that the dependence was not related to ORC function or replication initiation at the silencer. Formally, neither this study nor the preceding one has excluded the cell cycle requirement in silencing as being in G_2 rather than S. In principle, this issue should be resolvable with temperature-sensitive alleles that arrest cells in different stages of the cell cycle. However, such experimental conditions have proven difficult to exploit, as silencing per se is affected by temperature (*26*).

Nevertheless, the available data favor a role in S phase rather than in G_2 phase for establishing silencing. For example, mutations in *CDC7*, which encodes a protein kinase required for replication initiation, affect silencing (*27*). Similarly, mutations in the *Drosophila* gene encoding the proliferating cell nuclear antigen (PCNA), a processivity factor for replication, affect heterochromatic gene inactivation (*28*). If S phase is critical for establishment of silencing, what aspect of S phase is involved?

Silencing involves the assembly of a specialized repressive chromatin structure (*29*). The passage of a replication fork may be necessary to allow chromatin assembled in one state to be reassembled into another state. Likewise, the passage of a replication fork through *HMR* may be the critical event in allowing active chromatin to reassemble into repressed chromatin. The presence of Sir1p and perhaps other Sir proteins at a silencer may increase their local concentration and allow them to assemble into chromatin as the replication fork passes, nucleating the assembly of silenced chromatin. Of course, it remains possible that some other S phase event, perhaps a critical phosphorylation, is required for silencing.

The Sir1-independence of telomeric silencing allowed a critical test of whether ORC had any role in silencing beyond interactions with Sir1p. Indeed, both *ORC2* and *ORC5*, and by inference the entire ORC, were important for telomeric silencing. The result is surprising because, in contrast to natural telomeres, which usually have ORC binding sites near them, the artificial telomere used here was constructed without any ORC binding sites. How ORC affected telomeric silencing in the absence of an obvious nearby binding site is unknown. However, telomeres in many organisms including yeast are synapsed into a large structure, often near the periphery of the nucleus (*30*). It is conceivable that an ORC bound to an ACS of one telomere can, by virtue of its juxtaposition to a synthetic telomere, promote telomeric silencing by mechanisms akin to transvection in *Drosophila*. Alternatively, *orc* mutations may alter the timing of replication and thus affect telomeric silencing.

In summary, the role of ORC in silencing is independent of its role as a replication initiator. Its role in silencing at *HMR-E* is largely through Sir1p. In contrast, its role in silencing telomeres is rather different and may reveal new lessons about organizational principles in the nucleus.

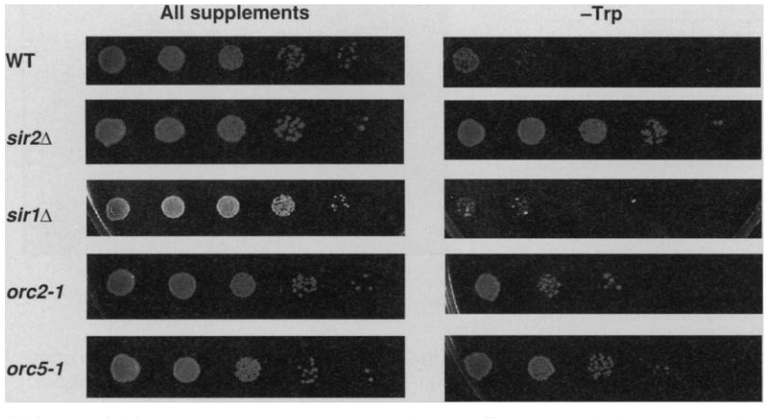

Fig. 4. *ORC2* and *ORC5* were required for telomeric silencing. Tenfold serial dilutions of strains bearing a telomeric copy of the *TRP1* gene were spotted onto plates supplemented with tryptophan (left) or lacking tryptophan (right). The strains used were JRY4469 (*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3Δ::LEU2 can1-100 TEL-VII-L::TRP1::URA3*) and its derivatives JRY4470 (*sir2Δ::LEU2*), JRY4506 (*sir1Δ::LEU2*), JRY4471 (*orc2-1*), and JRY4472 (*orc5-1*).

REFERENCES AND NOTES

1. S. P. Bell, R. Kobayashi, B. Stillman, *Science* **262**, 1844 (1993).
2. G. Micklem, A. Rowley, J. Harwood, K. Nasmyth, J. F. Diffley, *Nature* **366**, 87 (1993); M. Foss, F. J. McNally, P. Laurenson, J. Rine, *Science* **262**, 1838 (1993); S. Loo *et al.*, *Mol. Biol. Cell* **6**, 741 (1995).
3. C. A. Fox, S. Loo, A. Dillin, J. Rine, *Genes Dev.* **9**, 911 (1995).
4. S. Loo and J. Rine, *Annu. Rev. Cell. Dev. Biol.* **11**, 519 (1995).
5. A. H. Brand, G. Micklem, K. Nasmyth, *Cell* **51**, 709 (1987); W. Kimmerly, A. Buchman, R. Kornberg, J.

Rine, *EMBO J.* **7**, 2241 (1988).
6. F. J. McNally and J. Rine, *Mol. Cell. Biol.* **11**, 5648 (1991).
7. J. Abraham, K. A. Nasmyth, J. N. Strathern, A. J. Klar, J. B. Hicks, *J. Mol. Biol.* **176**, 307 (1984); J. B. Feldman, J. B. Hicks, J. R. Broach, *ibid.* **178**, 815 (1984).
8. D. H. Rivier and J. Rine, *Science* **256**, 659 (1992); unpublished data.
9. A. M. Miller and K. A. Nasmyth, *Nature* **312**, 247 (1984).
10. S. P. Bell, J. Mitchell, J. Leber, R. Kobayashi, B. Stillman, *Cell* **83**, 563 (1995).
11. C. T. Chien, S. Buck, R. Sternglanz, D. Shore, *ibid.* **75**, 531 (1993).
12. A *GAL4-SIR1* fusion gene was set under control of the constitutive promoter of the yeast alcohol dehydrogenase I, *ADH1*p [G. Ammerer, *Methods Enzymol.* **101**, 192 (1983)] in pRS316 (*31*). The *GAL4-SIR1* fusion plasmid (pCF117) contained *ADH1*p (Bam HI–Hind III from pJR1208), followed by *GAL4-SIR1* {Hind III–Bgl II from pKL5 [C. Chien, P. L. Bartel, R. Sternglanz, S. Fields, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9578 (1991)] containing the 5′ end of the gene} and the 3′ end of *SIR1* including the *SIR1* terminator (Bgl II–Hind III from pJR979).
13. Gal4 binding sites were introduced in place of the ACS of the synthetic *HMR* silencer (*6*). First, a Bam HI–Sph I fragment containing the ACS was excised from pJR1273. pJR1273 consisted of the Eco RI–Hind III fragment of *HMR* cloned into the Eco RI–Hind III sites of pUC18. This *HMR* allele also lacked *HMR-I*. In place of the excised ACS, annealed nucleotides that formed the Gal4 binding site were inserted (5′-GATCCCCCCTCGAGGATCTCGGAAGACTCTCC-TCCGGCTGATGCATG-3′). Plasmids were screened for multiple insertions, and isolates that had one, three, and five tandem insertions (pJR1584, pJR1614, and pJR1619, respectively) were used to replace the *hmrΔ::URA3* allele in JRY3933 (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 hmrΔ::URA3*) by one-step gene replacement [R. J. Rothstein, *Methods Enzymol.* **101**, 202 (1983)]. Correct integration was verified by DNA blot hybridization.
14. C. Fox, S. Loo, J. Rine, data not shown.
15. B. F. Pugh and R. Tjian, *Cell* **61**, 1187 (1990); *Genes Dev.* **5**, 1935 (1991; B. P. Cormack and K. Struhl, *Science* **262**, 244 (1993).
16. A methionine-regulated *GAL4-SIR1* fusion gene was obtained by inserting the Hind III fragment of pCF117 into Hind III–linearized pJR1588. pJR1588 is a derivative of pRS313 (*31*), which carries a Sal I–Eco RV fragment of the *MET3* promoter [H. Cherest, P. Kerjan, K. Y. Surdin, *Mol. Gen. Genet.* **210**, 307 (1987)] inserted at Xho I–Eco RV.
17. L. H. Hartwell, *J. Mol. Biol.* **104**, 803 (1976).
18. C. W. Jacobs, A. E. Adams, P. J. Szaniszlo, J. R. Pringle, *J. Cell Biol.* **107**, 1409 (1988).
19. F. Felici, G. Cesareni, J. M. X. Hughes, *Mol. Cell. Biol.* **9**, 3260 (1989).
20. T. Triolo and R. Sternglanz, *Nature* **381**, 251 (1996).
21. To create the Gal4-Orc2 and Gal4-Orc5 fusion proteins, we amplified the *ORC2* and *ORC5* open reading frames from yeast genomic DNA by polymerase chain reactions (PCR). The PCR primers used introduced Bam HI sites both 5′ and 3′ of both genes. The PCR products were cleaved with Bam HI and ligated into the Bam HI site of pRH98-1, thus setting them under control of the constitutive glyceraldehyde dehydrogenase (GPD) promoter and the phosphoglycerate kinase (PGK) terminator. pRH98-1 is a derivative of YCplac33 [R. D. Gietz and A. Sugino, *Gene* **74**, 527 (1988)], which has the Hind III–Xba I fragment of pG-1 [M. Schena, D. Picard, K. R. Yamamoto, *Methods Enzymol.* **194**, 389 (1991)] containing a GPD-PGK cassette inserted at the Hind III and Xba I sites. The resulting plasmids, pJR1637 (expressing *ORC2*) and pJR1638 (expressing *ORC5*), were able to complement the temperature sensitivity and silencing defect of *orc2-1* and *orc5-1*, respectively. Subsequently, a derivative of pRH98-1 was constructed that encoded the Gal4 DNA binding domain (residues 1 to 147). The *GAL4* sequence was obtained by PCR amplification from yeast genomic DNA. The primers were designed such that a Bgl II site was generated 5′ and a Bam HI site was created 3′ of the *GAL4* sequence. The PCR product was cleaved with Bgl II and Bam HI and inserted into the Bam HI site of pRH98-1, between *GPD*p and *PGK*, generating pJR1639. Bam HI fragments containing *ORC2* (pJR1637) and *ORC5* (pJR1638) were cloned into the unique Bam HI site of pJR1639, generating the Gal4(1–147)-Orc2 (pJR1640) and Gal4(1–147)-Orc5 (pJR1641) fusion plasmids.
22. J. Zhu, C. S. Newlon, J. A. Huberman, *Mol. Cell. Biol.* **12**, 4733 (1992).
23. O. M. Aparicio, B. L. Billington, D. E. Gottschling, *Cell* **66**, 1279 (1991).
24. D. E. Gottschling, O. M. Aparicio, B. L. Billington, V. A. Zakian, *ibid.* **63**, 751 (1990).
25. D. D. Dubey *et al.*, *Mol. Cell. Biol.* **11**, 5346 (1991).
26. O. M. Aparicio and D. E. Gottschling, *Genes Dev.* **8**, 1133 (1994).
27. A. Axelrod and J. Rine, *Mol. Cell. Biol.* **11**, 1080 (1991).
28. D. S. Henderson, S. S. Banga, T. A. Grigliatti, J. B. Boyd, *EMBO J.* **13**, 1450 (1994).
29. A. Hecht, T. Laroche, S. Strahl-Bolsinger, S. M. Gasser, M. Grunstein, *Cell* **80**, 583 (1995); A. Hecht, S. Strahl-Bolsinger, M. Grunstein, *Nature* **383**, 92 (1996).
30. F. Palladino *et al.*, *Cell* **75**, 543 (1993).
31. R. S. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989).
32. B. J. Brewer and W. L. Fangman, *Cell* **51**, 463 (1987).
33. We thank D. Gottschling for constructs and the members of our laboratory for stimulating discussions. Supported by a grant from the National Institutes of Health (GM31105) (J.R.), by postdoctoral fellowships from the California Division of the American Cancer Society and the Leukemia Society of America (C.A.F.), by postdoctoral grants from the Swiss National Science Foundation and the Janggen-Pöhn Foundation (A.E.E.-M.), and by a Howard Hughes Medical Research Institute predoctoral fellowship (S.L.). C.A.F. thanks M. D. Sheets for discussion and support. Core support was provided by a National Institute of Environmental Health Sciences Mutagenesis Center grant to B. N. Ames.

14 August 1996; accepted 11 April 1997

Orientation Selectivity in Pinwheel Centers in Cat Striate Cortex

Pedro E. Maldonado,*† Imke Gödecke,* Charles M. Gray, Tobias Bonhoeffer

In primary visual cortex of higher mammals neurons are grouped according to their orientation preference, forming "pinwheels" around "orientation centers." Although the general structure of orientation maps is largely resolved, the microscopic arrangement of neuronal response properties in the orientation centers has remained elusive. The tetrode technique, enabling multiple single-unit recordings, in combination with intrinsic signal imaging was used to reveal the fine-grain structure of orientation maps in these locations. The results show that orientation centers represent locations where orientation columns converge containing normal, sharply tuned neurons of different orientation preference lying in close proximity.

In recent years, optical imaging has enabled the investigation of neuronal response properties over large areas of the visual cortex in vivo (*1*–*3*). These experiments have revealed that orientation selectivity is not organized in parallel bands but in iso-orientation domains that are arranged radially in a pinwheel-like fashion (*4*). Optical imaging studies have shown that the magnitude of the orientation signal in the centers of these pinwheels is low (*1*, *3*, *4*), suggesting that the population of neurons in these locations might mainly consist of unoriented cells. However, because of their relatively low spatial resolution, imaging studies cannot reliably determine the physiological characteristics of individual neurons in these regions. We have previously reported that in some locations of cat striate cortex, adjacent cells display large differences in orientation preference (*5*). Because this is an alternative explanation for the low magnitude of the optical orientation signal, we conjectured that these regions may correspond to the pinwheel centers in the orientation preference map.

In five halothane-anesthetized adult cats, we used optical imaging based on intrinsic signals to record the orientation preference maps of visual areas 17 and 18 (*6*). The animals were stimulated with drifting square wave gratings of different orientations. The image of the visual cortical surface obtained in one experiment along with the corresponding "angle" and "polar" maps is shown in Fig. 1 (*7*). After obtaining these maps, we used tetrodes, which enable simultaneous and separable recording of small numbers of neighbouring neurons (*8*,

P. E. Maldonado, Max-Planck-Institute for Psychiatry, Am Klopferspitz 18A, 82152 München-Martinsried, Germany, and The Center for Neuroscience, University of California, Davis, CA 95616, USA.
I. Gödecke and T. Bonhoeffer, Max-Planck-Institute for Psychiatry, Am Klopferspitz 18A, 82152 München-Martinsried, Germany.
C. M. Gray, The Center for Neuroscience and the Section of Neurobiology, Physiology and Behavior, University of California, Davis, CA 95616, USA.

*These authors contributed equally to the work.
†To whom correspondence should be addressed at The Center for Neuroscience, University of California, Davis, CA 95616, USA. E-mail: pedro@chaos.ucdavis.edu