Research Article

Role of Nucleosomal Cores and Histone H1 in Regulation of Transcription by RNA Polymerase II

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The relation between chromatin structure and transcriptional activity was examined by in vitro transcription analysis of chromatin reconstituted in the absence or presence of histone H1. To maintain well-defined template DNA, purified components were used in the reconstitution of chromatin. Reconstitution of nucleosomal cores to an average density of 1 nucleosome per 200 base pairs of DNA resulted in a mild reduction of basal RNA polymerase II transcription to 25 to 50 percent of that obtained with naked DNA templates. This nucleosome-mediated repression was due to nucleosomal cores located at the RNA start site and could not be counteracted by the sequence-specific transcription activators Sp1 and GAL4-VP16. When H1 was incorporated into the chromatin at 0.5 to 1.0 molecule per nucleosome (200 base pairs of DNA), RNA synthesis was reduced to 1 to 4 percent of that observed with chromatin containing only nucleosomal cores, and this H1-mediated repression could be counteracted by the addition of Sp1 or GAL4-VP16 (antirepression). With naked DNA templates, transcription was increased by a factor of 3 and 8 by Sp1 and GAL4-VP-16, respectively (true activation). With H1-repressed chromatin templates, however, the magnitude of transcriptional activation mediated by Sp1 and GAL4-VP16 was 90 and more than 200 times higher, respectively, because of the combined effects of true activation and antirepression. The data provide direct biochemical evidence that support and clarify previously proposed models in which there is depletion or reconfiguration of nucleosomal cores and histone H1 at the promoter regions of active genes.

TO UNRAVEL THE MECHANISMS BY WHICH GENES ARE activated in eukaryotes, it is necessary to examine the factors that are involved in the pathway leading to initiation of transcription. Transcription of protein coding genes requires the general transcriptional machinery, which includes RNA polymerase II and several auxiliary factors (*1*). Synthesis of RNA by the general factors is regulated by sequence-specific DNA binding factors that interact with proximal promoter and enhancer elements (*2*). Chromatin also has a role in the regulation of gene activity (*3*). For example, the conversion of genes from a repressed state to a transcriptionally active or competent state is often accompanied by alterations in chromatin structure in vivo (*3, 4*). Moreover, variations in the expression or structure of the core histones in vivo in yeast may increase expression of a subset of genes (*5*).

The study of transcriptional activation entails the analysis of interactions between transcription factors and chromatin. For such experiments, it is necessary to perform in vitro transcription reactions with both the general and sequence-specific factors and to reconstitute the chromatin templates. With RNA polymerase II, reconstitution of chromatin results in repression of transcription in vitro (*6–9*). Moreover, the TATA box–binding factor (TFIID), transcription initiation complexes, or sequence-specific DNA binding factors can, when bound or assembled on the template before chromatin reconstitution, counteract repression of transcription that was mediated by the formation of chromatin (*8, 10–14*). However, with one exception (*9*), these earlier studies were performed with relatively crude chromatin reconstitution systems containing either crude or heat-treated *Xenopus* extracts. Furthermore, the specific roles of the nucleosomal cores and histone H1 in the regulation of RNA polymerase II transcription remain to be clarified.

We had observed that histone H1 is a potent repressor of transcription by RNA polymerase II and that sequence-specific transcription factors were capable of counteracting H1-mediated repression (antirepression) (*15*). In those studies, H1-DNA complexes were used as a model for the repressed state of chromatin. Although there are many aspects of H1 binding to naked DNA that resemble its interaction with chromatin (*15, 16*), whether or not H1-DNA complexes were a relevant model for chromatin was an issue of concern. We now describe our studies on the nucleosomal cores in the absence or presence of H1 in the context of basal and regulated transcription by RNA polymerase II.

Effect of nucleosomal cores on RNA polymerase II transcription. To study the relation between chromatin structure and gene activity, we examined the effect of the nucleosomal cores on transcription by RNA polymerase II. In previous studies on RNA polymerase II transcription with chromatin templates (*6–8, 10–13*), the distinction between the effects of the nucleosomal cores and histone H1 generally was not made. A notable exception was the investigation of Lorch *et al.* (*9*), in which repression of RNA polymerase II transcription was observed upon deposition of a nucleosomal core onto a 182-bp DNA fragment containing the adenovirus major late promoter. It was not known, however, whether the effect of a nucleosome on a short DNA fragment would be similar to that of nucleosomes on longer stretches of chromatin. To clarify this point as well as to provide a foundation for further studies on the incorporation of H1 into chromatin, we pursued an

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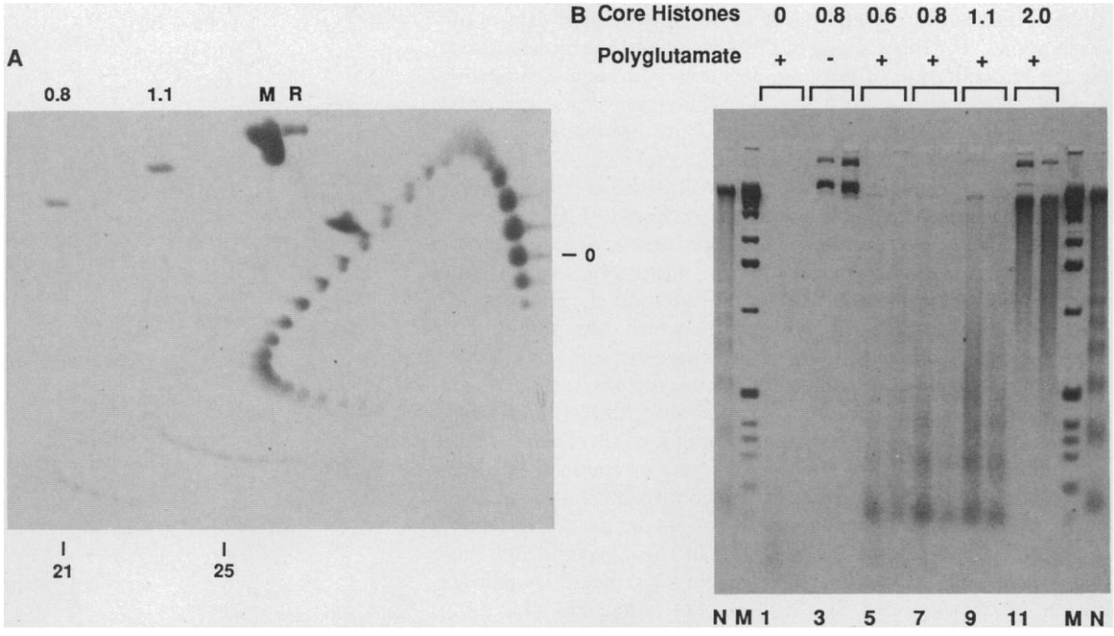

Fig. 1. Characterization of chromatin reconstituted from purified components. (**A**) Two-dimensional topological assay. Chromatin was reconstituted at mass ratios of core histone to DNA of 0.8 to 1.0 and 1.1 to 1.0, deproteinized (*39*), and then subjected to electrophoresis on a 1 percent agarose gel in the absence of chloroquine in the first dimension (top to bottom) and the presence of 4.1 μM chloroquine in the second dimension (left to right) (*19*). The DNA was visualized by staining with eithidium bromide. Lane M is the topological marker. Lane R is the relaxed, covalently closed circular marker. The numbers in the lower and right-hand margins indicate the linking numbers at those positions. (**B**) Micrococcal nuclease digestion of reconstituted chromatin. Reconstituted chromatin was partially digested with micrococcal nuclease (0.1 unit/ml; Sigma) at 37°C for 4 minutes (odd-numbered lanes) and 8 minutes (even-numbered lanes), deproteinized (*39*), and subjected to electrophoresis on a 1.5 percent agarose gel. Lanes N contain native calf thymus chromatin digested with micrococcal nuclease. Lanes M contain molecular mass markers (1-kb ladder; BRL). The presence or absence of polyglutamic acid (19 to 44 μg/ml, depending upon the mass ratio of core histone to DNA) is designated by the plus or minus signs. The ratios are also indicated.

analysis of RNA polymerase II transcription with nucleosomal templates.

To reconstitute the chromatin templates, we used purified components to maintain a well-defined system. The reconstitution reactions (*17*) contained polyglutamic acid as a deposition vehicle (*18*), purified core histones, plasmid DNA, and purified topoisomerase I to maintain the circular DNA in a relaxed state. To characterize the chromatin, two-dimensional topological analysis (*19*) and partial digestion with micrococcal nuclease (*20*) were performed (Fig. 1). In the presence of topoisomerase I, the deposition of one nucleosome on a closed circular DNA results in a change of −1 in the linking number (*21*). Thus, we measured the efficiency of nucleosome reconstitution by determining the total change in the linking number of the plasmids by two-dimensional gel electrophoresis under conditions that allowed resolution of each of the topoisomers (*19*), and found a quantitative deposition of nucleosomes (Fig. 1A). At a mass ratio of 0.8 to 1.0 of core histone to DNA there was an average of one nucleosome per 200 bp of

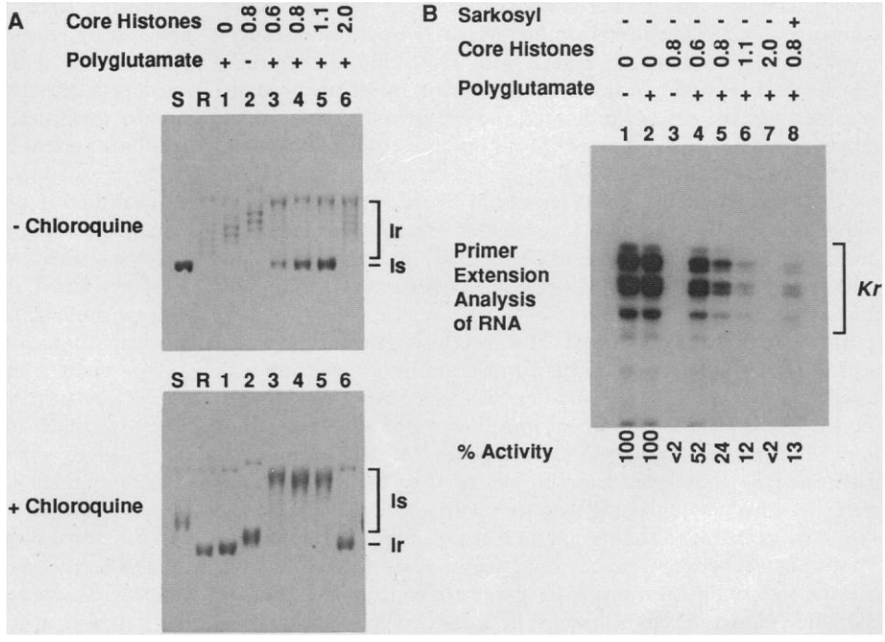

Fig. 2. In vitro transcription analysis of chromatin reconstituted with purified components. (**A**) Efficiency of chromatin reconstitution, as measured by supercoiling of relaxed, circular template DNA. Chromatin was reconstituted onto relaxed pKr plasmid DNA, which contains the proximal promoter region of the *Drosophila Krüppel* gene (*40*). The samples were deproteinized (*39*) and subjected to electrophoresis on 1 percent agarose gels either in the absence or in the presence of 4.1 μM chloroquine. The DNA was visualized by staining with eithidium bromide. Lanes S are the supercoiled DNA markers. Lanes R are the relaxed DNA markers. Lanes 1 to 6 contain the resulting DNA obtained after deproteinization of chromatin (*39*) that was reconstituted with the indicated ratios. The positions of relaxed circular (Ir) and supercoiled plasmid DNA (Is) are indicated. (**B**) In vitro transcription with the chromatin templates. Reconstituted chromatin (containing 100 ng of template DNA) was transcribed with a *Drosophila* nuclear extract (*41*), and RNA synthesis was detected by primer extension analysis (*25*). The reverse transcription products of *Krüppel* RNA are shown. The *Krüppel* promoter directs transcription from several sites clustered over a ten-base region (*25, 40*). (Lane 1) Naked DNA template; (lane 2) template DNA plus polyglutamic acid; (lane 3) template DNA plus core histones; (lanes 4 to 7) template DNA plus polyglutamic acid plus core histones at the indicated mass ratios of histone to DNA; (lane 8) same as lane 5, except that the detergent Sarkosyl was added to limit transcription to a single round (*40*). The transcriptional activity relative to naked DNA (= 100 percent) is indicated at the bottom of the figure.

DNA, which is roughly equivalent to the physiological density of nucleosomes. The supercoiling of DNA is not necessarily diagnostic for the reconstitution of nucleosomes, however, because subcomponents of chromatin, such as $(H3)_2(H4)_2$ tetramers, can also induce supercoiling of circular DNA in a manner that is indistinguishable from that of complete nucleosomes (*22*). Hence, the reconstituted chromatin was digested with micrococcal nuclease (*20*) to determine the nucleosomal repeat length (Fig. 1B). We found a nucleosomal repeat length of about 150 bp, an indication of short stretches of closely packed complete nucleosomes rather than a distribution of one every 200 bp (*23*). In addition, the polypeptide composition of reconstituted chromatin, which was purified by sucrose gradient centrifugation, was determined by SDS gel electrophoresis, and the four core histones were present in the normal stoichiometry. It thus appeared that complete nucleosomes were reconstituted from the purified core histones and polyglutamic acid.

To examine the effect of nucleosomal cores on transcription, we then performed in vitro transcription reactions with chromatin that was reconstituted with varying mass ratios of core histone to DNA in the absence of histone H1 (Fig. 2). In these experiments, the *Drosophila Krüppel* gene was transcribed with a standard *Drosophila* nuclear extract (*24*) that was depleted of histone H1 (*15, 25*). Nucleosomes were deposited quantitatively onto the template DNA (Fig. 2A) (*17, 23*). Then, in vitro transcription reactions with the chromatin templates revealed a progressive decrease in the amount of RNA synthesis that was proportional to the increase in the mass ratio of the core histone to DNA (Fig. 2B). At a ratio of 0.8 to 1.0, which corresponds to the physiological density of one nucleosome per 200 bp (Fig. 1A), the transcriptional efficiency was 24 percent relative to that of naked DNA (Fig. 2B, lanes 1 and 5). These results indicate that inhibition of transcription by nucleosomes increases progressively with the degree of chromatin reconstitution and that a physiological density of nucleosomal cores results in roughly fourfold repression of transcription relative to that of naked DNA.

In contrast, reconstitution of nucleosomes has been found to completely repress transcription by RNA polymerase II (*6–9*). Since it was possible that we had observed abnormally high levels of transcription with the chromatin templates because we used *Drosophila* templates and transcription factors rather than the more commonly used factors from mammalian cells, we performed experiments similar to those in Fig. 2 with HeLa (*26*) rather than *Drosophila* transcription extracts, the adenovirus major late promoter rather than the *Krüppel* promoter, and calf thymus core histones rather than *Drosophila* histones. In all instances, the results were identical to those shown in Fig. 2; hence, these data support the conclusion that transcriptional repression by nucleosomal cores is roughly proportional to the occupancy of the template DNA by nucleosomes. A qualitatively similar effect had also been observed in studies on RNA polymerase II and RNA polymerase III transcription with chromatin templates (*6, 14*).

Blockage of RNA polymerase II transcription by nucleosomes at the RNA start site. To clarify the mechanism of transcriptional repression by the nucleosomal cores, we considered two possible models. First, nucleosomes might impede but not block transcription. Alternatively, some of the templates may be completely inactivated by a static nucleosome located over the transcription start site, while the remaining templates with unoccupied start sites would be accessible to factors and therefore transcriptionally active. To distinguish between these two mechanisms, we used restriction enzyme accessibility as a probe for templates containing a nucleosome-free region in the vicinity of the transcription start site (*27*) (Fig. 3). If the chromatin was digested with the restriction enzyme Xba I, which cleaves the template DNA 10 bp downstream of the transcription start site, templates that do not have a nucleosome

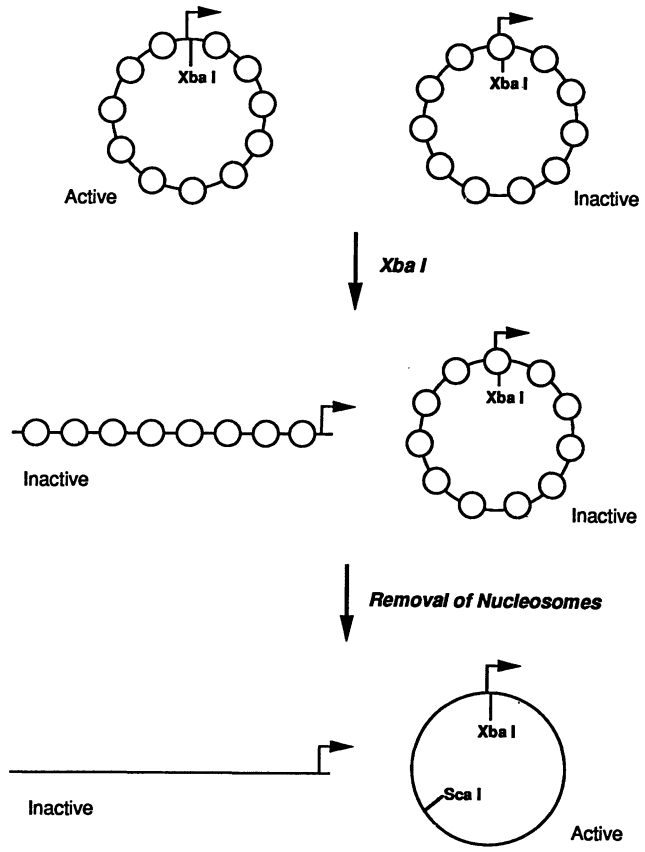

Fig. 3. Scheme for enrichment of templates containing a nucleosome located over the transcription start site.

located over the start site would be linearized and incapable of yielding a transcript that could be detected by primer extension analysis. The remaining circular Xba I–resistant templates with a nucleosome located over the transcription start site would then be subjected to in vitro transcription analysis. If the Xba I–resistant chromatin were transcriptionally inactive, then transcriptional blockage by a nucleosome over the RNA start site would be favored. Alternatively, if the Xba I–resistant chromatin had residual transcriptional activity that was proportional to the remaining intact circular templates, then transcriptional impedance, but not blockage, by a nucleosome would be likely.

These experiments were performed as follows. Chromatin was reconstituted at a mass ratio of core histone to DNA of 0.8 to 1.0 (one nucleosome per 200 bp; Fig. 1A) on the plasmid pSV-Kr, which contains the three 21-bp repeat elements of the SV40 early promoter fused upstream of the TATA box of the *Drosophila Krüppel* minimal promoter (*15, 25*). This plasmid is responsive to transcription factor Sp1 (*2*), which binds to five sites in the SV40 21-bp repeat elements. Next, the reconstituted chromatin was purified by sucrose gradient centrifugation (*28*). A portion of the sucrose gradient-purified chromatin was also subjected to salt gradient dialysis against buffer containing from 0.6 to 0.05 M KCl (*29*). Since there is a significant degree of nucleosome sliding at 0.6 M NaCl (*30*), we tested the effect of nucleosome sliding on the transcriptional properties of the chromatin. Then, the naked pSV-Kr DNA, the sucrose gradient-purified chromatin, and the salt gradient–dialyzed chromatin were subjected to digestion with Xba I, Sca I (which, as a control, linearizes pSV-Kr at a position located 1700 bp upstream of the transcription start site; Fig. 3), or no restriction enzyme (as a control). Finally, the digested templates were purified by sucrose gradient centrifugation to remove the

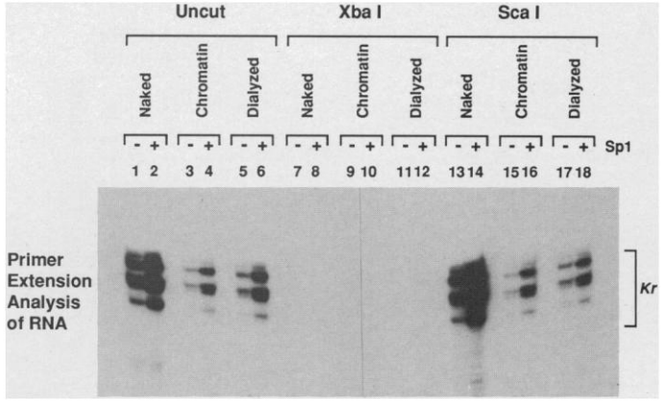

Fig. 4. Nucleosomes located at the transcription start site block RNA polymerase II transcription. Naked pSV-Kr template DNA (*15*), sucrose gradient-purified chromatin, and salt gradient-dialyzed chromatin were either unmodified or digested with Xba I or Sca I (see Fig. 3). The resulting samples (50 ng of DNA in each reaction) were then transcribed in vitro (*41*) with the soluble nuclear fraction (*42*) in the absence or the presence of transcription factor Sp1. The Sp1 was purified from HeLa cells (*43*), and the protein (15 ng, which corresponds to a 1.3 times molar excess of Sp1 monomers to Sp1 binding sites) was added as indicated. (Lanes 1 to 6) Undigested templates; (lanes 7 to 12) Xba I–digested templates; (lanes 13 to 18) contained Sca I–digested templates. The naked DNA was designated as "Naked"; the sucrose gradient-purified chromatin was designated as "Chromatin"; and the salt gradient-dialyzed chromatin was designated as "Dialyzed." The reverse transcription products of *Krüppel* RNA are shown.

restriction enzyme, and in vitro transcription reactions were carried out in the absence or presence of Sp1 (Fig. 4).

With the undigested templates, the basal transcription was four to five times lower when the chromatin was reconstituted (Fig. 4, compare lane 1 with lanes 3 and 5), as would be expected from the previous results (Fig. 2B, lanes 1 and 5). Upon digestion with either Xba I or Sca I, the naked DNA template was completely (>98 percent) linearized, whereas the chromatin templates were partially (30 to 45 percent) linearized because of blockage of digestion by nucleosomes. Digestion of the templates with Xba I led to almost a complete loss (less than 1 percent activity) of transcription from the naked DNA or chromatin templates (Fig. 4, lanes 7 to 12). In contrast, digestion with Sca I did not affect the amount of transcription (Fig. 4, lanes 13 to 18). Thus, linearization of the template at a position distant from the RNA start site did not result in inhibition of transcription. These data suggest that RNA polymerase II transcription is blocked by a nucleosome located in the vicinity of the RNA start site. The results also indicate that the transcriptional properties of the chromatin do not change significantly on salt gradient dialysis.

We had found that Sp1, GAL4-VP16, and the GAGA factor were able to counteract transcriptional repression that was mediated by histone H1 (*15*), and we therefore examined whether or not the sequence-specific factors were also able to counteract nucleosome-mediated repression. The Sp1-mediated increase in transcription varied from 2.5- to 3 times that obtained in the absence of the factor with either the naked DNA or chromatin templates (Fig. 4, compare lanes 1 and 2; 3 and 4; 5 and 6). These data indicate that Sp1 cannot counteract nucleosome-mediated repression after the cores have been assembled. Hence, there are distinct differences in the ability of Sp1 to counteract transcriptional repression that is mediated by either histone H1 or nucleosomal cores. Similar experiments with the GAL4-VP16 activator (*31*) yielded identical results.

It is possible that, by an unforeseen mechanism, Xba I digestion of the chromatin templates had resulted in complete inactivation of transcription, even though about 55 to 70 percent of templates had remained circular after treatment with the restriction enzyme. To address this possibility, we deproteinized the Xba I–digested chromatin and the resulting naked DNA templates were then subjected to in vitro transcription analysis (Fig. 3). An estimated 30 to 45 percent of the chromatin templates were linearized by Xba I (Fig. 5A). Deproteinization of the transcriptionally inactive, Xba I–digested chromatin resulted in recovery of 58 percent of the transcriptional activity relative to the naked DNA template (Fig. 5B, lanes 1 and 4). This level of transcription correlates well with the estimated 55 to 70 percent of the template DNA that was protected from Xba I

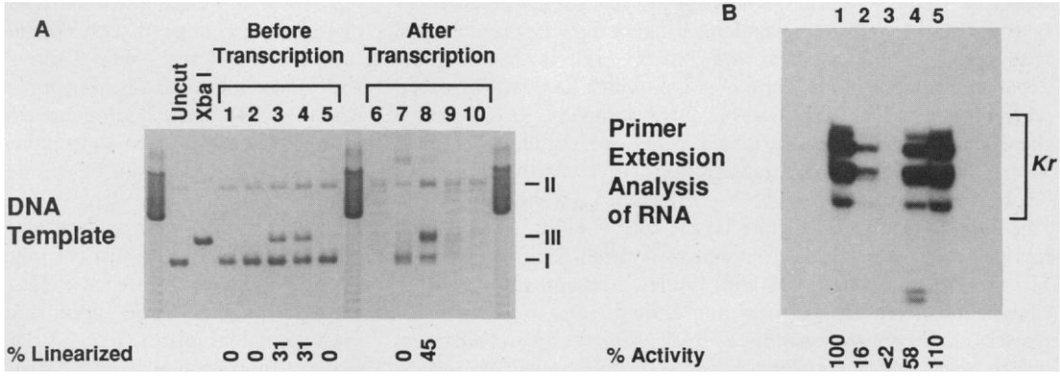

Fig. 5. Removal of nucleosomes restores transcriptional competence of chromatin with nucleosomes located over the RNA start site. Chromatin was reconstituted on pSV-Kr template at a mass ratio of core histone to DNA of 0.8 to 1.0 and then purified by sucrose gradient centrifugation (*28*). A fraction of the purified chromatin was digested with Xba I, and a portion of the Xba I–digested chromatin was deproteinized by treatment with proteinase K with subsequent extraction with phenol and chloroform. A portion of the intact sucrose gradient-purified chromatin was also deproteinized. These samples were then subjected to in vitro transcription analysis with the *Drosophila* soluble nuclear fraction (*42*). (**A**) Agarose gel electrophoresis of various chromatin and naked template DNA's. A portion of the template DNA's was subjected to gel electrophoresis before addition to the transcription reactions (lanes 1 to 5). Another portion of the template DNA's was subjected to gel electrophoresis after recovery from completed in vitro transcription reactions (lanes 6 to 10). Because of high concentrations of topoisomerase I in transcription extracts, there is significant relaxation of the template DNA's during transcription. The DNA was visualized by staining with eithidium bromide. Lanes designated "Uncut" and "Xba I" contained undigested and Xba I–digested plasmid markers. (Lanes 1 and 6) undigested naked DNA; (lanes 2 and 7) undigested chromatin; (lanes 3 and 8) Xba I–digested chromatin; (lanes 4 and 9) Xba I–digested and deproteinized chromatin; (lanes 5 and 10) undigested and deproteinized chromatin. The percentage of linearization is indicated at the bottom of the panel. The positions of nicked or relaxed circular DNA (form II), linear DNA (form III), and supercoiled DNA (form I) are denoted at the right margin. (**B**) In vitro transcription analysis. Reactions with the indicated templates (50 ng of DNA) resulted in transcripts that were detected by primer extension analysis (*25*). (Lane 1) Undigested naked DNA; (lane 2) undigested chromatin; (lane 3) Xba I–digested chromatin; (lane 4) Xba I-digested and deproteinized chromatin; (lane 5) undigested and deproteinized chromatin. Transcription activity is indicated at the bottom relative to that from the undigested, naked DNA template (= 100 percent). The reverse transcription products of *Krüppel* RNA are shown.

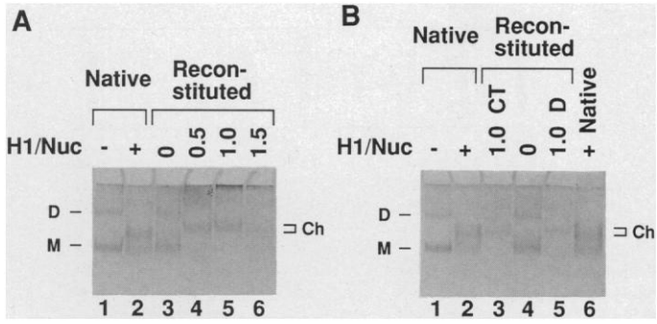

Fig. 6. Nucleoprotein gel electrophoresis of chromatin reconstituted with histone H1. Chromatin was reconstituted with purified core histones at a mass ratio of core histones to DNA of 8.0 to 1.0 to give an average of one nucleosome per 200 bp. The chromatin was purified by sucrose gradient centrifugation (*28*), and H1 was incorporated by salt gradient dialysis (*29, 34*) as indicated (in molecules of H1 per nucleosome). The samples were then digested with micrococcal nuclease to yield either core particles or chromatosomes, which were then analyzed by nondenaturing gel electrophoresis (*44*). Native core particles and chromatosomes from calf thymus were used as references. The location of monomeric core particles was designated "M"; the migration of H1-stripped dinucleosomes was designated "D"; and the migration of chromatosomes was indicated "Ch." Chromatosomes containing *Drosophila* H1 migrated in the gel slightly more slowly than chromatosomes containing calf thymus H1, probably because *Drosophila* H1 is slightly larger than calf thymus H1. (**A**) Comparison of native and reconstituted core particles and chromatosomes. *Drosophila* H1 was incorporated into the reconstituted chromatin at the indicated number of molecules of H1 per nucleosome. (Lane 1) Native calf thymus core particles; (lane 2) native calf thymus chromatosomes; (lane 3) reconstituted core particles; (lanes 4 to 6) reconstituted chromatosomes containing *Drosophila* H1. (**B**) Comparison of chromatosomes reconstituted with either *Drosophila* H1 or calf thymus H1. (Lane 1) Native calf thymus core particles; (lanes 2 and 6) native calf thymus chromatosomes; (lane 3) reconstituted chromatosomes with calf thymus H1; (lane 4) reconstituted core particles; (lane 5) reconstituted chromatosomes with *Drosophila* H1.

digestion by nucleosomes (Fig. 5A). Therefore, it is likely that the Xba I–mediated blockage of transcription from the chromatin templates was due to linearization of templates within a nucleosome-free region in the vicinity of the RNA start site. These data, in the strictest sense, indicate that a nucleosome positioned at the start site such that Xba I digestion is blocked also inhibits transcription initiation by RNA polymerase II, and the exact positioning of the nucleosomes relative to the RNA start site is not revealed by these experiments.

Sequence-specific antirepression of H1-mediated inhibition of transcription with nucleosomal templates. We next sought to incorporate histone H1 into the reconstituted chromatin to investigate the role of H1 in repression of RNA polymerase II transcription. In particular, we analyzed the transcriptional properties of H1-containing chromatin templates to examine both "true activation," in which sequence-specific transcription factors facilitate the inherent transcription process, and "antirepression," in which the sequence-specific factors counteract chromatin-mediated repression of basal transcription. In the absence of nucleosomal cores, H1 is a potent repressor of RNA polymerase II transcription, but complete repression of transcription requires one molecule of H1 per 30 to 45 bp of DNA (*15*), which is several times higher than the physiological level of one molecule of H1 per 200 bp (*32*). It was therefore debatable whether or not antirepression, which had been observed with transcriptionally repressed H1-DNA complexes (*15*), reflected the natural mechanisms by which genes are regulated in vivo. H1-mediated repression of transcription with chromatin templates has been demonstrated for RNA polymerase III transcription (*29, 33*); but with RNA polymerase II, the role of H1 in transcription repression has not yet been addressed with chromatin templates. Moreover, in previous work with crude chromatin reconstitution systems (*6–8, 10–13*), it was not known what proportion of transcription repression was due to nucleosomal cores and what was due to histone H1. Thus, it was necessary to examine the specific role of H1 in the regulation of transcription by RNA polymerase II with chromatin templates.

For such experiments, reconstitution of H1-containing chromatin was required. First, nucleosomal cores were deposited onto the DNA templates at a mass ratio of core histone to DNA of 0.8 to 1.0 (one nucleosome per 200 bp) and then purified by sucrose gradient centrifugation (*28*). To incorporate H1 into the chromatin, we used salt gradient dialysis (*29, 34*). For reactions including sequence-specific activators (either GAL4-VP16 or Sp1), both H1 and the activator protein were added together at the beginning of the dialysis to provide direct competition between H1 and the sequence-specific factors for the DNA templates (*35*).

The interaction between H1 and the reconstituted chromatin was characterized as follows. First, sucrose gradient centrifugation indicated that H1-containing chromatin sedimented more rapidly than chromatin having only nucleosomal cores. Then, SDS polyacrylamide gel analysis of the polypeptides in the sucrose gradient-purified chromatin revealed that the incorporation of H1 into the chromatin was quantitative. We also examined the nature of the interaction of H1 with the nucleosomes and found that there was quantitative formation of chromatosome-like structures when H1 was incorporated into the chromatin (Fig. 6A). The reconstituted chromatosomes were indistinguishable from the native chromatosomes (Fig. 6B). These findings suggested that the interaction of H1 with the reconstituted chromatin was similar to that with native chromatin, and we thus proceeded with in vitro transcription analysis.

First, in the absence of H1, there was a mild repression of basal RNA polymerase II transcription by the nucleosomal cores (Fig. 7A, lanes 1 and 3). The adenovirus E4 promoter was slightly less sensitive to nucleosomal repression than the *Krüppel* promoter, (Figs. 2B, 4, and 7B). When GAL4-VP16 was added to the nucleosomal templates, there was only a slight difference in the GAL4-VP16–mediated true activation with the naked DNA templates when compared with the nucleosomal templates (Fig. 7A, compare lanes 1 and 2 with 3 and 4). Similar results were obtained with transcription factor Sp1 (Fig. 7B, compare lanes 1 and 2 with lanes 3 and 4). These findings are consistent with the data presented in Fig. 4 and suggest that repression of transcription by the nucleosomal cores cannot be counteracted by the sequence-specific factors.

In vitro transcription analysis with the H1-containing chromatin templates revealed two distinct stages of transcriptional repression by H1. First, in the absence of H1, the amount of RNA synthesis was increased 12 times upon addition of GAL4-VP16 to the nucleosomal templates (Fig. 7A, lanes 3 and 4). Then, in the first stage of repression at 0.5 to 1.0 molecule of H1 per nucleosome, basal, but not GAL4-VP16-activated transcription was reduced to 1 to 4 percent of that obtained with chromatin to which H1 had not been incorporated (Fig. 7A, compare lanes 3, 5, and 7 to lanes 4, 6, and 8). With these H1-repressed nucleosomal templates, the magnitude of transcriptional activation by GAL4-VP16 was greater than 200 (Fig. 7A, lanes 5 and 6) because of the combined effects of true activation and antirepression. The absolute level of GAL4-VP16-activated transcription remained relatively constant (Fig. 7A, lanes 4 and 6) while basal transcription decreased measurably on incorporation of H1 into the chromatin (Fig. 7A; compare lanes 3 and 5). Transcriptional antirepression was also observed with Sp1 at 0.5 molecule of H1 per nucleosome (Fig. 7B). Therefore, with nucleo-

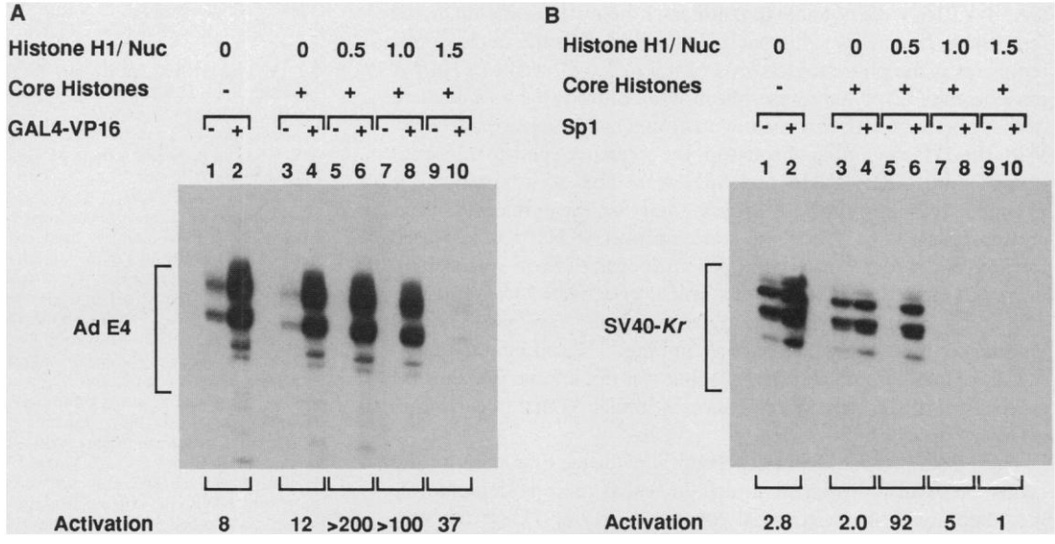

Fig. 7. Sequence-specific antirepression of histone H1-mediated inhibition of transcription by GAL4-VP16 and Sp1 with nucleosomal templates. The term "Activation" refers to the combined effects of antirepression and true activation with the chromatin templates (lanes 3 to 10) and to true activation only with the naked DNA templates (lanes 1 and 2). (**A**) GAL4-VP16. Chromatin was reconstituted on pG_5E4 (which contains 5 GAL4 binding sites upstream of the adenovirus E4 minimal promoter) (*45*) at a mass ratio of core histone to DNA of 0.8 to 1.0 and then purified by sucrose gradient centrifugation (*28, 34*). The purified chromatin was then subjected to salt gradient dialysis (*29*) from 0.6 M to 0.05 M KCl in the absence or presence of GAL4-VP16 (20 ng; three times molar excess of protein dimers per binding site) with variable amounts of purified histone H1 from *Drosophila* embryos (*15, 16*). The samples (50 nanograms of DNA each) were then subjected to in vitro transcription analysis with the soluble nuclear fraction (*42*). Lanes 1 and 2, naked DNA template; lanes 3 and 4, chromatin dialyzed in the absence of H1; lanes 5 to 10, chromatin dialyzed with the indicated amounts of H1 (given in molecules of H1 per nucleosome = 200 bp of DNA). The activation by GAL4-VP16 is given at the bottom. The reverse transcription products of adenovirus E4 RNA are shown. (**B**) Transcription factor Sp1. The experiments with Sp1 were done in a manner identical to that for GAL4-VP16, except that pSV-Kr template was used with transcription factor Sp1 (15 ng; 1.3 times molar excess of Sp1 monomers per binding site).

somal templates and physiological amounts of H1 (0.5 to 1.0 molecule per nucleosome) (*32*), Sp1 and GAL4-VP16 were able to counteract H1-mediated repression of basal RNA polymerase II transcription.

The second stage of H1-mediated repression was a sharp, nonlinear decrease in factor-activated transcription that occurred at 1.0 to 1.5 molecules of H1 per nucleosome. For instance, as H1 was increased from 1.0 to 1.5 molecules per nucleosome, GAL4-VP16–activated transcription decreased sharply (Fig. 7A, compare lanes 8 and 10). A similar decrease in Sp1-activated transcription was observed when H1 was increased from 0.5 to 1.0 molecule per nucleosome (Fig. 7B, compare lanes 6 and 8). Hence, transcription with the chromatin templates is highly sensitive to the levels of H1 in the chromatin, and the nonlinear decrease in transcription in the presence of varying amounts of H1 (see Fig. 7, A and B, compare lanes 4, 6, 8, and 10) is suggestive of a cooperative mechanism for repression of factor-activated transcription. We have also determined that the chromatin templates were soluble under the conditions of the transcription reactions (*36*), and thus, it is unlikely that insolubility and aggregation of the chromatin was responsible for the loss of transcription with the H1-containing templates.

Collectively, these findings suggest that H1 plays an important role in the repression of basal and activated RNA polymerase II transcription and that sequence-specific transcriptional activators function, in part, to counteract H1-mediated repression (antirepression) with chromatin templates. Yet, it was also possible that the antirepression effect was due to the formation of a complex between H1 and the transcription factor that prevented H1-mediated repression. To test this possibility, we performed experiments in which the concentrations of both H1 and GAL4-VP16 were varied and found that the concentration of GAL4-VP16 could be eight times above that used in the experiment shown in Fig. 7A without any detectable alteration in the amount of H1 required for repression. It is thus unlikely that GAL4-VP16-mediated antirepression was the simple consequence of complex formation between GAL4-VP16 and H1. If such a mechanism were true, then additional H1 would have been needed to repress transcription in the presence of the higher levels of GAL4-VP16.

Role of nucleosomal cores and histone H1 in the regulation of gene expression. We have focused on one stage in the activation of genes—the final release of chromatin-mediated transcriptional repression. We did not address the function of higher order structures, such as the 30-nm-diameter chromatin filament, locus boundary elements, and the nuclear matrix or scaffold, nor did we examine the role of nonhistone chromosomal proteins, such as high mobility group proteins, in the transcription process. When nucleosomes were reconstituted at an average density of 1 per 200 bp, there was mild (two to four times less) decrease in RNA polymerase II transcription relative to that with naked DNA templates, and the nucleosome-mediated repression was due to nucleosomal cores located at the RNA start site. This observation is consistent with other findings on transcriptional repression by nucleosomal cores with both RNA polymerase III and prokaryotic RNA polymerases (*9, 27, 37*). Two sequence-specific transcription factors, Sp1 and

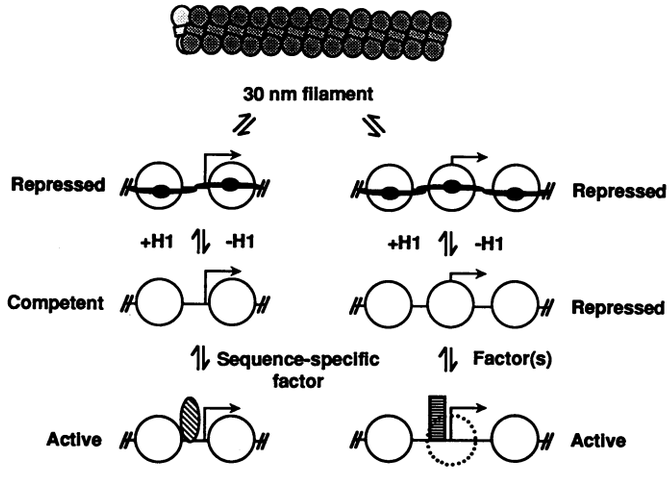

Fig. 8. A simple model for transcriptional activation.

GAL4-VP16, were not able to counteract the nucleosome-mediated repression. If, however, histone H1 was added to the nucleosomal templates at the physiological level of 0.5 to 1.0 molecule of H1 per nucleosome (*32*), basal transcription was reduced to 1 to 4 percent of that obtained with chromatin containing only nucleosomal cores. With the H1-containing chromatin, the sequence-specific transcription factors, GAL4-VP16 and Sp1, were able to counteract the H1-mediated repression, a process that we have referred to as antirepression (*15*). When the concentration of H1 was increased further to 1.0 to 1.5 molecules per nucleosome, there was a sharp decrease (more than 25 times less) in factor-activated transcription. This effect correlates with the transcriptionally repressed state of chicken erythrocytes, which have a combined H1 and H5 content of 1.3 molecules per nucleosome (*32*), but it is not known whether the mechanism of this general repression is similar to that occurring in avian erythrocytes.

These data provide direct biochemical evidence that support and clarify previously proposed models in which there is depletion or reconfiguration of nucleosomal cores and histone H1 at the promoter regions of active genes (*3, 4*). Moreover, the findings suggest that chromatin templates with physiological levels of nucleosomal cores and H1 can be used to study transcriptional activation by promoter- and enhancer-binding factors. In a schematic model for the steps involved in transcriptional activation (Fig. 8), inactive promoters were repressed either by a nucleosome located at the RNA start site or by histone H1 binding to linker DNA containing the RNA start site. Our data suggest that sequence-specific factors, such as Sp1 and GAL4-VP16, may be able to counteract inhibition of H1-repressed promoters by the antirepression mechanism. Yet, antirepression of nucleosome-mediated inhibition of transcription by either Sp1 or GAL4-VP16 was not observed. It is possible that some transcription factors can bind to DNA only during replication and chromatin assembly, whereas a specific subclass of factors may be able to displace or to cause reconfiguration of nucleosomes. For example, the binding of the glucocorticoid receptor to DNA that is simultaneously occupied by a positioned nucleosome in the mouse mammary tumor virus promoter has been well-characterized (*27, 38*), and the glucocorticoid receptor may mediate the reconfiguration of the positioned nucleosome prior to transcription initiation. The analysis of these and other interactions between chromatin and transcription factors should increase our understanding of the complex process of gene expression.

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17. Reconstitution of chromatin was carried out with the following purified components. Core histone octamers from calf thymus: (i) Calf thymus nuclei were prepared by blending 50 g of pulverized, frozen tissue in 50 mM tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1.2 M sucrose, 1 mM sodium metabisulfite, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 1 mM benzamidine and washing with nuclei washing buffer (NWB) consisting of 10 mM tris-HCl, pH 7.4, 3 mM $MgCl_2$, 350 mM sucrose, 1 mM sodium metabisulfite, 1 mM PMSF, 1 mM benzamidine; (ii) chromatin fragments were generated by partial digestion with micrococcal nuclease, and oligonucleosomes (6 to 18 nucleosomes) were purified by sucrose gradient centrifugation in the presence of 0.5 M NaCl to dissociate histone H1 (*46*); (iii) core histone octamers were then purified by hydroxylapatite chromatography as described (*47*) except that octamers were eluted in a single step from 0.3 to 2.5 M NaCl. Core histones from *Drosophila* embryos: (i) nuclei from *Drosophila* embryos were prepared as described (*24*) and washed twice with NWB; (ii) oligonucleosomes were prepared and core histones were purified as described for the calf thymus histones. The core histone concentrations were determined by the absorbance at 280 nm (for core histone, $A_{280\ nm} = 0.42$ for 1 mg/ml). Polyglutamic acid (Sigma P-4886; 50 to 100 kD) was chromatographed on Sephacryl S-300 HR, and the high molecular mass material in the void volume was pooled. The protein concentration of the polyglutamic acid was determined by amino acid analysis. Topoisomerase I was purified from *Drosophila* embryos (*48*). Chromatin was reconstituted as described (*18*). The reconstituted chromatin that was used in our work was prepared from core histones from calf thymus. Core histone octamers and polyglutamic acid were combined and dialyzed against transcription buffer for 16 hours, and the resulting polyglutamic acid-core histone octamer complexes were deposited onto relaxed, circular plasmid DNA in the presence of topoisomerase I.
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23. Although the micrococcal nuclease digestion revealed stretches of closely packed nucleosomes, the nucleosomes in general are probably distributed throughout the DNA templates in a random fashion because Stein and co-workers (*18*) showed by electron microscopy that polyglutamate-deposited nucleosomes are randomly distributed. In the reconstitution of the nucleosomes, we also observed the following. First, the core histones in the absence of polyglutamic acid formed an aggregate with the DNA that was refractory to micrococcal nuclease digestion (Fig. 1B, lanes 3 and 4). When the mass ratio of core histone to DNA was 2 to 1 in the reconstitution reactions, the resulting complexes were resistant to micrococcal nuclease digestion and did not yield a discernible oligonucleosome ladder or mononucleosome fragment (Fig. 1B, lanes 11 and 12). Therefore, chromatin that is reconstituted at a mass ratio of core histone to DNA of 2 to 1 may not have a normal nucleosomal structure, but might contain subnucleosomal particles such as the H3-H4 tetramer. In Fig. 2, we also observed abnormal behavior for chromatin that was reconstituted at a mass ratio of 2 to 1.
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28. Sucrose gradient centrifugation removes free histones as well as polyglutamic acid. Polyglutamic acid at concentrations 19 to 44 μg/ml would interfere with in vitro transcription with the soluble nuclear fraction (*42*), but not the standard nuclear extracts (*24*). Chromatin was purified by sucrose gradient centrifugation. Chromatin (0.5 ml containing 100 μg of DNA) was placed on a 13-ml linear sucrose gradient (5 to 30 percent, w/v) in 12.5 mM Hepes, K^+, pH 7.6, buffer containing 50 mM KCl, 0.05 mM EDTA, 0.5 mM DTT and 0.1 mM PMSF. The samples were centrifuged (Beckman SW41 rotor at 40,000 rpm) for 4 hours at 4°C. The chromatin peak was identified by absorbance at 260 nm.
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34. The salt gradient dialysis was performed at 4°C as follows. Solutions containing

sucrose gradient–purified chromatin (*17, 28*) consisted of DNA at 20 μg/ml, 0.6 M NaCl, 6.25 mM $MgCl_2$, 0.05 percent (v/v) NP-40, and histone H1 at concentrations ranging from 0.0 to 1.5 molecules per nucleosome [if 0.05 percent (v/v) NP-40 is not included in buffers containing H1, the H1 sticks to the plastic or glass vessels]. Sequence-specific transcription factors were added as indicated in the figure legends. The samples were subjected to dialysis (Pierce microdialyzer) against a linear salt gradient from 0.6 M KCl to 0.05 M KCl in buffer [12.5 mM Hepes, K^+, pH 7.6, 0.05 mM EDTA, 6.25 mM $MgCl_2$, 5 percent (v/v) glycerol, 0.05 percent (v/v) NP-40, 0.5 mM DTT, 0.5 mM benzamidine, 0.5 mM sodium metabisulfite, and 0.05 mM PMSF] for 12 hours at 1 ml/min. The samples were again dialyzed for 4 hours against the same buffer containing 0.05 M KCl, at 1 ml/min. The H1-containing chromatin was then subjected to in vitro transcription analysis. Upon salt gradient dialysis, the nucleosomal spacing increased slightly from 150 bp to about 155 bp, as judged by micrococcal nuclease digestion. The presence or absence of H1 during salt gradient dialysis did not, however, affect the nucleosomal spacing. Although polyglutamic acid has been used to deposit histone H5 into chromatin (*49*), we have found that it interfered with binding of H1 to chromatin. In such instances, it was necessary to add an excess (several times more) of H1 to achieve H1-mediated repression of transcription. When H1 was added directly to the sucrose gradient-purified chromatin in the absence of the salt gradient dialysis step, several times more H1 (four to eight molecules of H1 per nucleosome) was required to achieve only 70 to 90 percent repression of basal transcription, an indication of the need for salt gradient dialysis for efficient H1-mediated repression of basal transcription at 0.5 to 1.0 molecule of H1 per nucleosome.

35. We did not observe antirepression by either GAL4-VP16 or Sp1 when the factors were added to chromatin to which H1 was previously incorporated by salt gradient dialysis.
36. Chromatin that was subjected to salt gradient dialysis with 0.0, 0.5, 1.0, and 1.5 molecules of H1 per nucleosome was incubated under in vitro transcription conditions (*41*) in either the presence or the absence of the soluble nuclear fraction (*42*). The mixtures were centrifuged 14,000*g*, 10 minutes at room temperature, and the supernatants and sediments were analyzed by agarose gel electrophoresis (*39*). In all instances, more than 98 percent of the DNA was present in the supernatant. Hence, the H1-containing chromatin appeared to be soluble.
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41. Transcription experiments were performed at least two times to ensure reproducibility. Standard *Drosophila* embryo nuclear extracts that were deficient in histone H1 were prepared as described (*24*) through the polyvinyl alcohol precipitation step. The soluble nuclear fraction from *Drosophila* embryos, which is also deficient in H1, was prepared as described (*42*). Except for the experiment shown in Fig. 2B, the in vitro transcription experiments were done with the soluble nuclear fraction. We typically obtained about 6 percent template usage with the soluble nuclear fraction (*42*); the template DNA was a limiting component in the reactions (*42*). Transcription reactions were done as described (*24*) except for the following modifications. Naked DNA or chromatin template (containing 50 to 100 ng of DNA in a 10-μl volume) was incubated with or without sequence-specific DNA binding factor (2 μl) for 30 minutes at 21°C. The general transcription factors (in 12 μl), which were supplied from either the standard nuclear extract (3 μl) or the soluble nuclear fraction (6 μl), were added to the mixture (24 μl, total volume), which was then incubated at 21°C for 30 minutes. In this period, there is nearly complete assembly of transcription initiation complexes (*40*). Transcription was then initiated by added ribonucleoside triphosphates (NTP's) (6 μl) to a final volume of 30 μl, and the reactions proceeded for 30 minutes at 21°C. Synthesis of RNA was assayed by primer extension analysis and quantified by liquid scintillation counting of the appropriate gel slices (*24*). We consistently observed a greater activation by GAL4-VP16 when transcription initiation complexes are formed before addition of NTP's to initiate RNA synthesis compared to when NTP's are added immediately (<1 minute) after the general transcription factors. This modification of the reaction conditions accounts for the slight difference in the degree of activation observed with the naked DNA templates in the absence of H1 with GAL4-VP16 (~ eight times) relative to our previous studies (~2.5 times) (*15, 25*). A similar difference in the degree of activation by transcription factor Sp1 was not observed under the different reaction conditions.
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