NF- κ B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro

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NF- κ B is a potent inducible transcription factor that regulates many genes in activated T cells. In this report we examined the ability of different subunits of NF- κ B to enhance HIV-1 transcription in vitro with chromatin templates. We find that the p65 subunit of NF- κ B is a strong transcriptional activator of nucleosome-assembled HIV-1 DNA, whereas p50 does not activate transcription, and that p65 activates transcription synergistically with Sp1 and distal HIV-1 enhancer-binding factors (LEF-1, Ets-1, and TFE-3). These effects were observed with chromatin, but not with nonchromatin templates. Furthermore, binding of either p50 or p65 with Sp1 induces rearrangement of the chromatin to a structure that resembles the one reported previously for integrated HIV-1 proviral DNA in vivo. These results suggest that p50 and Sp1 contribute to the establishment of the nucleosomal arrangement of the uninduced provirus in resting T cells, and that p65 activates transcription by recruitment of the RNA polymerase II transcriptional machinery to the chromatin-repressed basal promoter.

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The NF- κ B transcription factor is an important regulator of immune and inflammatory response genes in activated T cells. NF- κ B exists in the nucleus as a mixture of various homo- and heterodimers of p65 (RelA) and p50 (KB1)-related subunits (for recent reviews, see Baeuerle and Henkel 1994; Siebenlist et al. 1994; Miyamoto and Verma 1995). In resting cells, NF- κ B proteins are sequestered in cytoplasmic complexes by interaction with members of the I κ B inhibitory protein family. Activation of cells by a variety of inflammatory stimuli, including mitogens, cytokines, and oxidative stress, induces phosphorylation and degradation of the I κ B proteins (Brown et al. 1995; Di Donato et al. 1995) and releases active NF- κ B heterodimers, which translocate to the nucleus to stimulate transcription of responsive genes. Whereas homodimers of p50 appear to be the predominant form of NF- κ B in resting cells, activated cells contain high levels of p50/p65 and related heterodimers, as well as homodimers of p65 (Ganchi et al. 1993). Transient expression assays reveal that the p65 subunit of NF- κ B is responsible primarily for transcriptional activation by NF- κ B (Schmitz and Baeuerle 1991; Ballard et al. 1992; Perkins et al. 1992), and a potent *trans*-activation domain was mapped to a carboxy-terminal region of p65

that is not shared with p50 (Schmid et al. 1994; Schmitz et al. 1994, 1995). In general, p50 does not activate transcription through NF- κ B sites in transient expression assays, although it has been shown to be capable of stimulating transcription on naked DNA templates in vitro through certain κ B-binding sites (Fujita et al. 1992; Kretzschmar et al. 1992; Lin et al. 1995). Consistent with the different *trans*-activation potentials of p50 and p65 seen in vivo, gene disruption experiments in transgenic mice reveal that the absence of p65 is significantly more detrimental to the regulated expression of tumor necrosis factor (TNF)-inducible genes (Beg et al. 1995) than is the loss of p50 (Sha et al. 1995).

Among the various well-characterized targets for activation by NF- κ B is the enhancer for the human immunodeficiency virus (HIV-1). The HIV-1 enhancer contains two tandem binding sites for NF- κ B that are recognized efficiently by at least four different Rel-related protein heterodimers as well as by homodimers of either p50 or p65 (for review, see Liou and Baltimore 1993). Several different NF- κ B subunit combinations have been shown to activate HIV-1 transcription strongly in vivo and in vitro (Fujita et al. 1992; Kretzschmar et al. 1992; Duckett et al. 1993; Lin et al. 1995), and various studies have reported that the NF- κ B-binding sites (Ross et al. 1991; Kim et al. 1993) as well as the NF- κ B proteins (Qian et al. 1994) are important for virus replication in peripheral

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blood lymphocytes and activated T-cell lines *in vivo*. The p65 subunit of NF- κ B binds to DNA and activates HIV-1 transcription cooperatively with Sp1 (Perkins et al. 1993, 1994; Li et al. 1994; Sif and Gilmore 1994), which binds to promoter sequences immediately downstream of the κ B sites (see Fig. 1A).

Activation of T cells is required to induce expression from HIV-1 proviral DNA. The induction of HIV-1 transcription has been shown to be accompanied by several changes in the local chromatin structure of the promoter for the integrated HIV-1 provirus. In particular, a nuclease hypersensitive site near the κ B elements is extended upon T-cell activation *in vivo*, and a nucleosome positioned near the RNA start site appears to become rearranged or disrupted upon activation (Verdin 1991; Verdin et al. 1993). These changes are likely to be mediated by the binding of factors to DNA because they occur within an hour, do not require ongoing transcription, and are not blocked by inhibitors of RNA polymerase II (Ver-

din et al. 1993). Consequently, NF- κ B can recognize its sites in regions of chromatin and may stimulate HIV-1 transcription in the absence of DNA replication in activated T cells. Overall, the structure of the integrated HIV-1 promoter (Verdin et al. 1993) resembles that described for the promoter of the integrated mouse mammary tumor virus (MMTV; Richard-Foy and Hager 1987). Specifically, both viruses contain a periodic array of nucleosomes in the regions flanking the promoter, nuclease-hypersensitive regions that overlap specific regulatory elements, and a positioned nucleosome in the vicinity of the RNA start site or TATA box. In addition, transcriptional induction of either provirus is accompanied by changes in the nuclease accessibility of the chromatin *in vivo*. Interestingly, expression of the integrated HIV-1 provirus appears to require a more extensive region of the enhancer than the one needed for activity in transient expression assays (Kim et al. 1993). Similarly, the MMTV promoter is regulated somewhat differently

Figure 1. The p65 (RelA) subunit of NF- κ B is a potent activator of HIV-1 chromatin templates *in vitro*. (A) Schematic diagram of individual transcription factor-binding sites in the HIV-1 enhancer and promoter (for review, see Jones and Peterlin 1994). (B) Analysis of transcription *in vitro* from chromatin HIV-1 templates. Purified proteins that were preincubated with HIV-1 DNA for 20 min on ice before nucleosome assembly are indicated above each lane. Where indicated, proteins were added at the following concentrations: Sp1 (20 nM), p50 (160 nM), and p65 (120 nM). α -Globin DNA was added after nucleosome assembly and serves as an internal control for RNA recovery. Naked DNA templates were not subjected to chromatin assembly and were preincubated with recombinant proteins (as indicated above each lane) at the following concentrations: Sp1 (50 nM), p50 (400 nM), and p65 (300 nM). RNAs were detected by primer extension; the numbers below each lane indicate the relative amount of RNA as determined by phosphorimager scanning. Transcription was carried out using HeLa nuclear extracts. (C) DNase I footprinting of HIV-1 chromatin templates. Binding of transcription factors to the chromatin templates from the reactions shown in B was assessed by DNase I footprinting. Aliquots of the reaction were removed before the addition of the nuclear transcription extract, digested with DNase I, and analyzed by primer extension. The location of specific transcription factor binding sites is indicated alongside the footprint.

when it is present on a stably replicating episome than it is in transient expression assays (Archer et al. 1992). It is likely that these differences can be attributed, at least in part, to the fact that proviral DNA is integrated into the genome and packaged into a transcriptionally repressed chromatin structure that is not created under the conditions of transient transfection assays (for reviews, see Felsenfeld 1992; Paranjape et al. 1994).

Recent studies have revealed important differences in the transcriptional properties of chromatin and nonchromatin DNA templates in vitro. For example, the complete *trans*-activation domain of an enhancer-binding protein is more likely to be required for transcription of chromatin templates than for transcription stimulation of naked DNA templates (Workman et al. 1991; Croston et al. 1992; Pazin et al. 1994). Furthermore, the magnitude of activation by transcription factors with chromatin templates is more similar to that seen in vivo than is the degree of stimulation seen with naked DNA templates, and chromatin templates are more likely than nonchromatin templates to require the specific *trans*-activators that are critical in vivo (Laybourn and Kadonaga 1991, 1992; Barton et al. 1993).

Using a chromatin assembly extract derived from *Drosophila* embryos (Kamakaka et al. 1993; Bulger and Kadonaga 1994; Pazin et al. 1994) in conjunction with HeLa or Jurkat T-cell nuclear transcription extracts, recently we demonstrated that the HIV-1 promoter can be activated strongly in vitro by proteins (LEF-1, Ets-1, and TFE-3) that bind to the distal region of the enhancer (Sheridan et al. 1995). Activation by LEF-1 and Ets-1 in vitro requires assembly of DNA into chromatin, consis-

tent with previous reports that the distal portion of the enhancer is much more important for expression of integrated proviral DNA than for HIV-1 transcription in transient assays (Kim et al. 1993). Here we extend this approach to analyze the ability of individual subunits of NF- κ B, alone or in combination with Sp1 and distal enhancer-binding proteins, to activate transcription from nucleosomal HIV-1 DNA in vitro. In addition, we examine the influence of NF- κ B and Sp1 on the chromatin structure of the HIV-1 promoter. Our findings indicate that functional interactions between transcriptional activators and the chromatin template are likely to play an important role in the regulation of transcription from the integrated HIV-1 provirus.

Results

Purified NF- κ B p65, but not NF- κ B p50, strongly activates nucleosome-assembled HIV-1 promoter DNA in vitro

To determine whether individual NF- κ B subunits can relieve chromatin-mediated repression of HIV-1 transcription in vitro, purified p50 and p65 proteins were preincubated with HIV-1 DNA before assembly of nucleosomes, and in vitro transcription reactions with HeLa cell nuclear extracts were carried out by using chromatin templates. As shown in Figure 1B, purified p65 was found to be a strong activator of transcription from the chromatin-assembled HIV-1 promoter. Moreover, the combination of Sp1 and p65 stimulated transcription synergistically in vitro (see also Figs. 2B and 3).

Figure 2. Specificity of p65 *trans*-activation on nucleosomal DNA in vitro. (A) Analysis of ability of p65 to activate transcription from the wild-type enhancer or from mutant enhancers containing site-specific alterations affecting either the 5' or 3' half of the two NF- κ B sites. Where indicated, the templates were preincubated with Sp1 (20 nM) or p65 (100 nM) before chromatin assembly. Transcription in HeLa nuclear extracts from the wild-type and NF- κ B mutant templates without chromatin assembly (naked DNA templates) is shown on the right. (B) Comparison of transcriptional activity of p50, p65, and p65ΔQ417 (a mutant form of p65 lacking the carboxy-terminal activation domain) on HIV-1 chromatin templates in vitro. The proteins that were preincubated with the HIV-1 DNA before chromatin assembly are listed above each lane. Where indicated, the concentration of purified or recombinant factors used in this experiment was Sp1 (10 nM), p50 (60 nM), p65 (50 nM), and p65ΔQ417 (60 nM).

Figure 3. Synergistic activation by p65 and transcription factors bound to the distal region of the HIV-1 enhancer in vitro. Analysis of the combinatorial effects of p65 and the transcription factors LEF-1, Ets-1, and TFE-3 in the presence or absence of Sp1. Transcription factors that were preincubated with the HIV-1 DNA template before chromatin assembly are listed above each lane. Where indicated, purified or recombinant proteins were added at the following concentrations: Sp1 (10 nM), p65 (70 nM), LEF-1 (70 nM), Ets-1 (75 nM), and TFE-3 (16 nM). The relative amounts of RNA synthesized from the HIV-1 promoter were determined by phosphorImager scanning of the gel.

With Sp1 and p65, the amount of transcription from the chromatin templates was $\sim 30\%$ of that observed with naked DNA templates under the same conditions. In contrast with these results with p65, little, if any, *trans*-activation was observed with preincubation of HIV-1 DNA with the p50 subunit of NF- κ B, regardless of whether it was tested alone or in combination with Sp1 (Fig. 1B). In these experiments, the magnitude of *trans*-activation of chromatin templates by p65 was typically ~ 20 -fold higher than that observed with p50. In addition, when both p50 and p65 were included in the reaction, the amount of transcription was slightly less than that observed with p65 alone, possibly because of competition between the transcriptionally active p65 homodimers and the transcriptionally inactive p50 homodimers for the available DNA-binding sites.

For comparison, we tested the effects of p50 and p65 on transcription in vitro from naked DNA templates (Fig. 1B). Neither p50 nor p65 activated transcription in vitro from naked DNA templates when tested at the same protein and DNA concentrations that were used with the chromatin templates (data not shown). Addition of 2.5-fold higher concentrations of p65 to the HeLa nuclear extracts resulted in a threefold stimulation of transcription, and we also observed a modest stimulation of transcription with high levels of the p50 subunit (Fig. 1B). Other groups have reported 3- to 15-fold activation of transcription by p65 in vitro with various re-

porter genes, as well as significant stimulation of transcription by the p50 subunit of NF- κ B (Fujita et al. 1992; Kretschmar et al. 1992; Lin et al. 1995). A detailed comparison of these results indicates that both the fold-activation as well as the selectivity of *trans*-activation by p65 is much higher with chromatin templates than with non-chromatin templates. Thus, the use of chromatin templates recreates in vitro more closely the differential activity of individual NF- κ B subunits that is seen in vivo (Perkins et al. 1993, 1994; Siebenlist et al. 1994). These results suggest that a significant component of the p65 *trans*-activation mechanism involves its ability to counteract nucleosomal repression.

To investigate the relationship between DNA binding and transcriptional activation, we examined the binding of Sp1 and the NF- κ B subunits to the chromatin templates. Aliquots of the chromatin templates that were used in the transcription experiments (Fig. 1B) were subjected to primer extension DNase I footprint analysis in the absence of transcription extract. As shown in Figure 1C, p50 bound with higher affinity than p65 to the HIV-1 NF- κ B sites on the nucleosomal templates. Therefore, the failure of p50 to activate transcription was not attributable to an inability to bind to chromatin, but instead was attributable to its relative inability to stimulate transcription from nucleosomal DNA. The relatively weak binding of p65 to the chromatin template was enhanced in the presence of Sp1 (Fig. 1C), which is consistent with previous reports that p65 and Sp1 bind cooperatively to DNA in vitro (Perkins et al. 1993, 1994; Sif and Gilmore 1994). In contrast, binding of p50 to the chromatin templates was not enhanced by Sp1. In these chromatin footprint experiments, we observed binding of p65 predominantly to the 3' half of the κ B sites, whereas Sp1 bound preferentially to its two high-affinity recognition sites (II and III). The differences in the binding of the two subunits of NF- κ B are consistent with the results of previous studies demonstrating that p50 binds to DNA more tightly than p65 and has a preference for the 5' half of the κ B site, whereas p65 binds predominantly to 3' half-sites (Siebenlist et al. 1994). We conclude that p65 and Sp1 bind cooperatively to chromatin templates, and that p50 is a poor activator of transcription, despite its ability to bind the templates with higher affinity than p65.

The HIV-1 κ B repeats and carboxy-terminal trans-activation domain of p65 are required to counteract nucleosomal repression in vitro

To determine the specificity of *trans*-activation by p65, we tested the ability of the protein to activate transcription from mutant HIV-1 promoters that contain alterations in the 5' and 3' half sites of the tandem κ B repeats. As shown in Figure 2A, point mutations in either the 5' or 3' half of the two NF- κ B sites specifically abolished *trans*-activation by p65 in vitro. In contrast, these mutations had no effect on transcription from naked DNA templates, or on *trans*-activation of the chromatin templates by Sp1. To characterize further the ability of p65

to counteract nucleosomal repression, we examined whether the p65 carboxy-terminal *trans*-activation domain was necessary for stimulation of transcription from the chromatin templates. A carboxy-terminal truncation mutant of p65 (p65 Δ Q417) that lacks the *trans*-activation domain (Schmitz et al. 1994) was purified and tested for its ability to stimulate transcription in vitro. As shown in Figure 2B, *trans*-activation by the p65 Δ Q417 protein was markedly reduced from that of the full-length p65 protein, and the combination of p65 Δ Q417 with Sp1 only slightly activated HIV-1 transcription. Overall, the activity of p65 Δ Q417 was comparable to that observed with p50, and significantly less than that of the intact p65 protein. DNase I footprint experiments with these same chromatin templates revealed that binding of p65 Δ Q417 was indistinguishable from that observed for p65, and both proteins bound DNA cooperatively with Sp1 (data not shown). Therefore, activation of chromatin templates by p65 requires its carboxy-terminal *trans*-activation domain as well as the specific κ B-binding sites in the HIV-1 enhancer.

NF- κ B activates HIV-1 transcription in conjunction with distal enhancer-binding factors

Next, we examined whether Sp1 and NF- κ B could act in conjunction with the distal enhancer-binding factors to regulate HIV-1 transcription in the context of a chromatin template. Although the distal region of the HIV-1 enhancer is not important for transcriptional activity in transient expression assays, both this region of the enhancer as well as the κ B elements are necessary for efficient virus replication in vivo (Kim et al. 1993). These results suggest that proteins bound to the upstream region of the enhancer can activate transcription in concert with NF- κ B in vivo. To assess this possibility, we asked whether the distal enhancer-binding factors, LEF-

1, Ets-1, and TFE-3 (which bind to the HIV-1 enhancer from positions -124 to -165 relative to the RNA start site; see Fig. 1A), could augment *trans*-activation by NF- κ B in vitro. As shown in Figure 3, strong synergy was observed between p65 and LEF-1, Ets-1, and TFE-3 at subsaturating concentrations of each protein. This synergy was not observed at significantly higher levels of p65 or the distal enhancer-binding factors, because either was independently capable of fully activating HIV-1 transcription at saturating concentrations (in the presence of Sp1). DNase I footprint experiments indicated that the binding of the distal enhancer-binding factors to the chromatin templates is strengthened in the presence of Sp1, despite the fact that their binding sites are separated by 60 bp (data not shown). We conclude that p65 can activate transcription in conjunction with other proteins bound further upstream on the HIV-1 enhancer.

NF- κ B and the distal enhancer factors can activate previously assembled chromatin templates in vitro

Transcription of the integrated HIV-1 provirus, which is assembled into chromatin, can be induced rapidly with T cell activation, possibly in a replication-independent manner. To recreate this effect in vitro, we tested whether transcriptional activation by NF- κ B could be observed if the activator were added to HIV-1 promoter DNA that had been assembled previously into chromatin. As shown in Figure 4, we found that the p65 subunit of NF- κ B as well as the distal enhancer proteins were capable of activating transcription from chromatin templates to a comparable extent when added either before or after nucleosome assembly. Similar results were obtained when Sp1 was added together with the enhancer factors after nucleosome assembly (data not shown). DNase I footprinting of the chromatin templates further revealed that the addition of the proteins to the tem-

Figure 4. NF- κ B and the distal enhancer factors can activate HIV-1 transcription after chromatin assembly. Analysis of the ability of p65 or the distal enhancer factors to activate HIV-1 transcription on previously assembled chromatin templates. The proteins listed above each lane were added either before or after nucleosome assembly, as indicated on the left. Proteins added before nucleosome assembly were allowed to bind to HIV-1 DNA for 20 min on ice before the addition of the chromatin-assembly extract, and proteins added after nucleosome assembly were introduced at 4.5 hr after assembly, and incubated with the chromatin templates for 30 min at 30°C before addition of the HeLa nuclear transcription extract. The final concentration of factors used in this experiment was Sp1 (16 nM), p65 (100 nM), LEF-1 (100 nM), Ets-1 (110 nM), and TFE-3 (24 nM).

plates either before or after nucleosome assembly did not affect the efficiency of DNA binding of any of the factors (data not shown). Therefore, p65 and the distal enhancer-binding factors are able to bind and activate transcription from preassembled chromatin templates *in vitro*, and consequently, these factors may act to relieve chromatin-mediated repression in the course of DNA replication-independent activation of the integrated HIV-1 provirus.

NF- κ B and Sp1 induce a reconfiguration of the chromatin structure at the HIV-1 promoter

To test the influence of the enhancer-binding proteins on the chromatin structure of the HIV-1 long terminal repeat (LTR), we examined the effect of factor binding on the regularity of the nucleosome spacing in the vicinity of the promoter region (Pazin et al. 1994; Tsukiyama et al. 1994). Bulk, native chromatin comprises nucleosomes that are approximately regular in spacing, and this regularity of spacing is examined by carrying out micrococcal nuclease digestion analysis of the chromatin (Noll and Kornberg 1977). In this assay, chromatin is digested partially with micrococcal nuclease, which creates double-stranded DNA cleavages in the linker DNA region between the nucleosomal cores. The resulting oligonucleosomal fragments are then deproteinized, and the DNA fragments are resolved by agarose gel electrophoresis. A distinct DNA ladder with a periodicity corresponding to the nucleosome repeat length is observed if the nucleosomes are spaced regularly. These studies were performed in the absence of transcription extract to eliminate any structural changes that might occur as a consequence of transcription.

We carried out a series of micrococcal nuclease digestion experiments with various combinations of Sp1 and NF- κ B proteins, transferred the DNA fragments from the agarose gel to nitrocellulose, and hybridized sequentially the blot to probes that correspond either to sequences in the HIV-1 promoter region ("specific promoter probe") or to sequences located ~1 kb upstream of the RNA start site (control plasmid probe; Fig. 5A). Examination of the micrococcal nuclease digestion ladders obtained by hybridization with the control probe (Fig. 5A, left) revealed that the chromatin consisted of regularly spaced nucleosome arrays and that the binding of the transcription factors to the chromatin did not disrupt the periodicity of the nucleosome spacing at a distant location from the promoter region (identical results were also obtained with another control probe that corresponded to sequences located ~800 bp downstream of the RNA start site; data not shown). Similarly, we found that the enhancer-binding factors did not affect the regularity of nucleosomal spacing of the bulk chromatin, as observed by ethidium bromide staining of the agarose gel (data not shown). In contrast, when the same blot was hybridized with the promoter probe (Fig. 5A, right), the periodicity of the nucleosome array was found to be disrupted upon the addition of Sp1 and NF- κ B proteins. Therefore, it appears that the binding of Sp1 with either p50 or p65

results in a specific disruption of the regularity of the nucleosome spacing in the HIV-1 promoter region, yet does not affect the properties of bulk chromatin beyond the vicinity of the promoter.

Because the pattern of DNase I hypersensitive sites also provides a general indication of the effects of transcription factors on the chromatin structure of genetic control regions (Gross and Garrard 1988), we then examined whether the DNase I hypersensitivity of the chromatin assembled *in vitro* was similar to that seen *in vivo* for the integrated HIV-1 provirus. In this experiment, the DNase I hypersensitivity of the region encompassing the HIV-1 RNA start site was examined by indirect end-labeling analysis (Nedospasov and Georgiev 1980; Wu 1980). As shown in Figure 5B (left), a weak DNase I hypersensitive site was observed with binding of Sp1 to the promoter. Addition of either p50 or p65 together with Sp1 induced a total of three hypersensitive sites, two of which were strong (HS-A and HS-B; Fig. 6A) and one of which was weak (HS-A'). These hypersensitive sites correspond well to DNase I-hypersensitive regions HS2 and HS3 that were identified *in vivo* in the analysis of the chromatin structure of the HIV-1 provirus (see summary depicted in Fig. 6A). Hence, binding of NF- κ B and Sp1 *in vitro* generates a DNase I hypersensitive region similar to that observed in this region of the HIV-1 proviral LTR *in vivo* (Verdin 1991; Verdin et al. 1993), which strongly suggests that these factors are involved in the generation of hypersensitive sites HS2 and HS3 *in vivo*.

To compare further the chromatin structure that is generated *in vitro* with that observed *in vivo*, we examined the translational positioning of the nucleosomes in the HIV-1 promoter region in either the presence or the absence of Sp1 and NF- κ B. In these experiments, the positioning of nucleosomes *in vitro* was characterized by using micrococcal nuclease digestion and indirect end-labeling techniques. As shown in Figure 5B (middle), the combination of Sp1 with either p50 or p65 induced a pattern of nucleosome positioning that was distinct from that of chromatin formed in the absence of the factors. Specifically, in the presence of Sp1 and NF- κ B, translationally positioned nucleosomes were observed in the vicinity of the transcription factor-binding sites. Moreover, the binding of Sp1 and NF- κ B proteins also induced the formation of micrococcal nuclease hypersensitive regions in the promoter that are similar to those observed *in vivo* (Verdin et al. 1993). As summarized in Figure 6A, the chromatin structure that is generated *in vitro* in the presence of Sp1 and NF- κ B proteins resembles the chromatin structure of the upstream proximal promoter region observed *in vivo* with the uninduced provirus (Verdin et al. 1993). These findings suggest that the chromatin structure of the HIV-1 provirus *in vivo* is established by the binding of transcription factors, rather than by the DNA sequence alone, and it seems likely that the transcription factors that bind both upstream and downstream of the Sp1 and NF- κ B sites are also involved in the formation of the chromatin structure of the entire HIV-1 promoter region that has been observed *in vivo*. The structural studies, when combined with the tran-

Figure 5. Binding of NF- κ B and Sp1 programs chromatin structure at the HIV-1 promoter in vitro. (A) Binding of NF- κ B and Sp1 disrupts the local nucleosomal array in the vicinity of the HIV-1 promoter. Aliquots of the chromatin templates used in Fig. 1B were treated by digestion with micrococcal nuclease and the nucleosomal arrays were analyzed by agarose gel electrophoresis and transferred to nitrocellulose filters. The filters were hybridized sequentially to a control plasmid DNA probe (1062 bp upstream of the promoter) to examine bulk chromatin, or to a probe overlapping the HIV-1 TATA box to examine the nucleosomal array in the vicinity of the promoter. Proteins that were prebound to the HIV-1 DNA before chromatin assembly are indicated above each lane. (B) Binding of NF- κ B and Sp1 induces positioning of nucleosomes near the HIV-1 promoter. The chromatin templates prepared for the transcription reactions shown in Fig. 1B were digested with micrococcal nuclease or DNase I, cleaved with *Nde*I (–1062) and *Sph*I (+765), resolved by agarose gel electrophoresis and transferred to a nitrocellulose filter. The panels were hybridized to a labeled primer located at position –1062 (DNase I panel) or +765 (MNase panels) as described in Materials and methods. The relative location of positioned nucleosomes is indicated with circles in the diagram to the right of the middle panel.

scriptional data, indicate that we have been able to recreate in vitro, to a large extent, key aspects of both the chromatin structure and the transcriptional regulation of the integrated HIV-1 provirus.

Discussion

In this study we examined the regulation of HIV-1 transcription in vitro by NF- κ B with chromatin templates under conditions wherein the nucleosomes can be mobilized in an ATP-dependent manner to facilitate transcriptional activation (Pazin et al. 1994; Tsukiyama et al. 1994). We find that binding of p50 and Sp1 does not activate HIV-1 transcription significantly, but does lead to a reconfiguration of the chromatin structure in the proximal promoter region to an arrangement that resembles that of the integrated provirus in vivo (summarized in Fig. 6A). Because p50 homodimers are prevalent in the nuclei of nonactivated T cells, these results suggest that p50 and Sp1 contribute to the formation of the “preactivated” chromatin structure of the uninduced HIV-1 provirus, which is characterized by the presence of strong

nuclease hypersensitive sites near the TATA box and a nucleosome that is positioned over the transcription start site (Verdin 1991; Verdin et al. 1993). In contrast to p50, we find that the p65 subunit of NF- κ B is a potent activator of transcription in vitro from the chromatin templates. As illustrated in Figure 6B, p65 may function to counteract the chromatin-mediated repression by recruitment of the RNA polymerase II transcriptional machinery to the DNA template through interactions with its *trans*-activation domain. Hence, in this model, a key step in the rapid transcriptional induction of HIV-1 is not the initial binding of factors, including Sp1 and p50, that function to reconfigure the chromatin structure to establish the preactivated, uninduced state, but rather the binding of p65 and the subsequent recruitment of the basal transcriptional machinery to the repressed chromatin template by the p65 *trans*-activation domain.

Chromatin assembly enhances the magnitude and specificity of activation by NF- κ B

Our results indicate that chromatin assembly has a pro-

Figure 6. Comparison of the chromatin structure induced in vitro upon binding of NF- κ B and Sp1 to the HIV-1 promoter with the structure observed previously for integrated HIV-1 proviral DNA in vivo. (A) The diagram labeled in vitro shows the relative location of DNase I hypersensitive sites and positioned nucleosomes that are observed with binding of purified NF- κ B and Sp1 in vitro (see Fig. 5); the diagram labeled in vivo shows the structure reported for integrated proviral DNA in the ACH-2 T cell line (Verdin et al. 1993). DNase I hypersensitive sites are shown with bars; ovals show the location of positioned nucleosomes (numbering is according to Verdin et al. 1993). (B) Model for chromatin remodeling and activation of transcription by NF- κ B and Sp1.

found effect on transcriptional activation by NF- κ B in vitro. Specifically, we find that both the magnitude of activation by p65 as well as the differential activity between the p50 and p65 subunits is much higher on nucleosomal templates than on naked DNA templates transcribed in vitro. Activation of chromatin templates by p65 was specific as it required both the κ B elements in the HIV-1 LTR, as well as the carboxy-terminal *trans*-activation domain of p65. The differential transcriptional activity between the two subunits that we observe with nucleosomal DNA is similar to that reported previously in transient expression assays carried out in mammalian cell lines (Schmitz and Baeuerle 1991; Ballard et al. 1992; Perkins et al. 1992). In contrast, the results from studies of NF- κ B activity in yeast cells (Moore et al. 1993), as well as previous in vitro transcription studies carried out with nonchromatin DNA templates (Fujita et al. 1992; Kretzschmar et al. 1992; Lin et al. 1995), suggested that p50 homodimers could stimulate transcription nearly as well as homo- or heterodimers containing p65 on the HIV-1 LTR. Our results suggest that transcription of chromatin templates differs qualitatively from that observed with nonchromatin templates, such that artificially high levels of transcriptional activation by p50 are observed with naked DNAs. Similarly, it has been shown that derivatives of the GAL4-VP16 activator protein that are transcriptionally inactive in vivo (and cannot activate nucleosomal DNA templates in vitro), do stimulate transcription of naked DNA templates in vitro (Pazin et al. 1994). Therefore, the use of chromatin templates significantly increases the stringency of *trans*-activation for both of these transcription factors. Although the direct target for the acidic activation domain of p65 is unknown, various transcription factors have been implicated as potential coactivators, including TATA-binding protein (TBP), transcription factor IIB (TFIIB), upstream stimulatory activity

(USA), and positive cofactor 1 (PC1) (Kretzschmar et al. 1992; Kerr et al. 1993; Xu et al. 1993; Schmitz et al. 1995). Given that our system is highly sensitive to the action of the p65 *trans*-activation domain, it will be interesting to learn whether these or different factors are critical for the activation of chromatin templates by p65.

Our results suggest that homodimers of p65 are sufficient to counteract chromatin-mediated repression. Addition of high levels of p50 inhibited activation by p65 in vitro, consistent with the repressive effects of p50 homodimers that have been observed in vivo (Schmitz and Baeuerle 1991). Although p65 homodimers have been detected in human T cells (Ganchi et al. 1993), and experiments with p50-deficient B cells support the possibility that p65 homodimers can play a role in gene activation (Sha et al. 1995), it is unlikely that significant levels of p65 homodimers coexist with homodimers of p50 in cells because these subunits greatly prefer to form heterodimers. Our findings support the hypothesis that the p50 subunit enhances binding of NF- κ B to the template, whereas the p65 subunit provides a potent *trans*-activation domain (for review, see Baeuerle and Henkel 1994). The biochemical approach described here should prove useful in quantifying the transcriptional activities of other members of the NF- κ B protein family, including c-Rel and its oncogenic counterpart, v-Rel, as well as a potentially oncogenic spliced derivative of p65, termed p65 Δ (for review, see Baeuerle and Henkel 1994). In addition, this approach will be useful in comparing the activity of NF- κ B with that of the very distantly related transcription factor, NF-AT, which is also present in high levels in activated T cells (Crabtree and Clipstone 1994), and which we have found recently to bind avidly to the κ B elements in the HIV-1 enhancer (A. Clausell, T. Hoey, and K. Jones, unpubl.).

Importantly, p65 activates transcription from chromatin regardless of whether it is added to the templates

before or after nucleosome assembly. The ability of NF- κ B to activate transcription from preassembled chromatin in vitro is particularly important because it is induced rapidly with T cell activation, and therefore, it must be capable of activating transcription in the absence of de novo DNA replication and nucleosome assembly in vivo. We find that the distal enhancer-binding proteins (LEF-1, Ets-1, and TFE-3) can also bind to chromatin and activate transcription in vitro when added after nucleosome assembly, and previous studies have shown the same for the model activator, GAL4-VP16 (Pazin et al. 1994), consistent with its ability to bind to GAL4 sites in nucleosomes and to reconfigure chromatin in vivo (Morse 1993). However, the ability to bind and activate preformed chromatin templates is not a universal feature of all transcription factors. For example, the CCAAT box-binding transcription factor (CTF/NF-1) and the Oct-1 transcription factor appear to be unable to recognize their binding site in MMTV chromatin in vivo in the absence of previous DNA-binding and chromatin reconfiguration by the glucocorticoid receptor (Archer et al. 1992; Mymryk and Archer 1995).

We find that NF- κ B activates HIV-1 transcription synergistically with the constitutively expressed T cell proteins LEF-1, Ets-1, and TFE-3, which bind to distal sequences upstream of the κ B sites in the HIV-1 enhancer. Although this distal region of the HIV-1 enhancer has been implicated to play an important role in *trans*-activation of integrated proviral DNA (Kim et al. 1993), only the NF- κ B and Sp1 sites have been found to be critical for HIV-1 transcription in transient transfection experiments. These observations suggest that factors bound to the distal region of the enhancer might be important only for expression of the virus from regions of chromatin, and consistent with this possibility, we have shown that these factors activate HIV-1 transcription in vitro from chromatin and not from nonchromatin templates (Sheridan et al. 1995). Therefore, a significant component of the activation potential of the T cell-enriched LEF-1 and Ets-1 proteins lies in their ability to counteract nucleosomal repression. Our results demonstrate that these factors bound upstream of the κ B elements can strongly potentiate transcription at subsaturating levels of the NF- κ B proteins, and this synergy may be very important in vivo under conditions where only limited amounts of the p65 subunit are available in the nucleus.

Effects of NF- κ B and Sp1 on chromatin structure at the HIV-1 promoter

Interestingly, the combination of Sp1 together with either subunit of NF- κ B was found to alter the local chromatin structure at the HIV-1 promoter and to induce the translational positioning of nucleosomes on the chromatin template (Fig. 5). As summarized in Figure 6A, the 5' boundary of one of the in vitro-positioned nucleosomes (termed nuc-1) overlaps the HIV-1 RNA start site and

Tat-responsive (TAR) element in a location that is similar to that of an in vivo-positioned nucleosome (also termed nuc-1) that was observed in T cell lines chronically infected with HIV-1 (Verdin et al. 1993). These results suggest that p50 and Sp1 participate in the positioning of nuc-1 in the provirus. Interestingly, nuc-1 appears to become rearranged or disrupted upon activation of T cells in vivo, concomitant with activation of transcription from the HIV-1 promoter, which suggests that it may play a regulatory role in viral gene expression (Verdin et al. 1993). Because disruption of nuc-1 does not require ongoing transcription, it may arise from the binding to nuc-1 of factors (other than NF- κ B) that are induced in activated T cells (El Kharroubi and Verdin 1994). In addition to their effects on nucleosome positioning, binding of Sp1 and NF- κ B was found to generate two strong DNase I hypersensitive sites in vitro (HS-A and HS-B), which correspond well with the two major hypersensitive sites, HS2 and HS3, observed in vivo (see Fig. 6A). Similar DNase I hypersensitive sites were observed upon binding of Sp1 with the distal enhancer-binding proteins (LEF-1, Ets-1, and TFE-3), although HS-A was slightly broader in the presence of the distal factors (either when tested alone or together with NF- κ B) than was observed upon binding of the NF- κ B subunits alone (data not shown). Taken together, these results indicate that several fundamental aspects of the local chromatin structure found in the integrated HIV-1 LTR can be established upon binding of two transcription factors Sp1 and NF- κ B, to the central region of the HIV-1 promoter.

As outlined in Figure 6A, we also detected several differences between the structure induced in vitro by Sp1 and NF- κ B and that which has been observed for integrated proviral DNA in vivo. First, we observe uniform spacing between nuc-1 and the downstream-positioned nucleosome (nuc-2) in vitro, whereas a slight gap in the spacing between nuc-1 and nuc-2 is observed in vivo. These results suggest that additional proteins bound to the region downstream of the RNA start site, possibly including AP-1-related transcription factors (El Kharroubi and Verdin 1994), may be critical for positioning of nuc-2. Similarly, we find that the in vitro location of nuc-0, a nucleosome positioned in the upstream region of the enhancer, differs slightly from that reported in vivo (Verdin et al. 1993). Addition of the distal enhancer-binding proteins (LEF-1, Ets-1, and TFE-3), either alone or in the presence of NF- κ B, extended the nucleosome-free region in this part of the enhancer and induced a new micrococcal nuclease hypersensitive site (M. Pazin, P. Sheridan, unpubl.). However, we were unable to discern the location of positioned nucleosomes in the presence of the distal factors, and consequently, it appears likely that additional factors that bind to the distal region of the enhancer play a role in establishing the position of nuc-0 in vivo. In spite of these differences in the flanking regions of the promoter, the chromatin structure we observe in the close vicinity of the HIV-1 TATA box is strikingly similar to that seen for the integrated provirus in unactivated T cells.

Chromatin remodeling is not sufficient to activate transcription

It is interesting that similar chromatin structures were formed upon binding of Sp1 with p65, which is a strong transcriptional activator, or with p50, which does not activate transcription significantly from chromatin templates. Thus, although the combination of Sp1 and p50 was relatively ineffective at activating HIV-1 transcription *in vitro*, these two proteins were capable of inducing chromatin reconfiguration, as evidenced by the disruption of the nucleosomal array in the HIV-1 promoter, the formation of DNase I hypersensitive sites, and the positioning of nucleosomes. Indeed, we did not detect any significant difference between the structure of transcriptionally active templates that were bound by p65 and Sp1, and those that were bound by p50 and Sp1. We conclude that the p65 activation domain does not influence the local chromatin structure, although chromatin assembly is necessary to observe its full effects *in vitro*. Similar results were also obtained in studies comparing transcriptionally active and inactive derivatives of GAL4-VP16, which were found to induce similar structures in chromatin *in vitro* (Pazin et al. 1994), and others have found that an "active" chromatin conformation is not sufficient for transcriptional activation *in vivo* (Morse 1993; Hirschhorn et al. 1995; Mymryk and Archer 1995). Although binding of transcription factors to nucleosomal arrays has been shown previously to be ATP independent, the subsequent remodeling of chromatin directed by these transcription factors is dependent on ATP and has been shown to be necessary for full transcriptional activation (Pazin et al. 1994; Tsukiyama et al. 1994). Taken together, these results indicate that factor-mediated reconfiguration of chromatin structure may be necessary, but is not sufficient, for transcriptional activation *in vivo* or *in vitro*. In the context of the regulation of HIV-1 transcription, these data suggest that p50 and Sp1 are involved in the establishment of the chromatin structure of the poised, yet transcriptionally inactive provirus, and that p65 activates transcription by relieving the chromatin-mediated repression of basal transcription through recruitment of the RNA polymerase II machinery (Fig. 6B).

In summary, the *in vitro* transcription system described here provides a rapid and sensitive approach to understanding the mechanism of transcriptional activation by p65 and other members of the NF- κ B family. Because synergistic activation is also observed among NF- κ B, Sp1, and other distal enhancer-binding factors, this approach may provide a useful model for understanding the mechanism of activation of integrated HIV-1 proviral DNA. In addition to the striking effects of chromatin on the activity of these proteins bound to the HIV-1 enhancer, chromatin structure may also influence the extent of transcription attenuation and pausing in the promoter-proximal region (Izban and Luse 1991), as well as the mechanism of *trans*-activation by the HIV-encoded transcription factor, Tat. For example, Jeang et al. (1993) have reported that the second exon of Tat-1,

which is highly conserved among viral isolates but is not required for *trans*-activation in transient expression assays, may be particularly important for activation of HIV-1 promoters that have been integrated stably into the genome. These findings raise the possibility that the second exon of Tat may encode a domain that is necessary for transcription of chromatin templates. Further studies with the biochemical approach described here should help to clarify the important effects of chromatin on the regulation of HIV-1 transcription from the integrated provirus.

Materials and methods*Plasmids and oligodeoxynucleotide primers*

HIV-1-luciferase (LUC) contains HIV-1 promoter sequences (ARV-2) from -340 to +80 in a luciferase vector (Sheridan et al. 1995). The sequence of the NF- κ B-binding site mutations are indicated in Figure 2A. An HIV-1 sequence primer (5'-GCTT-TATTGAGGCTTAAGCAGTGGG-3') was used for primer extension of *in vitro* synthesized RNA and for DNase I footprinting of the chromatin templates. Nucleosomal ladder disruption was measured with an HIV-1 promoter primer (-47/-15; 5'-GGCGTCCCTCAGATGCTGCATATAAGCAGCTGCTTTT-TGC-3') and a control primer (*Nde*I; 5'-GCACTCTCAGTCAATCTGCTCTGATGCCGC-3'). Indirect end-labeling was performed with the *Nde*I (shown for DNase I) and *Sph*I primers (shown for micrococcal nuclease; 5'-CGCAGGCAGTTCTATCGCGAAGGGCCACACCC-3').

Expression of recombinant proteins in bacteria

Recombinant LEF-1, Ets-1, and TFE-3 proteins were prepared as described previously (Sheridan et al. 1995). Plasmids containing the full-length cDNAs for the p50 and p65 Δ Q417 proteins were kindly provided by Dr. I.M. Verma (The Salk Institute). The p50 cDNA was subcloned into the pGSTag vector, expressed as a glutathione S-transferase fusion protein, and purified as described by Ron and Dressler (1992) using commercially available glutathione-Sepharose-4B affinity resin (Pharmacia). The carboxy-terminal activation domain mutant p65 Δ Q417 was prepared by digesting the full-length p65 cDNA with *Sma*I, subcloning the 1270-bp fragment into the pGd Δ SS vector, and expression in bacteria as a gene 10 fusion protein. The recombinant p65 Δ Q417 contains 10 amino acids of gene 10 protein at the amino terminus, followed by the intact amino terminus and entire Rel-homology domain of p65, and is truncated at the glutamine residue at amino acid 417. For expression of the p65 Δ Q417 protein, induced bacterial cell lysates were prepared by sonication in lysis buffer [20 mM HEPES (pH 8), 1 mM EDTA, 5 mM MgCl₂, 100 mM KCl, 2 mM DTT, 0.1 mM PMSF] and centrifugation at 20,000g for 1 hr to generate a viscous pellet. This pellet was resuspended in lysis buffer containing 1 M guanidine hydrochloride, centrifuged at 20,000g for 30 min, and the supernatant was dialyzed into HM buffer [25 mM HEPES (pH 8), 12.5 mM MgCl₂, 2 mM DTT, 0.1 mM PMSF] containing 100 mM KCl. This dialysate was spun at 17,000g for 15 min and applied to a 1.5-ml heparin-Sepharose CL-6B (Pharmacia) column. The column was eluted with a 10-ml HM 0.1–0.6 M KCl gradient. The p65 Δ Q417 protein eluted at ~0.32 M KCl and was dialyzed into HM 0.1 M KCl buffer, and was aliquoted and frozen before use.

Expression of recombinant p65 in mammalian cells

Full-length p65 protein was expressed and purified from recombinant vaccinia virus-infected HeLa cells. To construct the expression vector, the p65 open reading frame (ORF) was amplified using the polymerase chain reaction (PCR) to give a blunt-ended 5' end and a *Bam*HI site at the 3' end. The newly constructed ORF was ligated into the pTM1 vector (Moss et al. 1990; Elroy-Stein and Moss 1992), which was cut at the *Nco*I site, blunted, and then cut with *Bam*HI. This ligation put the start codon for p65 in a position to use the encephalomyocarditis virus leader for translation. The new plasmid, pTM65, places the reconstructed p65 ORF under control of the bacteriophage T7 promoter. This construct was recombined into the thymidine kinase locus of the WR strain of vaccinia virus. Selection and plaque purification was done on thymidine kinase-negative 143 cells using 5-bromodeoxyuridine (Mackett et al. 1984). Recombinant viruses (v65T7) were screened by Western blot using rabbit polyclonal antisera to p65. The virus was amplified on BSC-1 cells and then on HeLa-S cells before use.

For purification of p65, 10 L of HeLa-S cells were infected with v65T7 and vTF7-3 at a multiplicity of infection of 10 plaque-forming units. The cells were pelleted at 20 hr postinfection, and the whole-cell pellet was resuspended in five packed cell volumes of buffer C [25 mM HEPES (pH 8), 1 mM EDTA, 2 mM DTT, 0.5 mM AEBSE, 0.5 mM sodium metabisulfite, 0.1% NP40, and 20% glycerol] containing 0.5 M KCl. The cell suspension was then sonicated with a microtip, and the insoluble material (containing most of the p65 protein) was pelleted and solubilized with buffer C containing 0.1 M KCl and 7 M urea. The urea was gradually removed by step-wise dialysis against buffer C to a final concentration of 0.5 M urea and 0.1 M KCl over a period of 3 days. Active p65 protein that remained in the soluble fraction was then purified to apparent homogeneity using a biotinylated oligodeoxynucleotide DNA affinity column containing tandem copies of the κ B-binding site from the IgG κ light chain gene enhancer. The resuspended p65 protein was bound to the DNA affinity column in buffer C containing 0.5 M urea and 0.1 M KCl. After washing with buffer C containing 0.2 M KCl, p65 protein was eluted with buffer C containing 0.5 M KCl. Peak fractions containing purified p65 protein (0.5 mg/ml) were pooled and stored in the elution buffer.

Chromatin assembly

Chromatin was assembled with *Drosophila* S-190 extract, core histones, histone H1, and an ATP-regenerating system prepared as described previously (Kamakaka et al. 1993; Bulger and Kadonaga 1994). The HM0.1 M buffer/protein mixture was $\leq 15\%$ of the final reaction volume and contained the specified concentration of proteins as indicated in the figure legends. Sp1 was purified from either Jurkat or HeLa nuclei using sequential lectin and DNA-affinity chromatography, as described previously (Sheridan et al. 1995). Unless otherwise indicated in the text, the experiments shown here were carried out using lectin-purified Sp1 fractions, and the specific activity (relative footprinting units) of each fraction was determined empirically by comparison with commercially available recombinant Sp1 (Promega) in DNA gel shift and DNase I footprint assays. Typically, the factors were allowed to bind their sites on DNA for 10–20 min on ice before the addition of the S-190 assembly extract and purified histone components as described previously (Sheridan et al. 1995). For the experiment shown in Figure 4, "added after assembly" refers to reconstitution reactions in which the proteins were added after the templates were assembled for 4.5 hr, after which the individual enhancer-binding proteins were added (as

indicated in the legend to Fig. 4), assembly was continued for an additional 30 min, and then the reactions were subjected to *in vitro* transcription and structural analysis. All of the experiments presented here were done with templates containing physiological amounts of histone H1. Efficiency of assembly was monitored by micrococcal nuclease assays; typically, 10–12 nucleosomal repeats were visible on the chromatin templates.

In vitro transcription reactions

Nuclear transcription extracts were prepared from the HeLa-S3 cell line as described by Waterman and Jones (1990). Transcription from either naked plasmid DNA or chromatin templates was analyzed by primer extension as described previously (Sheridan et al. 1995). Briefly, 100 ng of plasmid DNA or 100 ng of chromatin templates (taken as a 20- μ l aliquot of the chromatin reconstitution reaction) was mixed together with 50 ng of α -globin DNA, and the templates were preincubated in a 100- μ l reaction with HeLa transcription extract and buffer before the addition of rNTPs. The resulting RNAs were detected by primer extension and quantified using a phosphorimaging scanner (Molecular Dynamics). The α -globin template was used as a control for sample recovery. Baseline transcription was determined by subtracting representative background scanner values from the detected HIV-1 value for the repressed templates (buffer alone). Relative transcription values were calculated by dividing this designated baseline value into the HIV-1 levels determined for each template with the various factor additions.

DNase I footprint analysis of chromatin templates

Transcription factor binding to chromatin templates was measured by DNase I primer extension footprinting, as described previously (Pazin et al. 1994). Unpurified chromatin was incubated with DNase I in the absence of transcription extract. The DNA was purified and aliquots were analyzed by primer extension with Vent (exo⁻) polymerase (New England Biolabs) and the HIV-1 primer. DNase I footprint analysis was performed on aliquots of the same chromatin templates that were used for the *in vitro* transcription reactions.

Chromatin structure analysis

Chromatin structure was determined by nuclease hypersensitivity mapping of DNase I-digested chromatin, nucleosome ladder disruption assays, and indirect end-labeling of micrococcal nuclease-digested chromatin, as described previously (Pazin et al. 1994). For DNase I hypersensitivity mapping, the aliquots of the same DNase I samples that were analyzed by primer extension footprinting were digested instead with *Nde*I and *Sph*I. The digests were analyzed by agarose gel electrophoresis, transferred to nitrocellulose, and sequentially hybridized to *Nde*I primer and *Sph*I primer (only the results obtained upon hybridization with the *Nde*I primer are shown). For ladder disruption analysis, unpurified chromatin was incubated with micrococcal nuclease in the absence of transcription extract. Aliquots of these micrococcal digests were analyzed by agarose gel electrophoresis, transferred to nitrocellulose, and sequentially probed with *Nde*I, -47/-15, and *Sph*I primers. For indirect end-labeling of micrococcal nuclease-digested chromatin, aliquots of the same samples used for ladder disruption assays were digested with *Nde*I and *Sph*I. The digests were analyzed by agarose gel electrophoresis, transferred to nitrocellulose, and sequentially hybridized to the *Nde*I and *Sph*I primers (only the *Sph*I hybridization is shown). Chromatin structure analysis was performed on aliquots of the same chromatin templates, in the absence of

HeLa nuclear extract, that were used in the transcription reactions.

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