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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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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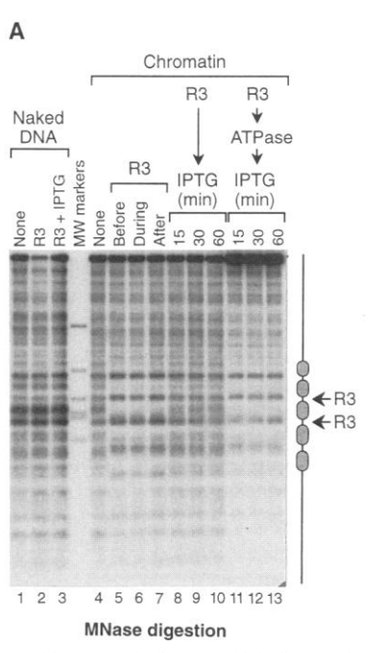
4) or the upstream activating sequence of the *GAL1-GAL10* intergenic region (5) of *Saccharomyces cerevisiae*, leads to specific positioning of adjacent nucleosomes. Sequence-specific DNA binding transcription factors such as GAGA factor, heat-shock factor, nuclear factor E2, Gal4p derivatives, and nuclear factor kappa B (NF-κB) (*6–10*) induce the rearrangement of nucleosome arrays in the vicinity of their DNA binding sites.

Although sequence-specific DNA binding proteins are able to establish the positioning of nucleosomes, it is not known whether they must continuously occupy their DNA sites to maintain specific nucleosome positioning. To investigate this question, we used the R3 mutant version of the *Escherichia coli lac* repressor as a sequence-specific DNA binding protein that can be dissociated upon the addition of the lactose analog isopropyl β-D-thiogalactopyranoside (IPTG). R3 protein binds specifically to the *lac* operator as a (monovalent) dimer rather than as a (bivalent) tetramer (*11*); unlike the wild-type *lac* repressor, R3 protein cannot promote the formation of DNA loops between pairs of binding sites. We have used R3 protein to induce specific nucleosome positioning in vitro and then observed the changes in chromatin structure that occur upon dissociation of R3 from chromatin after adding IPTG. We have also tested whether a nucleosome could be specifically placed between two appropriately spaced R3 binding sites (Fig. 1A).

To this end, we used a plasmid containing two *lac* operators separated by 183 base pairs (bp) (*12*). This plasmid was assembled into chromatin (*13*) in the presence or absence of purified R3 protein with the use of a crude *Drosophila* chromatin assembly extract containing adenosine triphosphate (ATP)–utilizing factors that facilitate the rearrangement of nucleosomes (*6*, *8*, *9*, *14*, *15*). The resulting products were subjected to partial micrococcal nuclease digestion and indirect end-labeling analysis (*9*, *16*, *17*). In the absence of R3 protein, the pattern of micrococcal nuclease digestion of chromatin resembled the digestion pattern seen with the corresponding naked DNA, which suggests that there was no preferred positioning of nucleosomes. In contrast, when R3 protein was added before, during, or after chromatin assembly, one nucleosome was found between the two *lac* operators and four other nucleosomes were specifically positioned in the flanking DNA, as indicated at the right of Fig. 1A. The ability of the R3 *lac* repressor to bind after chromatin assembly is consistent with the observation that the *lac* repressor can bind to chromatin in mammalian cells (*18*).

We then examined whether the positioning of nucleosomes in this array could be maintained upon dissociation of R3 protein from DNA with IPTG. With chromatin assembled in the presence of R3 protein, factor-induced nucleosome positioning was lost within 15 min of addition of IPTG, as the micrococcal digestion pattern reverted to that seen in the absence of R3 (Fig. 1A). Dissociation of R3 protein from DNA by IPTG was confirmed by deoxyribonuclease



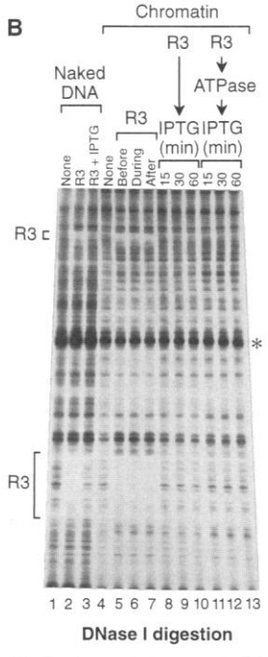


Fig. 1. R3 protein is required to establish and maintain nucleosome positioning. Chromatin was assembled onto a 3.2 kbp plasmid that contains two binding sites for the *lac* repressor (the 21-bp recognition sequence is identical to that of the wild-type *E. coli lac* O1 operator) separated by 183 bp of DNA (*12*). Chromatin assembly was performed with the *Drosophila* S-190 embryo extract (*9*, *13*). Purified R3 protein (50 nM) (*11*) and IPTG (0.5 mM) were included in the reaction, as indicated. All chromatin assembly and reconfiguration reactions were incubated for a total duration of 5.5 hours (chromatin assembly is complete in less than 4 hours), and the samples were subjected to indirect end-labeling analysis (*9*). For lanes 5 through 7, R3 protein was added (i) to naked DNA before chromatin assembly (lane 5; before), (ii) to the reaction medium at the onset of chromatin assembly (lane 6; during), or (iii) to newly assembled chromatin at 4.5 hours reaction time, after which the mixture was incubated for an additional hour under the same conditions (lane 7; after). For lanes 8 through 13, chromatin was assembled in the presence of R3, as for lane 6. Then, for lanes 8 through 10, IPTG was added at 15, 30, or 60 min before the termination of the reaction; whereas for lanes 11 through 13, apyrase [0.02 U/ml; ATPase (Sigma)] was added at 4 hours reaction time, and then IPTG was added 15, 30, or 60 min before the termination of the reaction. Lanes 1 through 3 show control reactions with naked DNA templates. Lane 4 shows chromatin in the absence of R3, IPTG, and apyrase. (**A**) Micrococcal nuclease digestion and indirect end-labeling analysis. The deduced positions of nucleosomes (ovals) and the locations of the *lac* operators (arrows) are indicated at right. (**B**) Primer extension DNase I footprinting analysis. Separate aliquots of the chromatin preparations characterized in (A) were subjected to DNase I digestion and primer extension footprinting (*9*). The locations of R3 binding sites are indicated by the brackets at the left of the autoradiogram. The asterisk denotes an artifact band that is not part of the footprint.

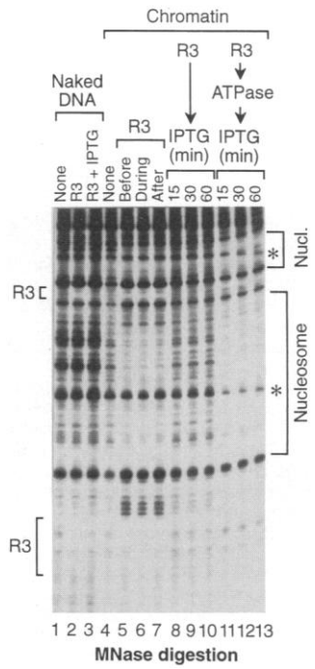


Fig. 2. High-resolution analysis of R3-induced nucleosome positioning. Aliquots of the chromatin preparations characterized in Fig. 1 were subjected in parallel to micrococcal nuclease digestion and primer extension footprinting (*9*). The samples shown in lanes 1 through 13 of each part of this figure correspond to the samples in lanes 1 through 13 of Fig. 1. The nucleosome footprint (Nucleosome) between the *lac* operators is denoted by a bracket, as is an adjacent footprint that is interpreted to be due to a flanking nucleosome (Nucl.). The asterisks denote artifact bands that are not part of the footprint.

I (DNase I) footprinting (Fig. 1B) in parallel with micrococcal nuclease digestion and indirect end-labeling analysis (*19*).

The nucleosome movement that occurred after dissociation of R3 protein from chromatin could be either spontaneous or facilitated by mobilization factors (*20*). *Drosophila* chromatin assembly extracts contain activities that facilitate nucleosome rearrangement in an ATP-dependent manner (*6*, *8*, *9*, *14*, *15*). We therefore tested whether the loss of specific nucleosome positioning that was observed upon dissociation of R3 protein would be affected by the depletion of ATP by apyrase, a plant adenosine triphosphatase (ATPase) and adenosine diphosphatase. After chromatin was assembled in the presence of R3 protein, apyrase was added to hydrolyze ATP, and IPTG was then added to dissociate R3. Micrococcal nuclease digestion and indirect end-labeling analysis revealed that the R3-induced chromatin structure remained unchanged for at least 60 min in the absence of ATP (Fig. 1A). DNase I footprinting analysis of separate aliquots of the same chromatin samples confirmed that the R3 protein had dissociated upon the addition of IPTG (Fig. 1B). The immobility of nucleosomes in the absence of ATP is consistent with the results of earlier studies of nucleosome mobility (performed in the absence of ATP-utilizing mobilization factors) with SV40 minichromosomes or with native rat liver chromatin fragments (*21*). Given the availability of ATP in most cells (at ~1 to 3 mM concentration), the data collectively suggest that a DNA binding factor is continuously required for the maintenance of specific nucleosome positioning and support a view of chromatin in cells as dynamic and mobile rather than static.

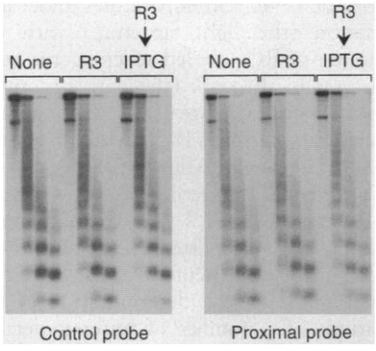


Fig. 3. The binding and dissociation of the *lac* repressor does not disrupt the periodicity of nucleosomal arrays. Chromatin was assembled in a 5-hour reaction period under conditions described in the legend to Fig. 1. Where indicated, the R3 *lac* repressor (50 nM) was present during chromatin assembly. "IPTG" denotes that IPTG (0.5 mM) was added to the reaction 15 min before the termination of the reaction. The resulting chromatin was treated with micrococcal nuclease, and DNA was purified and subjected to Southern blot analysis (*9*). The blot was hybridized sequentially to oligonucleotide probes complementary to DNA immediately adjacent (~60 bp) to the *lac* operators (proximal) or ~900 bp away from the nearest *lac* operator (control).

Primer extension analysis of chromatin after partial digestion with micrococcal nuclease (Fig. 2) (*22*) revealed a distinct nucleosome footprint between the *lac* operators that is dependent on the binding of R3 to chromatin but not to naked DNA (Fig. 2). Moreover, the footprint of an adjacent nucleosome is also apparent at the top of the autoradiogram. Consistent with the results obtained with the indirect end-labeling studies (Fig. 1A), the nucleosome footprints disappeared upon dissociation of R3 with IPTG in the presence, but not in the absence, of ATP (Fig. 2, compare lanes 8 through 10 with lanes 11 through 13). Thus, these data support the conclusions that a nucleosome is positioned between the two *lac* operators and that, as long as the chromatin remodeling system is active, R3 protein is continuously required to maintain this nucleosome positioning.

We then analyzed the effect of R3 binding on neighboring nucleosomes (Fig. 3). We probed a Southern (DNA) blot of micrococcal nuclease–digested chromatin with oligonucleotides complementary to DNA 60 and 900 bp away from the *lac* operators. In the vicinity of both probes, the nucleosomes remained in periodic arrays upon binding and dissociation of R3 protein. The periodicity of the nucleosome arrays was not affected by the active repositioning of nucleosomes after R3 dissociation.

In the absence of R3 protein, nucleosomes are in extended periodic arrays, spaced about 175 bp apart but not in register with a particular DNA sequence. Upon addition of R3 protein, the five nucleosomes in the vicinity of the R3 binding sites are repositioned in such a way that one nucleosome is located between the two *lac* operators. Upon dissociation of R3 with IPTG, the nucleosomal arrays revert, in an ATP-dependent process, to a state that does not exhibit correspondence with any specific DNA sequence. The ability to direct and confine one and perhaps more nucleosomes to a specific location by addition of a dissociable protein is likely to be useful for the study of chromatin structure and function.

The ability of R3, a prokaryotic sequence–specific DNA binding protein, to program changes in chromatin structure is similar to activities that have been described for eukaryotic DNA binding proteins. Positioning of nucleosomes by other factors that do not contain a transcriptional activation domain has been observed [truncated Gal4p fragments (*9*), NF-κB p50 (*10*), and a truncated form of NF-κB p65 (*10*)]. Thus, if a DNA binding factor in a transcriptionally inactive state (for example, lacking a transcriptional activation domain or bound by a repressor that masks the activation domain) can bind to its DNA site, it can induce a change in the chromatin structure of that gene, perhaps in preparation for a later activation event that will signal the onset of transcription. DNA binding and chromatin reconfiguration need not immediately lead to transcriptional activation. Inducible but quiescent genes have been observed in vivo to be bound by DNA binding proteins and to possess a reconfigured chromatin structure before transcriptional induction (*23*); and it has also been seen in vitro that factor binding and chromatin reconfiguration contribute to, but are not sufficient for, transcriptional activation (*9*, *10*).

The maintenance of specific nucleosome positioning by a DNA binding factor requires the continuous presence of that factor, due to nucleosome movement that is mediated by ATP-utilizing mobility factors. These results are consistent with the continuous requirement for a DNA-bound transcriptional activator for the maintenance of transcriptional activity in vivo (*24*). Furthermore, the easily reversible changes in nucleosome positioning (within 15 min of addition of IPTG) reported here are consistent with demonstrations of rapid (within 15 to 30 min) and reversible alterations of chromatin structure in vivo (*25*). We suggest that nucleosomes are not static and immovable barriers to DNA binding proteins such as transcriptional regulatory factors, but rather are dynamic components of gene structure that accommodate, with the assistance of ATP-utilizing mobilization factors, the functions of sequence-specific DNA binding proteins.

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23. With other factors and in different contexts, an activation region might be required for binding to the chromatin template, especially in cases of cooperative binding of transcription factors with other proteins that is mediated through interactions with the transcriptional activation domain. In addition, we do not imply that all sequence-specific DNA binding proteins (either prokaryotic or eukaryotic) must necessarily alter chromatin structure.
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Light-Induced Structural Changes in Photosynthetic Reaction Center: Implications for Mechanism of Electron-Proton Transfer

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High resolution x-ray diffraction data from crystals of the *Rhodobacter sphaeroides* photosynthetic reaction center (RC) have been collected at cryogenic temperature in the dark and under illumination, and the structures were refined at 2.2 and 2.6 angstrom resolution, respectively. In the charge-separated $D^+Q_AQ_B^-$ state (where D is the primary electron donor (a bacteriochlorophyll dimer), and Q_A and Q_B are the primary and secondary quinone acceptors, respectively), Q_B^- is located approximately 5 angstroms from the Q_B position in the charge-neutral (DQ_AQ_B) state, and has undergone a 180° propeller twist around the isoprene chain. A model based on the difference between the two structures is proposed to explain the observed kinetics of electron transfer from $Q_A^-Q_B$ to $Q_AQ_B^-$ and the relative binding affinities of the different ubiquinone species in the Q_B pocket. In addition, several water channels (putative proton pathways) leading from the Q_B pocket to the surface of the RC were delineated, one of which leads directly to the membrane surface.

The primary processes of photosynthesis, the conversion of electromagnetic energy (light) into chemical energy, are mediated by an integral membrane protein-pigment complex called the reaction center (RC) in which a sequence of photoinduced electron and proton-transfer reactions take place (*1*, *2*). Our knowledge of these processes was greatly enhanced through the determination of the three-dimensional structure of the RC from two purple photosynthetic bacteria: *Rhodopseudomonas viridis* (*3*, *4*) and *Rhodobacter sphaeroides* (*5*–*8*). In previous structure determinations, the primary reactants were in the neutral state, that is, no electron transfer (charge separation) had taken place. Several independent experimental findings, however, point toward a structural change accompanying charge separation (*9*–*15*). A particularly dramatic effect was observed (*11*) when the rate of electron transfer in RCs that were frozen under illumination was compared with that of those frozen in the dark. The rate of the electron transfer from the primary ubiquinone Q_A^- to the secondary ubiquinone Q_B was increased by several orders of magnitude when RCs were frozen under illumination, that is, in the charge separated state, as compared to RCs frozen in the dark.

We have now determined the structural changes accompanying charge separation in the RC and used them as a basis for a model to explain changes in the kinetics of electron transfer observed on freezing. The changes were obtained by comparing the structure of RCs in single crystals cooled to cryogenic (~90 K) temperatures under illumination (the light structure) with the structure of RCs cooled to cryogenic temperatures in the dark (the dark structure). In our experiments, we used tetragonal crystals of *Rb. sphaeroides* R-26, which had been obtained earlier by Allen (*16*). Our crystals diffracted at cryogenic temperatures to higher resolution (1.9 Å in the dark state) than previously reported RC crystals. Data collection and refinement to 2.2 Å resolution has led to the determination of the positions of a number of water molecules that provide several possible pathways for protons from the aqueous phase to the Q_B pocket. Two of these proton "channels" are delineated in detail; one of these has been reported (*8*). The importance of water molecules for proton transfer to reduced Q_B is discussed below.

RCs from *Rb. sphaeroides* R-26 were isolated and purified (*17*). Crystallization conditions were similar to those described (*16*). Crystals grew in 1 to 3 weeks to a thickness of 0.1 to 0.2 mm in space group $P4_32_12$ (unit cell dimensions $a = b = 140.1$ Å and $c = 271.6$ Å), with two RCs in the asymmetric unit (Table 1). Typical crystals

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