ecules was FLP-mediated recombination. FLP recombinase catalyzes the entire recombination reaction, requiring no other proteins (*26*) and leaving no substrates for repair synthesis. Also, no repair-coupled synthesis of the excised *HMR* molecules was detected by transient resistance to Dpn I cleavage. Thus, it was unlikely that replication-coupled process contributed to the quantitative silencing observed here. We are unaware of any evidence that mutations or damage occurs more readily on circular plasmids in yeast than on the chromosome.

In addition to offering a new mechanistic insight on silencing, these data place renewed importance on the role of proteins involved in DNA replication, such as PCNA, Rfc1p, Asf1p, Dna2p, and CAF-1, in silencing. An important challenge is to learn how those proteins affect silencing when silencing can be mechanistically divorced from both replication initiation and from the passage of a replication fork. PCNA left behind on a previously replicated template can mark that template as "competent" for CAF-1–dependent chromatin assembly (*33*). If PCNA from the previous cell cycle remains associated with *HMR* upon entering the subsequent G_1 phase, it may be excised with *HMR* and therefore available to establish heterochromatin. The efficiency of silencing observed here would require that some feature of *HMR* causes retention of PCNA. Alternatively, these proteins may have a role in silencing other than in its establishment (*18*). For example, once heterochromatin is established at a locus, it must be maintained throughout that cell cycle and duplicated in each subsequent cell cycle. Indeed, recent data underscore the dynamic nature of heterochromatin composition in vivo, even on nonreplicating DNA molecules (*3*). Perhaps proteins like CAF1 and PCNA have a replication-coupled role in the inheritance of heterochromatin at *HMR*, or possibly in its maintenance.

The results of this study have reframed the essential outstanding issues in establishing heterochromatin. One goal now is to learn what replication-independent event happens in this cell cycle window that is essential for silencing. The second goal is to uncover how replication proteins play a role in silencing when replication itself is not required. Both questions should provide fundamental insights into how cells assemble specific structures of chromatin in a spatially and temporally organized manner.

References and Notes

1. J. Z. Dalgaard, A. J. Klar, *Nature* **400**, 181 (1999).
2. X. Bi, J. R. Broach, *Mol. Cell. Biol.* **17**, 7077 (1997).
3. T.-H. Cheng, M. R. Gartenberg, *Genes Dev.* **14**, 452 (2000).
4. A. Schmid, K.-D. Fascher, W. Hörz, *Cell* **71**, 853 (1992).
5. S. Loo, J. Rine, *Ann. Rev. Cell Dev. Biol.* **11**, 519 (1995).
6. A. Hecht, T. Laroche, S. Strahl-Bolsinger, S. M. Gasser, M. Grunstein, *Cell* **80**, 583 (1995).
7. S. Imai, C. M. Armstrong, M. Kaeberlein, L. Guarente, *Nature* **403**, 795 (2000).
8. K. A. Gardner, J. Rine, C. A. Fox, *Genetics* **151**, 31 (1999).
9. T. Triolo, R. Sternglanz, *Nature* **381**, 251 (1996).
10. A. M. Miller, K. A. Nasmyth, *Nature* **312**, 247 (1984).
11. D. H. Rivier, J. L. Ekena, J. Rine, *Genetics* **151**, 521 (1999).
12. M. Foss, F. J. McNally, P. Laurenson, J. Rine, *Science* **262**, 1838 (1993).
13. S. Loo *et al.*, *Mol. Biol. Cell* **6**, 741 (1995).
14. G. Micklem, A. Rowley, J. Harwood, K. Nasmyth, J. F. X. Diffley, *Nature* **366**, 87 (1993).
15. J. S. Smith, E. Caputo, J. D. Boeke, *Mol. Cell. Biol.* **19**, 3184 (1999).
16. M. S. Singer *et al.*, *Genetics* **150**, 613 (1998).
17. P. D. Kaufman, R. Kobayashi, B. Stillman, *Genes Dev.* **11**, 345 (1997).
18. S. Enomoto, J. Berman, *Genes Dev.* **12**, 219 (1998).
19. P. Kaufman, personal communication.
20. Z. Zhang, K. Shibahara, B. Stillman, *Nature* **408**, 221 (2000).
21. C. A. Fox, A. E. Ehrenhofer-Murray, S. Loo, J. Rine, *Science* **276**, 1547 (1997).
22. S. P. Bell, J. Mitchell, J. Leber, R. Kobayashi, B. Stillman, *Cell* **83**, 563 (1995).
23. A. Dillin, J. Rine, *Genetics* **147**, 1053 (1997).
24. C. A. Fox, S. Loo, A. Dillin, J. Rine, *Genes Dev.* **9**, 911 (1995).
25. J. R. Broach, F. C. Volkert, in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Genome Dynamics, Protein Synthesis and Energetics*, J. R. Broach, J. R. Pringle, E. W. Jones, Eds. (Cold Spring Harbor Laboratory Press, Plainview, NY, 1991), vol. I, pp. 297–331.
26. L. Meyer-Leon, C. A. Gates, J. M. Attwood, E. A. Wood, M. M. Cox, *Nucleic Acids Res.* **15**, 6469 (1987).
27. A. L. Kirchmaier, J. Rine, data not shown.
28. Y.-C. Li, T. H. Cheng, M. R. Gartenberg, *Science* **291**, 650 (2001).
29. S. G. Holmes, J. R. Broach, *Genes Dev.* **10**, 1021 (1996).
30. Web fig. 1, Web table 1, and supplemental text are available at *Science* Online at www.sciencemag.org/cgi/content/full/291/5504/646/DC1.
31. K. L. Friedman, M. K. Raghuraman, W. L. Fangman, B. J. Brewer, *J. Cell Sci. Suppl.* **19**, 51 (1995).
32. J. Abraham, K. A. Nasmyth, J. N. Strathern, A. J. S. Klar, J. B. Hicks, *J. Mol. Biol.* **176**, 307 (1984).
33. K.-i. Shibahara, B. Stillman, *Cell* **96**, 575 (1999).

34. We thank J. Broach for pFV17; J. Gin and L. Rusché for technical assistance; P. Garber, L. Rusché, and P. Kaufman for helpful discussions; and L. Rusché, P. Kaufman, M. Botchan, and B. Meyer for critical review. Supported by NIHF32GM19392 (A.L.K.) and NIHGM31105 (J.R.).

14 August 2000; accepted 19 December 2000

Establishment of Transcriptional Silencing in the Absence of DNA Replication

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Transcriptional repression of the silent mating-type loci in *Saccharomyces cerevisiae* requires a cell cycle–dependent establishment step that is commonly assumed to involve DNA replication. Using site-specific recombination, we created a nonreplicating DNA ring in vivo to test directly the role of replication in establishment of silencing. Sir1 was tethered to the ring following excision from the chromosome to activate a dormant silencer. We show here that silencing can be established in DNA that does not replicate. The silenced ring adopted structural features characteristic of bona fide silent chromatin, including an altered level of DNA supercoiling and reduced histone acetylation. In addition, the process required silencing factors Sir2, Sir3, and Sir4 and progression between early S and M phases of the cell cycle. The results indicate that passage of a replication fork is not the cell-cycle event required for establishment of silencing in yeast.

Silencing of large chromosomal domains involves specialized, heritable chromatin structures that repress transcription in a gene-independent fashion. The silent *HM* mating-type loci of budding yeast (*HMR* and *HML*) represent well-studied examples of this type of transcriptional control (*1*). Silencing of *HMR* and *HML* is governed by cis-acting sequences, known as the *E* and *I* silencers, which flank both loci and consist of various combinations of sites for Rap1p, Abf1p, and the multisubunit origin recognition complex (ORC). Despite an essential role for ORC in initiation of DNA replication, substantial evidence indicates that this is not its function at silencers. Only a subset of silencers act as chromosomal replication origins and *orc* mutants have been isolated that are defective in replication initiation but not silencing (*2*, *3*). Instead, the function of ORC at silencers appears to be recruitment of Sir1 (*4*–*6*), which, along with the other silencer binding proteins, facilitates incorporation of Sir2, Sir3, and Sir4 into a heterochromatin-like structure, termed silent chromatin. Indeed, artificially

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tethering Sir1 to a nonfunctional silencer that lacks ORC sites restores silencing (*6*).

DNA replication, nonetheless, has long been implicated in silencing. In classic experiments by Miller and Nasmyth, de novo establishment of the silent state was shown to occur during passage between G1 and M phases of the cell cycle, a period during which DNA replication occurs (*7*). More recently, CAF1, a replication-coupled chromatin assembly factor, was shown to provide a contributory role to silencing of the mating-type loci (*8*), and to be critical for a related form of silencing at telomeres [reviewed in (*9*)]. An additional link was provided by the finding that certain replication mutants suppress loss of silencing caused by a mutated silencer (*10*, *11*). One hypothesis supported by the available evidence was that establishment of silencing requires passage of a replication fork.

To examine the role of DNA replication in silencing directly, we asked whether silencing could be established in a nonreplicating DNA ring formed by site-specific recombination. To this end, the chromosomal *HMR* locus was modified to contain *RS* target sites for the *Zygosaccharomyces rouxii* R recombinase (Fig. 1A). In addition, the *I* silencer of the locus was removed and the *E* silencer was replaced with a synthetic construct (*lexOEB*) that functions as a conditional silencer. *lexOEB* contains multiple sites for the bacterial DNA binding protein LexA and single sites for Rap1 and Abf1 (*12*). The silencer is nonfunctional because it lacks binding sites for ORC (*6*). Without these sites, recombination produces a nonreplicating DNA ring that contains a transcriptionally active copy of the **a** mating-type genes (Fig. 1A). Following excision, the ring-borne conditional silencer can be activated by expression of a LexA-Sir1 fusion protein (*13*).

Quantitative Southern blots were used to demonstrate that the excised DNA ring did not replicate (Fig. 1B). Ring amounts were measured following a 1-hour period for recombinase expression (*14*). At times corresponding to doublings in cell density, the amount of ring decreased by factors of two relative to a chromosomal marker (*HML*), irrespective of whether LexA-Sir1 or LexA was expressed. Similar results were obtained with cultures of synchronously growing cells as they progressed from early S to early M phase of the cell cycle (*15*). These data indicate that DNA ring content remained constant (i.e., did not replicate) as chromosomal DNA content increased.

The amount of **a**1 transcript from the unrecombined chromosomal locus was measured to determine whether the LexA-based silencing system conferred repression. **a**1 is a short-lived message and thus serves as a sensitive indicator of the onset of transcriptional silencing (*7*). Northern blots showed that induction of LexA expression produced little change in amounts of **a**1 transcript (Fig. 2A). Expression of LexA-Sir1, by contrast, reduced the **a**1 transcript by an order of magnitude, thereby demonstrating that the synthetic silencer can exert conditional repression in a chromosome.

To test whether silencing can be established in nonreplicating DNA, the synthetic silencer was activated following DNA ring formation. The **a**1 transcript disappeared rapidly when LexA-Sir1 was expressed (Fig. 2A). The transcript diminished slowly when LexA was expressed, but this apparent loss was a consequence of the reduction in nonreplicating template relative to a replicating chromosome (see above). Normalizing the amount of transcript for changes in template copy number showed that LexA-Sir1 caused a time-dependent reduction of transcript, whereas LexA did not (Fig. 2B). These results collectively demonstrate that replication of the ring is not required for establishment of transcriptional repression.

Conditional silencing systems have been used previously to show that de novo establishment of silencing occurs sometime during progression between the early S and early M phases of the cell cycle (*6*, *7*). To test whether silencing in the absence of DNA replication is subject to similar cell-cycle constraints, our assay was performed in cells that were blocked either in early S by using hydroxyurea [an inhibitor of ribonucleotide reductase (*16*)], or in early M by using nocodazole [an inhibitor of microtubule polymerization (*17*)]. Following cell-cycle arrest, LexA-Sir1 was induced, and expression of the ring-borne **a**1 was evaluated. LexA-Sir1 did not significantly reduce amounts of **a**1 transcript in cells blocked in either early S (Fig. 3, lanes 1 and 2) or early M phase (lanes 3 and 4).

The experiment was repeated in cells that progressed incrementally through the cell cycle. Following an initial arrest in either hydroxyurea or nocodazole, LexA-Sir1 was expressed and cells were released into fresh medium containing a subsequent cell cycle–blocking agent, or into medium containing no agent at all. In this

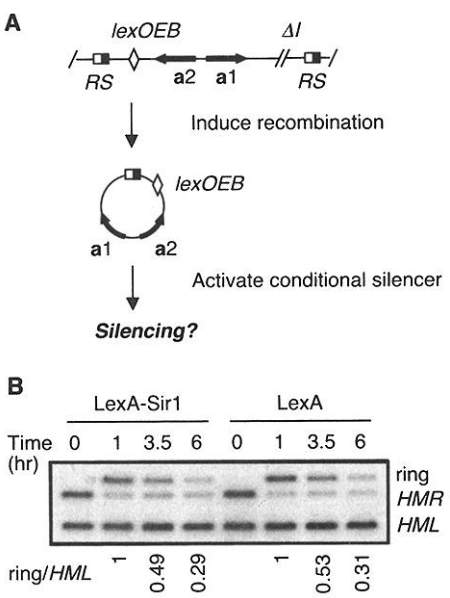

Fig. 1. (**A**) A system to test establishment of silencing in the absence of DNA replication. Thirty-four base pair (bp) *RS* sites (half-filled boxes) and a conditional synthetic silencer *lexOEB* (diamond) were incorporated within an *HMR* chromosomal construct that lacked native silencers, such that recombination yielded a 2.6-kb nonreplicating DNA ring (*12*). Following excision, LexA-Sir1 or LexA was induced, and expression of the ring-borne **a**1 gene was evaluated. (**B**) Extrachromosomal rings do not replicate. Recombination was initiated in strain YCL49 *MATα Δhmr::RS-6lexOEB***a**1**a**2*-RS ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 Δlys2 Δbar1::hisG Δsir1::loxp-kanMX-loxP*, containing a *GAL1::R* expression vector pHM153^{LEU2} (*19*), and either pMET3P-LexASIR1^{HIS3} or pMET3P-LexA^{HIS3}, as described in (*14*). Total cellular DNA was isolated by the glass bead method, and equal quantities were loaded in each lane. Blot was hybridized with a probe that binds identical sequences in the ring and *HML*. The ratio of the two signals (ring/*HML*) at 1 hour was normalized to 1. The values represent the average of three independent trials (SD = ±0.05).

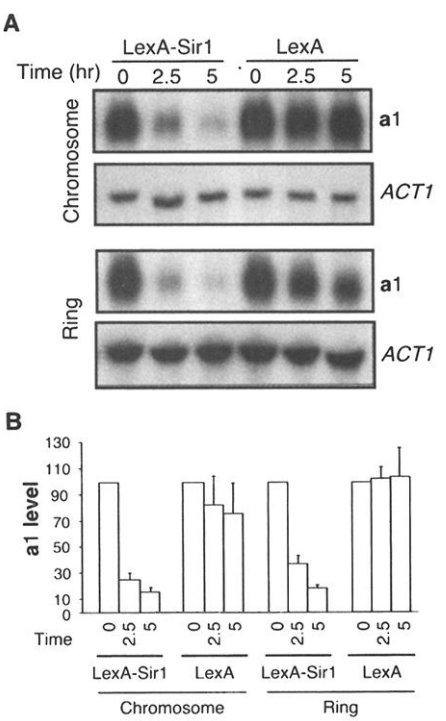

Fig. 2. (**A**) Silencing by LexA-Sir1 in a chromosome and in a nonreplicating DNA ring. Strain YCL49 containing either pMET3P-LexASIR1^{HIS3} or pMET3P-LexA^{HIS3} and either pRS425 (for chromosome) or pHM153^{LEU2} (for ring) was grown as described in (*14*). At various times following induction of the LexA constructs, total RNA was harvested (*19*) and equal amounts were loaded. Blots were hybridized sequentially with **a**1 and *ACT1* probes. (**B**) Quantification of **a**1 mRNA. **a**1 was measured relative to *ACT1* (three independent trials) and then normalized to account for differences in template copy, by using data obtained from Southern blots (see Fig. 1). In each series, the amount of **a**1 at time 0 was set to 100%.

case, **a**1 transcript was eliminated when cells progressed between early S and M (Fig. 3, lane 6). Control experiments verified that this repression required LexA-Sir1 (lane 5). Conversely, the **a**1 transcript persisted, albeit in reduced amounts, when cells progressed from M to early S (lane 10). The **a**1 gene was silenced, however, when cells were released from M phase into medium that lacked hydroxyurea, presumably because cells passed through a subsequent cell cycle (lane 12). Together, the data indicate that passage between early S and M is required for silencing, even when the template does not replicate.

Transitions between silenced and nonsilenced chromatin states were previously detected by measuring changes in the level of DNA supercoiling (*18*–*20*). To test whether repression in the absence of replication is also accompanied by changes in DNA supercoiling, topoisomer distributions of the excised rings were examined by chloroquine gel electrophoresis. Supercoiling standards for the repressed and nonrepressed states were obtained by expressing LexA-Sir1 or LexA continuously before DNA excision. Accordingly, the ring was found to be more negatively supercoiled when LexA-Sir1 was expressed (Fig. 4, lanes 1 and 2). When DNA rings were examined in the absence of a LexA fusion protein, the level of supercoiling matched that of the nonsilent standard (compare lanes 2, 3, and 6). Induction of LexA following ring formation did not alter the level of DNA supercoiling. Induction of LexA-Sir1, however, caused a time-dependent shift in supercoiling that coincided with the rate of **a**1 transcript loss. After 5 hours, the level of supercoiling closely matched the silent supercoiling standard (compare lanes 1 and 5). These supercoiling shifts demonstrate that establishment of silencing in a nonreplicating template is accompanied by alterations in chromatin structure.

Sir2, Sir3, and Sir4 form a protein complex that is required for transcriptional repression of the silent mating-type loci. Therefore, the experiment described above was repeated in mutants lacking *SIR2* and *SIR4*, and it was found that DNA supercoiling was not affected by LexA-Sir1 expression (Fig. 4). A Δ*sir3* strain behaved similarly (*21*). These results indicate that establishment of silencing on a nonreplicating template is *Sir*-dependent.

Histones H3 and H4 within silenced chromosomal domains are distinct from their bulk chromatin counterparts in that specific lysines within the amino-terminal tails are not acetylated (*22*). Chromatin immunoprecipitations were therefore performed to determine whether establishment of silencing in the absence of replication was accompanied by changes in histone acetylation. Antibodies specific for tetra-acetylated H4 or the nonacetylated H3 were used to precipitate formaldehyde-crosslinked chromatin and quantitative polymerase chain reaction (PCR) was used to ascertain the extent of ring isolation (Fig. 5A). The data were plotted as the multiple of change (fold increase or decrease) in histone acetylation state upon induction of a LexA construct (Fig. 5B). When LexA-Sir1 was expressed, the amount of nonacetylated H3 associated with the rings increased, with a concomitant decrease in the amount of acetylated H4. Expression of LexA, however, did not produce a significant change in histone acetylation. These results demonstrate that amounts of acetylated histone decrease upon establishment of silencing, even in a template that does not replicate. Furthermore, the data suggest that preassembled nucleosomes can be substrates for deacetylases. Sir2 was recently shown to be a histone deactylase that is dependent on NAD (the oxidized form of nicotinamide adenine dinucleotide) [reviewed in (*23*)], and it is likely that the enzyme modifies histone tails to establish silencing.

In this report and the accompanying report (*24*), extrachromosomal DNA rings were used to demonstrate that transcriptional silencing could be established in a nonreplicating template. That appropriate changes in histone acetylation and DNA supercoiling occurred during this Sir-dependent process verified that a bona fide silent chromatin structure was formed. Furthermore, silencing of ring-borne genes was subject to the same cell-cycle constraints as the native locus, in-

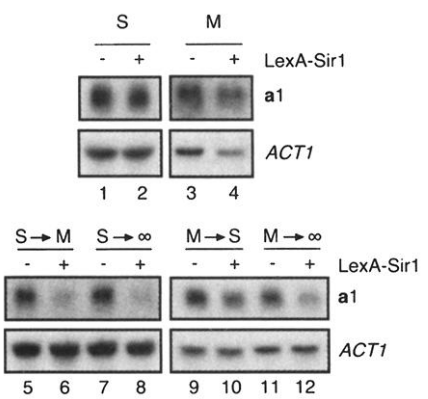

Fig. 3. Silencing in nonreplicating rings requires passage between early S and M phase. Strain YCL21 Δ*mat::TRP1* Δ*hmr::RS-3lexOEB***a**1**a**2-*RS ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* Δ*lys2* Δ*bar1::hisG* Δ*sir1::loxP-kanMX-loxP* containing pHM153 and pMET3P-LexASIR1^{HIS3} was grown in 2% galactose for 1 hour before the addition of hydroxyurea (Cf = 0.2 M) or nocodazole (Cf = 10 μg/ml). After a 3-hour period at 25°C for arrest, cells were pelleted, washed, resuspended in fresh medium containing the appropriate drug (or no drug, as indicated by ∞), and cultured for an additional 3 hours at 25°C in the presence or absence of LexA-Sir1 expression. In lanes 1 and 2, cells arrested initially with hydroxyurea were resuspended in medium containing 0.5 M hydroxyurea. In lanes 3 and 4, cells arrested initially with nocodazole were resuspended in 10 μg/ml nocodazole and 10 μg/ml benomyl. Initial and terminal arrests were confirmed by visual inspection of cell morphology.

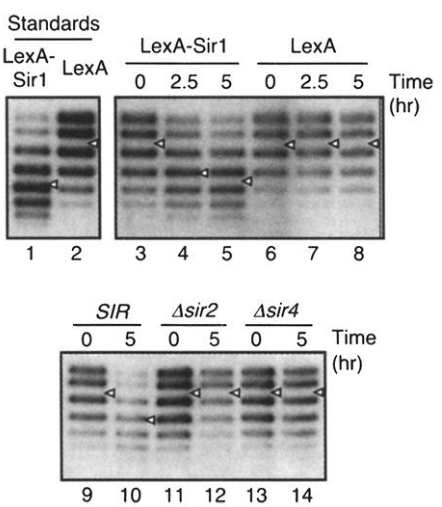

Fig. 4. DNA supercoiling changes upon induction of silencing. DNA samples isolated by the spheroplasting method were subject to electrophoresis in buffer containing 1.5 μg/ml chloroquine (*19*). More negatively supercoiled topoisomers migrated faster. Centers of the topoisomer distributions are marked with arrowheads. Controls in lanes 1 and 2 were obtained by growth in SC-Leu-His-Met medium containing raffinose, followed by 1 hour in similar medium containing galactose. Δ*sir2::klURA3* and Δ*sir4::klURA3* derivatives of YCL49 were used for lanes 11 to 12 and lanes 13 to 14, respectively.

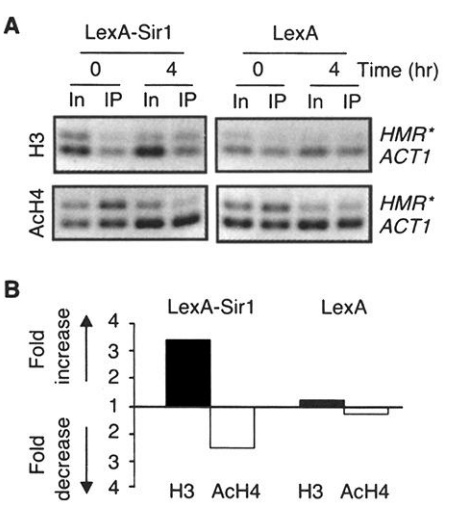

Fig. 5. Histone acetylation changes upon induction of silencing. (**A**) Chromatin immunoprecipitations. Antibodies from Upstate Biotechnology (Lake Placid, New York) were used according to (*32*) with some modifications (*33*). For ring-specific PCR, primers flanked the *RS* site such that recombination was required to generate a product, termed *HMR**. In, input; IP, immunoprecipitate. (**B**) Quantification of changes in acetylation state. The *HMR** signal in precipitated samples was measured relative to *ACT1* and normalized to the same ratio in the input lane. Data are plotted as the multiple of change in normalized precipitable *HMR** following a 4-hour induction of either LexA construct.

dicating that the normal pathway for establishing silencing was not bypassed. Taken together, the results demonstrate that establishment of silencing involves something other than DNA replication during passage between early S and M phase.

A simple model to account for the findings is that activity of a critical silencing component, such as a Sir protein or silencer binding protein, is regulated in a cell cycle–dependent manner. Posttranslational modification of the protein(s), for example, might be required to initiate silent chromatin assembly. Necessary silencing activities might also be acquired by regulated synthesis of additional proteins or degradation of existing inhibitors. Although Sir proteins are not known to fluctuate during the cell cycle, their quantities are limiting, and redistribution of the factors from one location could influence silencing at another. Intriguingly, pools Sir3 and Sir4 were recently shown to partially disperse from their telomeric locations in mitotic cells (*25*).

A second model to account for the findings is that silencing requires chromatin remodeling or assembly factors that are not strictly associated with replication forks. According to this view, the factors might be targeted by silencers to specific locations where they would function sometime between early S and M phase. Alternatively, the presumed factors might promote global changes in chromatin structure during this period (e.g., mitotic condensation) that could also benefit silencing. Under certain conditions, such factors might also destabilize silent chromatin in a cell cycle–dependent manner. Indeed, transcriptionally repressed sequences from *HML* reactivated following excision from the chromosome in a replication-independent fashion during passage from G1 to M (*18*). Silencing of a telomere-proximal reporter gene was also shown to be least effective in cells arrested in M phase (*26*).

Recent work has linked CAF1 to heterochromatin in mammalian cells. The replication-coupled chromatin assembly factor associates with HP1 and accumulates in heterochromatic regions long after replication is complete (*27*–*29*). The factor also localizes to replication foci, where it is targeted by interaction with proliferating cell nuclear antigen (*30*). If the role for CAF1 in yeast silencing is distinct from its role at replication forks, a mechanism must exist to target the factor to silenced domains.

Last, the experiments performed here do not necessarily exclude a contributory role for replication in establishment of silent chromatin. Although silencing can occur in nonreplicating DNA, it is possible that passage of a replication fork accelerates the establishment process. This kinetic difference may be beneficial when silencing must occur within a rapid developmental program or in rapidly dividing cells.

References and Notes

1. S. Loo, J. Rine, *Annu. Rev. Cell Dev. Biol.* **11**, 519 (1995).
2. D. D. Dubey *et al.*, *Mol. Cell. Biol.* **11**, 5346 (1991).
3. A. Dillin, J. Rine, *Genetics* **147**, 1053 (1997).
4. C.-T. Chien, S. Buck, R. Sternglanz, D. Shore, *Cell* **75**, 531 (1993).
5. T. Triolo, R. Sternglanz, *Nature* **381**, 251 (1996).
6. C. A. Fox, A. E. Ehrenhofer-Murray, S. Loo, J. Rine, *Science* **276**, 1547 (1997).
7. A. M. Miller, K. A. Nasmyth, *Nature* **312**, 247 (1984).
8. S. Enomoto, J. Berman, *Genes Dev.* **12**, 219 (1998).
9. A. J. Lustig, *Curr. Opin. Genet. Dev.* **8**, 233, (1988).
10. A. Axelrod, J. Rine, *Mol. Cell. Biol.* **11**, 1080 (1991).
11. A. E. Ehrenhofer-Murray, R. Kamakaka, J. Rine, *Genetics* **153**, 1171 (1999).
12. A LexA-based conditional silencer was obtained by replacing the Bam HI–Sph I fragment containing the ORC site in the synthetic *HMR* silencer of pJR1273 (*6*) with a PCR-amplified fragment containing 12 overlapping LexA sites from plasmid pAA6 (*31*). A Stu I–Xho I fragment containing the modified synthetic silencer and adjacent **a** genes was cloned between the *RS* sites in plasmid hmr::rHMR (*19*) to yield pLCY8. A similar procedure was used to create pLCY7, which contains six overlapping LexA sites. pLCY7 and pLCY8 were used to create strains YCL21 and YCL49, respectively, by regenerating the *HMR* locus in appropriately modified strains [see (*15*) for details].
13. A LexA-Sir1 expression vector (pMET3P-LexASIR1^{HIS3}) was obtained by replacing the Eco RV–Pst I fragment encoding the GAL4-binding domain and NH_2-terminus of *SIR1* (amino acids 1 to 136) in pJR1811 (*6*) with the LexA gene (Hind III–Xma I) from pBTM116 (*31*). The resulting chimera is expressed from the *MET3* promoter, which is induced by Met depletion. A vector expressing only LexA (pMET3P-LexA^{HIS3}) was obtained by deleting the remainder of the *SIR1* gene.
14. Cells were precultured overnight in SC-Leu-His containing 2% dextrose and 2 mM Met and then were inoculated into a similar medium containing raffinose (initial OD = 0.02). When the culture reached mid-log, galactose was added to a final concentration of 2% to induce recombinase expression. One hour later, cells were pelleted, washed, and resuspended in galactose-containing medium that lacked Met to induce expression of the LexA chimeras (*13*). Maximal excision (>80%) occurred within 1 hour, as noted previously (*19*).
15. Supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/291/5504/650/DC1
16. L. H. Hartwell, *J. Mol. Biol.* **104**, 803 (1976).
17. C. W. Jacobs, A. E. M. Adams, P. J. Szaniszlo, J. R. Pringle, *J. Cell Biol.* **107**, 1409 (1988).
18. X. Bi, J. R. Broach, *Mol. Cell. Biol.* **17**, 7077 (1997).
19. T.-H. Cheng, Y.-C. Li, M. R. Gartenberg, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5521 (1998).
20. T.-H. Cheng, M. R. Gartenberg, *Genes Dev.* **14**, 452 (2000).
21. Y.-C. Li, M. R. Gartenberg, unpublished observations.
22. M. Braunstein, R. E. Sobel, C. D. Allis, B. M. Turner, J. R. Broach, *Mol. Cell. Biol.* **16**, 4349 (1996).
23. L. Guarente, *Genes Dev.* **14**, 1021 (2000).
24. A. Kirchmaier, J. Rine, *Science* **291**, 646 (2000).
25. T. Laroche, S. G. Martin, M. Tsai-Pflugfelder, S. M. Gasser, *J. Struct. Biol.* **129**, 159 (2000).
26. O. M. Aparicio, D. E. Gottschling, *Genes Dev.* **8**, 1133 (1994).
27. K. Marheineke, T. Krude, *J. Biol. Chem.* **273**, 15279 (1998).
28. A. Taddei, D. Roche, J.-B. Sibarita, B. M. Turner, G. Almouzni, *J. Cell Biol.* **147**, 1153 (1999).
29. N. Murzina, A. Verreault, E. Laue, B. Stillman, *Mol. Cell* **4**, 529 (1999).
30. K. Shibahara, B. Stillman, *Cell* **96**, 575 (1999).
31. A. Ansari, M. R. Gartenberg, *Mol. Cell. Biol.* **17**, 7061 (1997).
32. P. B. Meluh, J. R. Broach, *Methods Enzymol.* **304**, 414 (1999).
33. Whole-cell lysates were cleared with protein A agarose before antibody addition, according to the manufacturer's recommendation, to reduce nonspecific binding of chromatin. Antibodies for nonacetylated H3 (40 μl) and acetylated H4 (5 μl) were incubated with the precleared lysate for 6 hours. Twenty times as much lysate was used for immunoprecipitated samples as for input samples. Images of ethidium bromide–stained agarose gels were captured by charge-coupled device camera (UVP, Cambridge, UK) and quantified with IPlab Gel software (Signal Analytics, now Scanalytics, Fairfax, VA). Linear responses were obtained for the range of template concentrations used. Primer sequences are as follows: RING-1 (5′-GGCAAAGGCGATCCTCTAG-3′); RING-3b (5′-CAAACTTTGAGAGAAATATGTCTTTC-3′); ACT1-3 (5′-CTTCCACGTCCTCTTGCAT-3′); ACT1-3c (5′-GCGTGAAAAATCTAAAAGCTGATG-3′).

34. We thank M. Pierce, R. Kamakaka, and C. Fox for materials; M. Pierce and D. Gross for technical advice; and N. Walworth, A. Ansari, and D. Reinberg for critical comments on the manuscript. This work was funded by a grant from the NIH (GM51402).

5 September 2000; accepted 6 December 2000

Role of Importin-β in Coupling Ran to Downstream Targets in Microtubule Assembly

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The guanosine triphosphatase Ran stimulates assembly of microtubule asters and spindles in mitotic *Xenopus* egg extracts. A carboxyl-terminal region of the nuclear-mitotic apparatus protein (NuMA), a nuclear protein required for organizing mitotic spindle poles, mimics Ran's ability to induce asters. This NuMA fragment also specifically interacted with the nuclear transport factor, importin-β. We show that importin-β is an inhibitor of microtubule aster assembly in *Xenopus* egg extracts and that Ran regulates the interaction between importin-β and NuMA. Importin-β therefore links NuMA to regulation by Ran. This suggests that similar mechanisms regulate nuclear import during interphase and spindle assembly during mitosis.

In interphase of the cell cycle, the guanosine triphosphate (GTP)–bound form of the small guanosine triphosphatase, Ran, is concentrated in the nucleus, whereas its guanosine diphosphate (GDP)–bound form predominates in the cytoplasm (*1*). This gradient of