Heart J. **130**, 806 (1995)] under anesthesia (50 mg of ketamine per kilogram of body weight and 10 mg/kg xylazine) indicated that shortening fractions for the SH-HF rats (37 ± 3%) were significantly ($P < 0.01$) reduced relative to those of three 17- to 18-month-old Sprague-Dawley controls (57 ± 4%). Peak systolic blood pressure for the SH-HF rats was 164 ± 11 mm Hg versus 108 ± 10 mm Hg for the controls ($P < 0.02$). All of the failing rats had ascites, hydrothorax, and hepatic and pulmonary congestion; three also had large, well-resolved atrial thrombi. None of the controls exhibited these generally accepted signs of congestive heart failure.

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29. In our study we report $[Ca^{2+}]_i$ transient data as both F/F_0 and as Ca^{2+}-spark occurrence. To count Ca^{2+} sparks activated by I_{Ca}, the line-scan images were contrast-enhanced and displayed on a video monitor and visually counted. We also applied a spark-counting software algorithm (H. Cheng, unpublished data) to verify the result. The algorithm includes dividing the image by the fluorescence signal before depolarization, then low-pass filtering with a 1.5-μm, 15-ms spatiotemporal window. Spark events were objectively identified as discrete, isolated regions where fluorescence intensity exceeds the baseline mean intensity by $3\sigma^2$, where σ^2 is the variance of the baseline fluorescence. The results of both computer and visual spark-counting methods were in close agreement, with differences of less than 10%.
30. SR-enriched vesicles were isolated by differential centrifugation from whole hearts from control and hypertensive rats as described [C. A. Tate *et al.*, *J. Biol. Chem.* **260**, 9618 (1985)]. The fusion of microsomes into planar lipid bilayers revealed functional RyRs with properties similar to those of native RyRs; the analysis of single-channel kinetics was done as described [A. J. Lokuta, T. B. Rogers, W. J. Lederer, H. H. Valdivia, *J. Physiol. (London)* **487**, 609 (1995)]. Bilayers were composed of 50% phosphatidylethanolamine and 50% phosphatidylcholine (25 mg/ml in *n*-decane). The cis solution consisted of 300 mM $CsCH_3SO_3$, 10 mM Mops (pH 7.2), and EGTA:Ca^{2+} admixtures necessary to set free $[Ca^{2+}]$ to 100 nM and 10 μM. The trans solution was 50 nM $CsCH_3SO_3$ before SR fusion and 300 mM after fusion. Binding of [^{3}H]ryanodine to SR microsomes was performed for 90 min at 36°C in medium containing 0.2 M KCl, 10 mM NaCl, 1 mM AMP-PCP (a nonhydrolyzable analog of adenosine triphosphate), 1.6 mM $MgCl_2$ (0.6 mM free Mg^{2+}), 10 mM Na-Hepes (pH 7.2), and 20 μM $CaCl_2$ (10 μM free Ca^{2+}). The reaction was terminated by rapid filtration of samples. Nonspecific binding was determined in the presence of 10 μM ryanodine and has been substracted. Smooth lines are fits to data points with the formula $B = B_{max} \cdot [Ry]/([Ry] + K_d)$, where B is the specific binding and K_d is the dissociation constant. Protein concentration was determined by the Bradford method.
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Cell Cycle–Dependent Establishment of a Late Replication Program

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DNA replication origins in chromosomes of eukaryotes are activated according to a temporal program. In the yeast *Saccharomyces cerevisiae*, activation of origins in early S phase appears to be a default state. However, *cis*-acting elements such as telomeres can delay origin activation until late S phase. Site-specific recombination was used to separate origin from telomere in vivo, thereby demonstrating that the signal for late activation is established between mitosis and START in the subsequent G_1 phase. Once set, the signal can persist through the next S phase in the absence of the telomere. Establishment of the temporal program and of initiation competence of origins may be coincident events.

Eukaryotic chromosomes are organized into blocks of DNA sequence that replicate early in S phase interspersed with blocks that replicate late. Each temporal block is presumably replicated by the coordinate activation of clusters of replication origins (*1*). The functional significance of this temporal regulation remains open to speculation. A demonstrated correlation between actively transcribed genes and early replication in higher eukaryotes has led to the suggestion that early replication may be a prerequisite for the activation of gene transcription (*2*). Straying from the normal temporal program of replication may have deleterious consequences for development—for example, the fragile X syndrome is associated with delayed replication of the *FMR1* locus (*3*).

Saccharomyces cerevisiae chromosomes also contain early- and late-replicating domains, and yeast replication origins that are activated late in S phase have been identified recently (*4*–*6*). Although there has been much progress in understanding the molecular steps leading to the activation of replication origins (*7*), the molecular basis for the temporal program is less clear. The picture that is emerging is that yeast replication origins are activated early in S phase by default. Late activation, in those instances in which it has been found, is not an intrinsic property of the origins involved, but is imposed by *cis*-acting elements that are separable from the origin (*6*, *8*, *9*).

One example of a chromosomal element that affects origin activation time is the telomere—proximity to a telomere can cause late activation of origins (*8*, *10*). How the telomere effects a delayed schedule of origin activation is unknown, but the parallel phenomenon of telomeric position effect on transcription (*11*) suggests the involvement of a special chromatin structure propagated from the telomere. A notable feature of transcriptional silencing is its heritability: Silenced chromatin can be maintained and clonally inherited through several cell division cycles and, at least in the silent mating–type cassettes, maintenance of the repressed state can be independent of its initial establishment (*12*).

We now asked, first, if maintenance of the signal for late origin activation is also separable from its establishment by the telomere, and second, when in the cell cycle the telomeric late replication program is established. To address these questions, we used inducible, site-specific recombination to remove a subtelomeric late origin from its chromosomal location in vivo (Fig. 1). The excised circle, lacking determinants of late origin activation, is expected to replicate early in S phase unless the late replication signal originally established by the telomere persists after excision. Excising the origin at different times in the cell cycle would reveal if the telomeric late replication program is stably maintained for more than one round of the cell cycle or if it is reestablished in every cell cycle. For example, the origin can be removed at START, when the cell commits entry into another round of the cell cycle, and the cells then allowed to proceed through S phase. If the previously late origin now becomes active early in S phase, then the telomere must be needed after START and possibly through S phase to maintain the late replication program. If the origin remains late, then the program must be established before START and maintained through S phase in the absence of the telomere.

The origin excision cassette was constructed (*13*) in a strain that contains three copies of the "R" recombinase from *Zygosaccharomyces rouxii* under control of the GAL1 promoter (*14*) integrated in tandem at the *LEU2* locus on chromosome III. Tar-

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get sites for the recombinase were inserted on either side of the late-activated origin *ARS501* (*5*) to create the cassette. One endpoint of the cassette is adjacent to the *RAD3* locus on chromosome V, ~23 kb centromere-proximal to *ARS501*. The other endpoint is located ~7 kb distal to *ARS501* (Fig. 1).

The kinetics of site-specific recombination were examined by inducing expression of the recombinase gene in G_1. Cells grown in minimal medium with acetate as the carbon source were arrested at START in G_1 by treatment with α-factor. Galactose was added to the culture to induce transcription of the recombinase gene and thereby initiate recombination at the *ARS501* cassette, and samples were removed for analysis at hourly intervals thereafter. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis (*15*) of whole chromosomes showed that, as expected, the band corresponding to full-length chromosome V (~575 kb) disappeared over time after the addition of galactose, while a faster migrating band appeared (Fig. 2). Excision of the cassette was efficient (>90% in 5 hours; Fig. 2), and half-maximal excision occurred in 2 to 2.5 hours. Restriction mapping by Southern (DNA) hybridization revealed the predicted junction fragment and confirmed the circular topology of the excised fragment (*16*).

Analysis of the timing experiments that follow depended on two prerequisites. First, integration of the recombinase target sites to create the excision cassette must not reduce or eliminate *ARS501* activity. Second, replication of the excised cassette must be initiated at *ARS501* and not at cryptic origins that might be uncovered in the excised circle. To confirm that these conditions were met, we examined replication intermediates at *ARS501* by two-dimensional (2D) agarose gel electrophoresis (*17*). S-phase DNA was collected from untreated control cells and from cells in which recombination had been induced in G_1. The DNA was cut with Xba I, subjected to 2D agarose gel electrophoresis, and hybridized to an Xba I fragment containing *ARS501*. A bubble-to-Y pattern expected for an asymmetrically located, active origin (Fig. 3A) was seen in both the uninduced and the induced sample (Fig. 3, B and C). The dearth of intermediates in the ascending portion of the Y-arc in the induced sample indicates that the origin is highly active, as passive replication of the fragment by an origin located elsewhere on the excised circle would have caused an increase in the signal in that portion of the Y-arc. We conclude that initiation at the chromosomal *ARS501* was extremely efficient and that replication of the excised circle was driven predominantly, if not exclusively, by *ARS501*. The activity of *ARS501* was not perturbed by either the creation of the cassette or by its subsequent excision; the replication timing results for *ARS501* that follow therefore reflect the activation time for the origin.

Does the telomere set up the late activation program at *ARS501* before the cells enter S phase? We anticipated that if so, *ARS501* excised in G_1 would remain late-activated in the subsequent S phase, provided that the signal set up by the telomere persisted through S phase. Accordingly, the *ARS501* cassette was excised in G_1 at START, and its replication time in the ensuing S phase was determined by density transfer experiments (*18*). The replication time of a sequence (measured as t_{rep}, the time of half-maximal replication) can vary from culture to culture, hampering direct comparisons between experiments. Therefore, we show both the absolute replication time of *ARS501* and its replication index (Table 1), which is much less subject to experimental variation (*6*).

The control, chromosomal copy of *ARS501* replicated in the second half of S phase, with a replication index of 0.61 (Fig. 4A and Table 1). After excision in G_1, *ARS501* replicated with an index of 0.52, slightly earlier than the control experiment but still in the second half of S phase (Fig. 4B and Table 1). These results were confirmed in a second, independent experiment (Table 1). In contrast, after long-term growth of the cells (more than 20 generations) following excision of the cassette, *ARS501* replicated early in S phase, with a replication index of –0.02 (that is, slightly

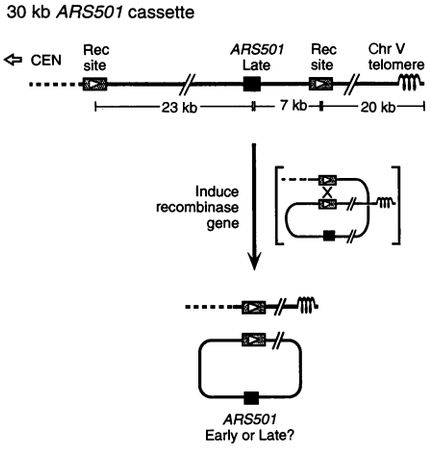

Fig. 1. Experimental rationale. The 30-kb cassette (top) was constructed by insertion of two target sites for the "R" recombinase in direct orientation at the indicated locations near the right end of chromosome V (*13*).

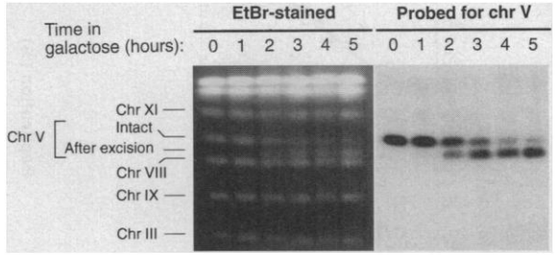

Fig. 2. Excision of *ARS501* by site-specific recombination in vivo. Image of an ethidium bromide (EtBr)–stained CHEF gel (left) and of an autoradiogram of a Southern blot (right) demonstrate excision of the 30-kb *ARS501* cassette. Cells grown in 1% sodium acetate as the carbon source were arrested in G_1 by treatment with α-factor (200 nM), and dry galactose was added to the culture medium (final concentration of 2% w/v). Samples were collected at hourly intervals thereafter for 5 hours, and the chromosomes were separated by CHEF gel electrophoresis (*15*). The gel was stained with ethidium bromide and photographed (left), then blotted onto a nylon membrane and probed for chromosome V with a fragment of the *RAD4* gene, centromere-proximal to the excision cassette (right). Bands corresponding to the various chromosomes (Chr) are indicated.

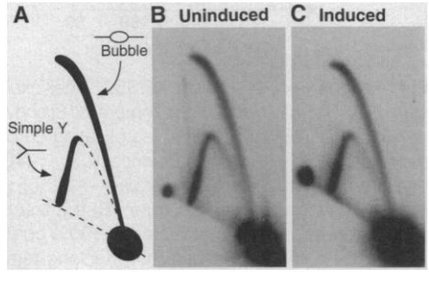

Fig. 3. Activation of *ARS501* on the excised cassette. Cells were arrested with α-factor, and excision of the cassette was induced in one-half of the culture by addition of galactose. After 5 hours, glucose was added to both portions; 30 min later, the cells were shifted to 37°C, released from the α-factor block by treatment with Pronase, and synchronized further at the *cdc7* block. The cells were then released from the *cdc7* arrest, and samples were collected at 1-min intervals for 30 to 35 min. DNA was extracted (*28*) from pooled samples, cut with Xba I, and examined by 2D gel electrophoresis (*17*). The gel used for the first dimension was made of 0.4% agarose in 1× TBE, and that of the second dimension was 1.1% agarose in 1× TBE containing 0.3 μg/ml ethidium bromide. (**A**) A cartoon depicts the bubble-to-Y 2D gel pattern expected for an active origin asymmetrically located in the fragment being examined. DNA from (**B**) control and (**C**) galactose-treated cells was hybridized to a probe fragment corresponding to *ARS501* (*5*).

earlier than *ARS1*, our marker for early replication) (Fig. 4C and Table 1).

Because the excised origin replicated late in the first S phase after excision but early in S phase after long-term growth, there must be a switch in replication timing that occurs some time after the first S phase. We entertained two possibilities: that the telomeric signal for late activation has to be established in every cell cycle, or that the origin, once programmed for late activation, remains in that state for many cell cycles but has some low probability of switching to early activation in every cell cycle. This second possibility is akin to the silencing of gene transcription seen at the silent mating type loci and at telomeres (*19*). In the first scenario, it should be possible to define the execution point for establishment of the telomeric position effect—a time in the cell cycle before which the late program is not yet established. Excision of the origin before this point in the cell cycle should result in its early activation in the following S phase; excised after this point, the origin should remain late-activated in the first S phase. Because origins appear to undergo a key transition to a prereplicative form at mitosis [(*20*); see below], we chose mitotic arrest as a starting point for the following experiments. Cells grown in isotopically dense culture medium were initially synchronized with α-factor, then arrested in G_2/M by treatment with nocodazole, an inhibitor of tubulin polymerization. Excision of the origin was induced by treatment with galactose. The cells were then washed and resuspended in isotopically light medium lacking nocodazole. After further synchronization at the *cdc7* block, the cells were allowed to enter S phase, and samples were collected for density gradient centrifugation as usual. The arrests at various stages of the experiment and the progress through S phase were confirmed by flow cytometry (*16*).

Excision of the origin in G_2/M resulted in a switch in its activation time to early in the following S phase, with a replication index of 0.05 (Fig. 5 and Table 1). In the control experiment, unexcised, chromosomal *ARS501* replicated late in S phase as expected (*16*). We conclude that the telomeric late replication program had not yet been established at G_2/M, the time of origin excision. An alternative possibility is that the excised circle initially was programmed for late replication but that the signal for late replication decayed by the time the cells reached S phase. This possibility seems unlikely because the interval between the excision step and the start of S phase was the same in this experiment as in the G_1 excision experiment (2.5 to 3 hours at 37°C), yet the observed replication times in the two experiments were very different. The slight delay in activation of the excised origin in this experiment (replication index of 0.05) compared with its activation time after steady-state growth (replication index of −0.02) may be attributed to incomplete excision of the cassette in nocodazole [~85% (*16*)].

Taken together, our observations demonstrate that the telomeric position effect on replication is established between mitosis and START. Once established, the late replication signal can be maintained through S phase even if the telomere was removed at START. The maintenance of the signal through S phase is reminiscent of previous observations on the silencing of transcription by telomeres—when Prp1, a transcription factor needed for *URA3* expression, is produced in G_1, it fails to de-repress a silenced copy of *URA3* until late S or G_2 (*21*). Presumably, a stable, inaccessible chromatin state (*22*) is not disrupted until then. Although it is not clear what elements, if any, are common to the telomeric silencing of transcription and the telomeric late replication effect, it is possible that the replication effect is also caused by a chromatin structure that is set up by the telomere but subsequently maintained through S phase in its absence. An alternative but not mutually exclusive possibility is that the subnuclear localization of the telomere is the primary cause of the late replication effect. Telomeres are believed to be localized to the nuclear periphery (*23*); perhaps origins located in that portion of the nucleus are targeted for late S-phase activation. The slightly earlier replication of the origin after excision at START (Fig. 4B) is consistent with either model—a slow loss of chromatin structure over time after excision or slow diffusion of the excised circle away from the nuclear periphery.

One difference between the telomere's effect on transcription and its effect on replication timing is the genomic distances involved—the origin can be affected over a distance of at least 27 kb from the telomere, whereas transcriptional silencing normally extends only about a tenth that distance (*24*). The early replication of *ARS501* after excision at G_2/M suggests another difference: unlike transcriptional silencing, which is clonally inherited for several cell cycles (*11*), the late replication signal has to be reestablished in every cell cycle.

The G_2/M phase transition appears to be a time when the cell prepares its DNA replication origins for the next round of

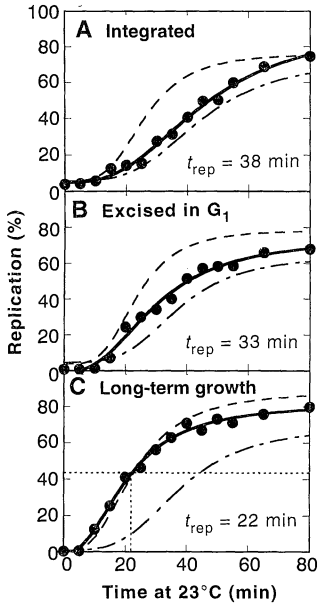

Fig. 4. Replication kinetics of *ARS501* after excision in G_1. Density transfer experiments (*18*) show replication of *ARS501* (●), early-replicating chromosomal *ARS1*-adjacent sequence [– – –; (*4*)], and late-replicating *RAP1* sequence [— · —; (*6*)] in (**A**) the control, uninduced culture; (**B**) the first S phase after excision of the cassette (≥90% complete) at the α-factor block; and (**C**) after long-term growth of a clone containing excised 30-kb cassette (*13*). The t_{rep} for *ARS501* is indicated [illustrated in (C) as a dotted line]. The data points for the early and late markers show the same low level of scatter as the experimental sample and have been omitted for clarity.

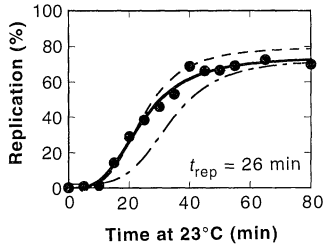

Fig. 5. Replication kinetics of *ARS501* after excision at G_2/M. Excision was ~85% complete in this experiment. Symbols are as in Fig. 4.

Table 1. Replication index of chromosomal and excised *ARS501*. Numbers in parentheses indicate values obtained from a second, independent experiment.

Condition of *ARS501*	Replication index*
Unexcised, chromosomal	0.61 (0.63)
Excised in G_1, first S phase	0.52 (0.51)
Excised in G_2/M, first S phase	0.05 (0.13†)
Excised, long-term growth	−0.02

*The replication index for *ARS501* was calculated by comparing its t_{rep} (time of half-maximal replication) with those of the early and late markers. The early marker (*ARS1*) was assigned a replication index of 0.00 and the late marker (*RAP1*) was assigned an index of 1.00; the replication index of *ARS501* is the *ARS1-ARS501* interval as a fraction of the *ARS1-RAP1* interval. †Excision was 70% complete.

initiations. Footprint analysis of origins shows that they are bound thoughout the cell cycle by the origin recognition complex (ORC), a six-subunit protein (*25*). At mitosis, the ORC is joined by one or more additional factors, resulting in an expanded footprint that lasts until S phase (*20*). The formation of this prereplicative complex depends on the *CDC6* gene product, which is essential in S phase (*26*). Coincident with the formation of the prereplicative complexes, the Mcm2, Mcm3, and Mcm5 proteins, which are also involved in replication initiation, are localized to the nucleus, where they remain until S phase (*27*). Thus, crucial steps in the decision of whether to fire an origin in S phase occur between the preceding mitosis and the end of G_1. Our results raise the possibility that the decision of when during S phase to fire the origin occurs at the same time.

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13. The starting strain was R3-ZDIR, which was derived from RM14-3a [*MATa cdc7-1 bar1 his6 leu2-3,112 trp1-289 ura3-52* (*4*)] by integration at *leu2* of three copies of a plasmid containing P_{GAL}-recombinase [made by deleting the 2-μm autonomously replicating sequence from plasmid pHM153 (*14*)]. One copy of the 65-bp recombinase target site [H. Araki *et al.*, *J. Mol. Biol.* **225**, 25 (1992)] was integrated in the "R14" fragment (*5*), ~50 kb from the right end of chromosome V, with *TRP1* as the selectable marker; the other copy of the recombinase site was integrated at the EcoR I site at the "R4/R5" junction, ~20 kb from the telomere with *URA3* as the marker. Upon excision of the cassette, the *TRP1* marker is left behind on the chromosome while *URA3* is retained on the circle. For the experiment on long-term growth of cells with the excised circle, cells were plated on minimal medium lacking uracil after induction of recombination. Clones with the excised circle were identified by CHEF gel electrophoresis and by Southern blotting, probing for the expected junction fragment on the circle. To overcome poor growth arising from loss of the circle (which contains essential genes like *RAD3*), we integrated a centromere (*CEN5*) in the *URA3* gene on the circle. Integrants were selected by growth on plates containing 5-fluoroorotic acid, which selects against expression of *URA3* [J. D. Boeke, F. LaCroute, G. R. Fink, *Mol. Gen. Genet.* **197**, 345 (1984)], and correct integration of the centromere fragment was confirmed by Southern blotting and hybridization.
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18. Density transfer experiments were based on previously described methods (*4*). Cells were grown for more than seven generations at 23°C in 0.1% (w/v) sodium [^{13}C]acetate and 0.01% (w/v) [^{15}N]ammonium sulfate as the sole carbon and nitrogen sources, respectively, and arrested in G_1 by treatment with α-factor (200 nM) for approximately one population doubling time (~8 hours). If the *ARS501* cassette was to be excised in G_1 (Fig. 4), the culture was filtered, washed, and resuspended in isotopically normal minimal medium containing 1% sodium acetate, 0.5% ammonium sulfate, and α-factor, and split in half. Galactose (final concentration of 2%) was added to one portion; the other portion was the control, uninduced sample. After 5 hours, glucose was added to both portions (final concentration of 2%); 30 min thereafter, the cultures were shifted to 37°C and treated with Pronase, whereupon the cells progressed to the *cdc7* block. The cells were released into S phase by shifting the culture to 23°C; samples were collected through S phase and processed for CsCl density gradient centrifugation (*5*). For excision at the nocodazole block, cells grown in isotopically dense medium were synchronized with α-factor as before, and nocadazole was added to the culture (final concentration of 10 μg/ml) before release from the α-pheromone block. Once the culture had arrested as predominantly large-budded cells, it was split in two and galactose was added to one portion. After 5 hours, both portions were filtered, washed, and resuspended in isotopically normal minimal medium containing 2% glucose and incubated at 37°C until the cells reached the *cdc7* block. The cells were then released into S phase and samples were collected as before. Small samples were also collected at various steps through the whole procedure and analyzed by flow cytometry to confirm the cell-cycle arrests (*16*).
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Nucleosome Mobility and the Maintenance of Nucleosome Positioning

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To study nucleosome mobility and positioning, the R3 *lac* repressor was used with an adenosine triphosphate (ATP)–dependent chromatin assembly system to establish the positioning of five nucleosomes, with one nucleosome located between two R3 *lac* operators. When R3 protein was dissociated from DNA with isopropyl β-D-thiogalactopyranoside, the R3-induced nucleosome positions remained unchanged for at least 60 minutes in the absence of ATP but rearranged within 15 minutes in the presence of ATP. These results suggest that nucleosomes are dynamic and mobile rather than static and that a DNA binding factor is continuously required for the maintenance of nucleosome positioning.

Transcription, replication, recombination, and repair in eukaryotes involve the interaction of proteins with DNA packaged into chromatin. Nucleosomes are often located at distinct positions in regions of genomes that regulate replication and transcription, and this specific positioning is typically altered as genes are activated or repressed (*1*). The analysis of factors that influence the organization of chromatin is essential to elucidating the function of DNA binding regulatory factors in the context of a chromatin template.

One interesting facet of chromatin structure is the relation between the binding of sequence-specific DNA binding proteins and the translational positioning of nucleosomes in nonrepetitive DNA (*2*). For example, the binding of factors to specific DNA elements, such as the α2 operator (*3*,

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