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On the origin of a silencer

Andrew Dillin and Jasper Rine

Although there are several compelling pieces of evidence suggesting that transcription can promote DNA replication, there are few studies that indicate a role for DNA replication in controlling transcription. Here, we discuss the role of the *HMR-E* silencer, an origin of replication that plays a crucial role in the regulation of expression of mating-type genes in the yeast *Saccharomyces cerevisiae*.

ELEGANT STUDIES with several different bacteriophage have established a role for transcription in the vicinity of phage origins of replication as a prerequisite for the initiation of DNA replication[1]. Recent evidence extends this ancient relationship to eukaryotes with the discovery of a transcriptional activator in the function of an origin of replication in the yeast *Saccharomyces cerevisiae*[2]. In contrast to the large amount of evidence that has established the role of transcription in promoting replication, there have been relatively few studies indicating a role for DNA replication in controlling the expression of genes. Examples of these studies include the observation that heterochromatin and fragile sites are among the last sequences in a genome to be replicated, although the cause-and-effect relationship between late replication and repression is unknown[3]. Genetic experiments have established that a specialized origin of replication in *S. cerevisiae* plays a critical role in regulating gene expression. In this review, we describe recent and ongoing experiments with this regulatory element and the proteins that act through it. These experiments offer an unusually favorable opportunity to explore potential links between those mechanisms that govern the replication of a eukaryotic genome and those that control its expression.

In *S. cerevisiae*, mating type is determined by alternative alleles of the mating type locus *MAT*. Cells with the *MAT***a** allele have the **a** mating type and cells with the *MAT*α allele have the α mating type. In addition, cells harbor cryptic copies of these mating-type genes at two additional loci, known as *HML* and *HMR*, which are many kilobases from *MAT*. The mating-type genes at *HML* and *HMR* are never expressed in wild-type cells. Rather, these loci serve as a source of genetic information that, upon transposition to *MAT*, cause *S. cerevisiae* to switch from one mating type to the other. *HML* and *HMR* are kept inactive, or silenced, through the combined action of flanking regulatory elements, known as silencers, and several different proteins, some of which bind the silencers (Fig. 1).

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How are *HML* and *HMR* silenced?

The process of silencing can be broken down into two distinct components: the establishment of the silenced state and its maintenance. These two processes are at least partially separable. The SIR1 protein, encoded by one of the four *SIR* genes, is primarily involved in establishing the repressed state but not in maintaining it. *SIR2*, *SIR3* and *SIR4* seem to be primarily involved in the maintenance of the silenced state, although some role for these genes in establishment is still possible[4]. The silencing of *HML* and *HMR* therefore reflects the combined action of processes that establish the repressed state and processes that maintain it. The efficiency with which the silenced state is maintained and inherited is sufficiently high that the establishment mechanism need not operate in every cell in a population. This point will become important later when we consider whether processes occurring in only a fraction of cell cycles can contribute to the phenotype of every cell in the population.

The mechanism of silencing involves the assembly of a specialized chromatin structure at *HML* and *HMR*, as evidenced by the ability of mutations in the genes for histones H3 and H4 to cause a loss of silencing[5–9]. Silenced chromatin is inaccessible to a variety of proteins, including RNA polymerases, DNA methylases, DNA repair enzymes and restriction endonucleases[10–15]. Heterochromatin is defined cytologically as highly condensed regions

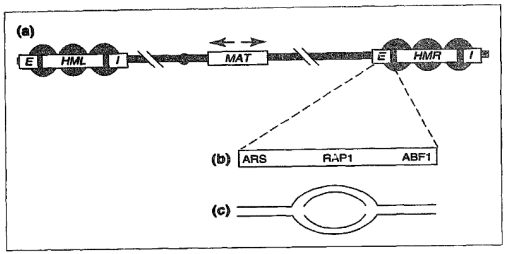


Figure 1

Schematic representation of *S. cerevisiae* chromosome III. **(a)** *MAT*, *HML* and *HMR* are the three loci that contain the regulatory genes for mating-type determination. *MAT* is near the centromere and *HML* and *HMR* are near the ends of the chromosome. Information at *MAT* is expressed (green arrows), whereas *HML* and *HMR* are kept silent (red bubbles) by the combined action of the silencer elements *E* and *I* and several proteins that play critical roles in silencing. **(b)** The *HMR-E* silencer is expanded to show the identity and order of the three modules required for its function. ARS is the sequence required for origin function. RAP1 is the sequence element bound by the RAP1 protein. ABF1 is the sequence element bound by the ABF1 protein. **(c)** Shows the function of *HMR-E* as an origin of replication.

on chromosomes that block expression of nearby genes. Heterochromatin includes condensed regions at centromeres, telomeres and, occasionally, regions within chromosome arms. In *S. cerevisiae*, the chromosomes are too small to allow heterochromatin to be visualized. Nevertheless, the inactivation of genes at the telomeres requires many of the same genes as does *HML* and *HMR*[16]. Thus, silenced chromatin and heterochromatin are likely to have many molecular similarities.

Of the silencer elements that flank *HML* and *HMR*, the *HMR-E* silencer is by far the most thoroughly studied[17]. This silencer consists of three functional modules. Two of the modules correspond to binding sites for RAP1 and ABF1, two common regulatory proteins that individually activate transcription of a variety of other genes[18]. The third module consists of an autonomously replicating sequence (ARS) consensus sequence (Fig. 1). Somehow, the combination of these three modules causes silencing, whereas none of the three elements on its own cause silencing.

ARS elements are defined as DNA sequences that, when cloned into a plasmid, can promote the autonomous replication of plasmids. ARS elements share an 11 base pair sequence motif known as the ARS consensus sequence, which is required for ARS activity[19]. Although all *S. cerevisiae* silencers have ARS elements, not all of these are authentic chromosomal origins of replication[20]. However, the *HMR-E* silencer is among those that, in its normal chromosomal location, is an origin of replication[21].

The origin recognition complex (ORC) is a six-protein complex that binds to the ARS consensus sequence of all ARS elements, including the *HMR-E* silencer. ORC may in fact be the protein responsible for regulating the initiation of replication at *S. cerevisiae* origins, based on the ATP dependence of ORC binding and the nature of the ORC footprint[22]. Genetic experiments provide strong evidence both that ORC is the initiator protein and that ORC plays a critical role in silencer function. For example, temperature-sensitive (ts) mutations in both *ORC2* and *ORC5*, which encode the second and fifth largest subunits of the ORC complex, respectively, cause derepression of *HML* and *HMR* at the permissive temperature, and loss of viability at the restrictive temperature[23,24]. In addition, even at the permissive temperature, replication initiation is reduced at both the *HMR-E* origin and at the *ARS1* origin[25,26]. Finally, the replication defect of plasmids carrying an *S. cerevisiae* origin in *orc*^{ts} mutants grown at low temperatures can be suppressed by cloning additional origins into the plasmid[24]. Thus, the replication defect in *orc* mutants is limited to replication initiation and does not affect elongation[27].

A further indication of a connection between replication and silencing is provided by an elegant temperature-shift experiment using a *sir3*^{ts} allele[28]. These experiments reveal that upon shift from the nonpermissive to the permissive temperature, cells must pass through the S phase of the cell cycle to re-establish *HML* and *HMR* in the silenced state. Although these studies do not identify the molecular event in S phase required to restore silencing, the existence of an origin of replication as part of the *HMR-E* silencer implicates some aspect of DNA replication in establishing the silenced state.

What is the relationship between the origin and silencing functions?

Formally, there are four possible models for the relationship between the origin and silencing functions of the *HMR-E* silencer: first, DNA replication initiation at the silencer may be a necessary prerequisite for its function as a silencer; second, the association of the two functions at the silencer may be a coincidence, not reflecting any mechanistic connection; third, *HMR-E* may function as a silencer despite an antagonistic effect caused by replication initiation; and finally, replication initiation at the *HMR-E* origin may occur only in silenced chromatin. Fortunately, the fourth model can be excluded by the ability of the *HMR-E* silencer to function as an origin of replication in *sir* mutants, which lack all silencing ability[21]. However, it is difficult to determine which of the remaining three models (Fig. 2) is nearer the truth. Below we discuss the evidence for and against each of these models and, where appropriate, suggest additional experiments that would help distinguish among the models.

Model 1: DNA replication initiation is essential for silencing

The strongest predictions of this model are that mutations in the silencer that block its function as an origin of replication should also render it deficient as a silencer (Fig. 2). In addition, mutations in genes encoding proteins that bind the silencer and that are required for its origin function should decrease both replication initiation and silencer function. These predictions have been fulfilled at two levels. First, studies with a synthetic version of the *HMR-E* silencer indicate that mutations in either the ARS consensus sequence

or the RAP1-binding site block the ability of this element to function either as an origin of replication or as a silencer[29]. Second, as mentioned earlier, at the permissive temperature *orc2*[ts] and *orc5*[ts] mutations reduce silencer function and reduce replication initiation at the *HMR-E* silencer and at other *S. cerevisiae* origins of replication[23,24]. At face value, the combined replication and silencing defects caused by *orc* mutations would seem to connect the role of ORC in replication initiation to its role in silencing. However, it should be noted that the study identifying *orc* mutations was restricted to a study of ts mutations[23]. Thus, *ORC* alleles that retained replication function yet were defective for silencing would not have been identified.

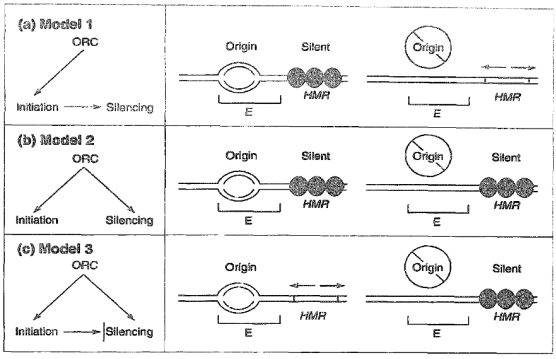


Figure 2

Three models for the function of ORC in transcriptional silencing. Bubbles at *HMR-E* indicate replication initiation and green arrows above *HMR* signify transcription from the divergent promoters of a1 and a2 at *HMR*. Red bubbles at *HMR* indicate transcriptional repression of that locus. The *HMR-I* element is not shown. **(a)** In model 1, the role of ORC in silencing depends upon its function as a replication initiator. **(b)** In model 2 the role of ORC in silencing is separable from its role in replication. **(c)** In model 3, ORC is required separately both for replication initiation and for silencing. However, replication initiation at the silencer is antagonistic to silencing. The evidence supporting and conflicting with each view is described in the text.

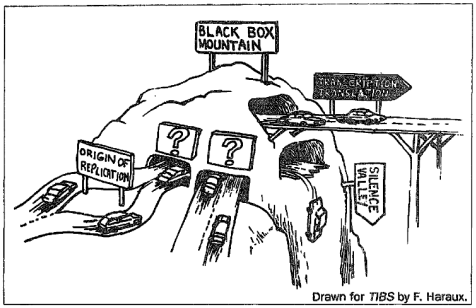
Evidence against a requirement for replication initiation at the *HMR-E* silencer for silencing has come from the properties of special alleles of *ORC5*. A simple approach to determining whether *ORC* genes have separable roles in silencing and replication is through reversion analysis of a ts mutation defective in both processes. If revertants of one phenotype can be found that do not affect the other phenotype, and vice versa, then the two functions may be separable. Indeed, three independent intragenic revertants have been characterized that restore viability to strains with the *orc5-1*[ts] mutation. By molecular analysis of origin function, all of these mutations restore replication initiation at the *HMR-E* silencer yet, strikingly, none of these mutations restores silencing[25].

Taken together, these observations indicate that replication initiation at the *HMR-E* silencer is not sufficient for silencing *HMR*. However, these results do not establish that silencing at *HMR* is independent of replication initiation at *HMR-E*. For example, the replication machinery that initiates at *HMR-E* may have an intrinsic activity that leads to the assembly of a specialized chromatin structure behind the two replication forks. The identification of *ORC* alleles that block replication initiation at *HMR-E* yet still silence *HMR* would be strong evidence for the separability of replication initiation and silencing. However, such alleles have not yet been described. Reversion analysis of the *orc2-1*[ts] mutations has yet to identify revertants that separate the two phenotypes.

Model 2: the association of silencing and replication initiation is a coincidence

According to this model, the ability of ORC to function as a replication initiator and to contribute to silencing reflect distinctly different functions of the ORC complex. In addition to the phenotype of *orc5-1* intragenic revertants, further evidence supporting this view comes from analysis of the *HML-E* and *HML-I* silencers, which flank *HML* and are required to maintain that locus in the silenced state. Although ORC is required for silencing both *HML* and *HMR*, no replication initiation has been observed at either *HML-E* or *HML-I*, although they both contain ARS elements[20]. These data would seem to indicate that ORC contributes to silencing of *HML* independently of its function as an initiator. However, there are limitations to this interpretation. As discussed above, silencing consists of an establishment phase and a maintenance phase. If replication initiation at a silencer were to have a role in establishing silencing but not in its maintenance, then the frequency of the establishment event, and hence of replication initiation at *HML-E*, may be below the threshold of detection in the published studies. One test of this view would be to evaluate replication initiation at the *HML-E* and *HML-I* silencers in a *sir3*[ts] temperature shift experiment in which all cells in the population establish repression simultaneously.

A further indication that the association between replication and silencing may be a coincidence comes from considering at which point in the cell cycle ORC is required for silencing and for replication initiation. Because ORC is the replication initiator, the role of ORC in replication is presumably manifest at the beginning of S phase. By contrast, in mitotically arrested *orc*[ts] mutants, a shift from the permissive to the restrictive temperature results in derepression of *HMR* in the absence of cell division[25]. A simple interpretation of this result is that one function of ORC may be continuously required for maintaining the silenced state, and a separate function of ORC may be periodically required during S phase for replication initiation.



Drawn for *TIBS* by F. Haraux.

Independent evidence that replication initiation at the *HMR-E* silencer may be a coincidence comes from analysis of special alleles of the *HMR-E* silencer combined with GAL4–SIR1 fusion proteins[30]. In these studies, a substantial portion of the *HMR-E* silencer is replaced with multiple binding sites for the DNA-binding domain of the *S. cerevisiae* transcriptional activator GAL4. In cells expressing fusion proteins consisting of SIR1 tethered to the GAL4 DNA-binding domain, these multiple GAL4-binding sites can mediate silencing of the flanking *HMR* locus.

These observations suggest a general model for how replication and silencing functions of ORC could be separable. The role of ORC in silencing may be, through protein–protein contacts with RAP1 or ABF1, to provide a surface that recruits SIR1, and perhaps additional proteins, to the silencer. In this model, *HMR-E* provides sequence features needed for ORC to initiate replication whereas *HML-E* does not. Nevertheless, both sites allow ORC to participate in recruitment of other proteins crucial to silencing. Although it is formally possible that the multiple GAL4-binding sites function as an origin of replication under these circumstances, it seems unlikely. Nevertheless, it is difficult to provide a clear interpretation of this experiment owing to the presence of the *HMR-I* element. *HMR-I*, which is downstream of the *HMR* locus and only a few kilobases away from *HMR-E*, binds the ORC complex and is a functional chromosomal origin of replication (D. H. Rivier and J. Rine, unpublished). Thus, regardless of how the GAL4–SIR1 fusion protein contributes to silencing, it cannot provide a critical test of the role of ORC or replication initiation in silencing unless tested in a strain lacking the ARS at *HMR-I*.

Model 3: *HMR-E* is a silencer despite its ability to function as an origin of replication

In this model, replication initiation at a silencer is antagonistic to silencing, and silencers have an ability to reduce the impact of this antagonism. Although there has been no critical test of this model, several observations are consistent with this view. First, replication initiation at *HMR-E* occurs in only a fraction of cell cycles. Perhaps the establishment of the silenced state can only occur in those cell divisions in which replication initiation fails to initiate at *HMR-E*.

One way to think about antagonism models is based upon considering what ORC might interact with during replication initiation compared with what it might interact with during silencing. For example, during replication initiation ORC is likely to interact with the DBF4–CDC7 kinase at functional origins[31,32] and possibly a helicase and a DNA polymerase holoenzyme as well. These interactions may exclude the ability of ORC to interact with RAP1 and ABF1 to promote silencing. This model offers a potential explanation for the seemingly paradoxical effect of *cdc7* mutations on silencing. *CDC7* encodes a protein kinase required for the initiation of mitotic DNA replication[33,34]. Strains containing a mutation in both the RAP1- and ABF1-binding sites in the natural *HMR-E* silencer are defective in silencing. *cdc7* mutations can restore silencing in cells containing this double-mutant silencer[35]. According to this antagonism model, reduced CDC7 kinase function may destabilize a complex that includes ORC, allowing ORC greater opportunity to participate in silencing. Moreover, part of the function of RAP1 and ABF1 bound to the silencer may be to reduce or modulate replication initiation at the silencer. In models in which replication initiation is required for silencer function, it is difficult to see how reduced CDC7 function would restore silencing. The antagonism model would be more strongly supported if other mutations affecting replication initiation were to increase silencing, or if mutations that decrease silencing of *HML* were found to activate replication initiation from the *HML-E* or *HML-I* silencer.

Perspectives

Here we have discussed how silencing and replication initiation may be connected. The ORC complex is clearly central to both processes, but how ORC functions in the two processes remains unresolved. In one model, silencing is completely dependent upon the initiation function of ORC; in a second model, the functions of ORC in silencing and replication are distinctly separable; and in a third model, the role of ORC in replication initiation is not only separable from its role in silencing, but may also function at the expense of silencing. At this point, the first model seems most fragile, leaving the second and third models largely intact.

One outcome of these experiments will be the elucidation of how silencing *can* work in strains with artificial silencers and the proper assortment of mutant genes. This reductionist approach is a crucial step in identifying the processes and players of silencing, but may not give an accurate view of the complete process inside the cell. For example, if ORC can contribute to the establishment of the silent state independently of replication initiation at *HMR-E*, how is the silenced state inherited by both daughter cells following replication?

A second unresolved issue is whether all replication initiation events are born equal with respect to silencing. The *HML* and *HMR* loci are among the last sequences to be replicated in S phase[3], and presumably *HMR-E* and *HMR-I* are late-firing origins of replication. Is late firing *per se* important for

silencing? An answer to this question will be required before the phenotype of a replication-competent, silencing-defective allele of the *HMR-E* silencer can be fully understood.

On a broader perspective, the involvement of ORC in silencing provides a wonderful opportunity to explore potential links between the mechanisms that replicate a genome and those that control its expression. The exquisite sensitivity of mating phenotypes in yeast combined with the ability to study gene expression with single-cell resolution ensure that a complete understanding of the mechanism of silencing in living cells is achievable in the foreseeable future.

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The RNP domain: a sequence-specific RNA-binding domain involved in processing and transport of RNA

Kiyoshi Nagai, Chris Oubridge, Nobutoshi Ito, Johanna Avis and Phil Evans

The RNP domain is found in a number of proteins involved in processing and transport of mRNA precursors. The crystal structure of a complex between the U1A spliceosomal protein and its cognate RNA hairpin at 1.92 Å resolution reveals the molecular basis of sequence-specific RNA recognition by the RNP domain.

IN LIVING CELLS, RNA usually exists as a complex with proteins, forming ribonucleoprotein particles (RNPs). The RNP (ribonucleoprotein) motif [also known as the RNA recognition motif (RRM) or the RNP consensus-sequence-type (RNP-CS) RNA-binding domain (RBD)] is the most common RNA-binding motif and is found in over 200 distinct RNA-binding proteins[1–3]. These proteins include messenger RNA (mRNA) precursor-binding proteins (hnRNPs)[2], components of the pre-mRNA splicing machinery[4] and proteins that bind to the polyadenylate tail[5,6] or the 5′-cap (I. Mattaj *et al.*, unpublished) of mRNA. The correct assembly of these proteins to their target RNA is essential for the excision of introns, correct processing of the 3′-end of pre-mRNA, and the transport and translation of mature mRNA, and hence provides additional steps of gene regulation following transcription. Some proteins in the RNP family are involved in the selection of alternative splice sites and RNA metabolism, and play essential roles in development[7,8].

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The RNP motif of RNA-binding proteins

Of the proteins found so far that contain the RNP motif (Fig. 1a), some contain only a single copy of the motif, while others contain as many as four copies (see Fig. 1b for examples)[1,2]. The distinct feature of this motif is two short sequences, referred to as RNP1 (RNP octamer) and RNP2 (RNP hexamer),