Stable co-occupancy of transcription factors and histones at the HIV-1 enhancer

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To investigate mechanisms yielding DNase I-hypersensitive sites (DHSs) at gene regulatory regions, we have initiated a biochemical analysis of transcription factor binding and nucleosome remodeling with a region of the human immunodeficiency virus 1 (HIV-1) 5' long terminal repeat (LTR) that harbors constitutive DHSs *in vivo*. *In vitro* reconstitution of an HIV-1 5' LTR fragment into nucleosome core particles demonstrates that Sp1, NF- κ B1, LEF-1, ETS-1 and USF can gain access to their binding sites in HIV-1 nucleosomal DNA. The factor-bound mononucleosomes resist histone displacement from the DNA by the chromatin remodeling activity, SW1-SNF, or the histone chaperone, nucleoplasmin, suggesting that the binding of these factors to nucleosomal HIV-1 sequences forms a stable complex that includes the underlying histones. However, when the HIV-1 5' LTR fragment is incorporated into a nucleosomal array, Sp1 and NF- κ B1 binding produce regions of enhanced DNase I sensitivity specifically at the HIV-1 nucleosome. These regions resemble the observed *in vivo* DHSs, yet the HIV-1 nucleosome remains intact even in the presence of nucleoplasmin. Thus, the constitutive DHSs identified at the HIV-1 enhancer in native chromatin may reflect the presence of a ternary complex composed of transcriptional activators, histones and DNA.

Keywords: chromatin remodeling/histone displacement/HIV-1/nucleosome binding/transcriptional regulation

Introduction

The intimate association of histone and non-histone proteins with eukaryotic DNA forms a highly condensed complex termed chromatin. The fundamental subunit of chromatin is the nucleosome core, which consists of 146 bp of DNA wrapped 1.8 times around an octamer of core histones (reviewed in van Holde *et al.*, 1995). Nucleosomes negatively regulate gene expression by restricting access of the transcriptional machinery to the DNA (for review, see Owen-Hughes and Workman, 1994). As a result, the chromatin structure at enhancer and promoter regions is often reconfigured prior to, or concurrently with, the induction of gene transcription (reviewed in Felsenfeld, 1996). These changes frequently are identified as an increase in sensitivity at specific DNA sequences to digestion by the nuclease DNase I, and are therefore

termed DNase I-hypersensitive sites (DHSs) (Gross and Garrard, 1988). Importantly, the induced binding of upstream activator proteins has been linked directly to the formation of DHSs, implicating the sequence-specific interactions of transcriptional regulators in the remodeling of chromatin structure (Elgin, 1988).

It has been largely assumed that DHSs in native chromatin are free of histones. Early *in vivo* studies by McGhee *et al.* (1981) demonstrated that a histone-free DNA fragment is released from the DHS at the 5' end of the chicken β -globin gene by restriction endonuclease cleavage. Moreover, biochemical analyses have revealed that the binding of transcription factors to nucleosomes, in concert with histone chaperones or chromatin remodeling activities, can lead to displacement of the underlying histones from the DNA (reviewed in Steger and Workman, 1996). However, *in vivo* studies with the mouse mammary tumor virus (MMTV) promoter suggest that histones are still present at the glucocorticoid receptor-induced DHS (Bresnick *et al.*, 1992; Truss *et al.*, 1995). In addition, transcription factors and nucleosomes have been found to co-occupy the active albumin enhancer (McPherson *et al.*, 1993). Thus, DHSs in cellular chromatin may vary in histone composition at different regulatory elements and/or at different stages of gene activity.

The human immunodeficiency virus type 1 (HIV-1) 5' long terminal repeat (LTR) is an excellent model system to investigate biochemically the mechanisms of nucleosome remodeling, since the chromatin organization for the integrated HIV-1 provirus has been well characterized. Verdin *et al.* (1993) identified positioned nucleosomes on the HIV-1 5' LTR by nuclease digestion of intact nuclei from the persistently infected ACH2 (T cell) and U1 (macrophage) cell lines. Under basal conditions expressing little viral RNA, DHSs are detected at –232 to –130 (DHS 2), –65 to –6 (DHS 3), and from 201 to 265 (DHS 4) (see also Verdin, 1991). In addition, micrococcal nuclease (MNase) treatment determined nucleosome locations of –415 to –255 (nuc-0) and –3 to 141 (nuc-1), with arrays of nucleosomes occupying the DNA upstream of nuc-0 and downstream of DHS 4. Although the DNA between nuc-0 and nuc-1 resists MNase digestion, and is large enough to accommodate a nucleosome, these authors proposed that this region is nucleosome free, given that DHSs 2 and 3 lie within it. Treatment of ACH2 and U1 cells with cytokines or phorbol esters, which greatly stimulate HIV-1 gene expression, induces hypersensitivity to both DNase I and MNase in the region encompassed by nuc-1 (Verdin *et al.*, 1993). This alteration occurs independently of transcription, suggesting that the disruption or displacement of nuc-1 is a prerequisite of HIV-1 gene expression, rather than a consequence. Interestingly, hyperacetylation of total cellular histones also reconfigures nuc-1 at a step preceding HIV-1 transcriptional activation,

further implicating the remodeling of chromatin structures in overcoming nucleosome-mediated repression (Van Lint *et al.*, 1996).

The regulatory sequences and transcriptional activators necessary for HIV-1 gene expression and replication have been studied intensively (for review, see Gaynor, 1992; Jones and Peterlin, 1994). Approximately the first 200 bp of sequence directly upstream of the transcription start site have been defined as the enhancer/promoter region. This domain, which is positioned between *nuc-0* and *nuc-1*, contains recognition elements for the sequence-specific DNA-binding proteins Sp1, NF- κ B, LEF-1, ETS-1 and USF. The Sp1 and NF- κ B sites are absolutely essential for proviral promoter activity, while mutations within the LEF-1 and ETS-1 sequences diminish viral growth in T cells exhibiting a more differentiated developmental state (Kim *et al.*, 1993). In agreement with these *in vivo* results, Sheridan *et al.* (1995) nicely demonstrated *in vitro* that the transcriptional activities of LEF-1 and ETS-1 on HIV-1 nucleosome-reconstituted templates require Sp1. Using the same nucleosome assembly system derived from *Drosophila* embryos, Pazin *et al.* (1996) presented data displaying chromatin modeling by Sp1 and NF- κ B when these factors are pre-incubated with the HIV-1 DNA template before nucleosome reconstitution. Finally, the importance of Sp1 and NF- κ B in HIV-1 regulation is realized further by *in vivo* footprinting of the HIV-1 enhancer/promoter region in U1 cells. This analysis reveals occupancy of the two upstream Sp1 elements and the downstream NF- κ B site, whereas no binding is detected by LEF-1, ETS-1 and USF, regardless of the transcriptional state of the proviral promoter (Demarchi *et al.*, 1993).

We began our studies by asking: can the transcriptional activators targeting the HIV-1 enhancer/promoter initiate the formation of a nucleosome-free region by binding to their sites in pre-formed nucleosomal DNA? To approach this question biochemically, yet remain faithful to the native HIV-1 chromatin organization, HIV-1 DNA templates were constructed which, upon assembly into nucleosomes *in vitro*, reflect the nucleosome positioning observed *in vivo*. We find that HIV-1 nucleosome core particles withstand histone dissociation by the chromatin remodeling activity, yeast SWI-SNF complex, or the histone-binding protein, nucleoplasmin, when specifically bound by Sp1, NF- κ B1, LEF-1, ETS-1 and USF. However, binding by these proteins increases DNase I sensitivity specifically within an HIV-1 nucleosome located in an array of nucleosomes. Sp1 and NF- κ B1 generate sites of increased DNase I cleavage reflecting *in vivo* DHSs 2 and 3, consistent with data indicating that only these two proteins occupy their sites on the proviral enhancer/promoter. In addition, nucleosome remodeling by Sp1 and NF- κ B1 occurs without displacement of the underlying histones, providing biochemical evidence that DHSs can be produced by ternary complexes made up of transcription factors, DNA and histones.

Results

Transcriptional activators targeting recognition elements within the HIV-1 5' LTR can gain access to their binding sites in nucleosomal DNA

We hypothesized that the constitutive DHSs, positioned between *nuc-0* and *nuc-1* (i.e. from -254 to -4) in

integrated HIV-1, represent a disrupted or displaced nucleosome in the viral genome. Furthermore, we reasoned that the interactions of sequence-specific DNA-binding proteins with this region of the HIV-1 enhancer/promoter are responsible for reconfiguring the chromatin structure. To test this idea, HIV-1 5' LTR sequences were reconstituted into nucleosome core particles *in vitro*, and incubated with the transcriptional activators having defined binding sites within the sequence. Biochemical reconstitution of histones onto DNA fragments significantly greater than nucleosome length (i.e. 146 bp) generally produces a heterogeneous population of core particles, since most DNA sequences lack an inherent ability to position nucleosomes *in vitro*, and large fragments allow the histone octamer to adopt multiple positions on the DNA. Because the translational position of the histone octamer relative to activator-binding sites can greatly affect factor binding (Owen-Hughes and Workman, 1994), we sought to minimize nucleosome position effects by restricting the HIV-1 sequence to nucleosome length. Thus, a 151 bp DNA fragment from -199 to -49 in the HIV-1 5' LTR was chosen for nucleosome reconstitution, so that a nucleosome on this sequence would be in-frame with the nucleosomal phasing established by *nuc-0* and *nuc-1* (Figure 1A). This sequence contains DNA-binding sites for the transcriptional activators Sp1, NF- κ B, LEF-1, ETS-1 and USF.

Binding reactions with the HIV-1 mononucleosomes and the activators Sp1, NF- κ B1 (i.e. p50-p50 homodimers), LEF-1, ETS-1 and USF were carried out in a highly purified system, so that observed effects could be attributed to defined components (Figure 1B). Electrophoretic mobility shift analysis (EMSA) reveals that the HIV-1 DNA fragment efficiently reconstitutes into mononucleosome cores (Figure 1C). Each of the transcription factors produces shifted complexes with the nucleosome core particles which migrate differently from the corresponding factor-DNA complexes, indicating that the nucleosomal complexes are composed of DNA, activator protein and histones. Consistent with the fact that the DNA contains two Sp1 recognition elements, two ternary complexes are detected with Sp1 bound to the HIV-1 mononucleosomes. NF- κ B1, which also targets two binding sites in the HIV-1 core particles, readily binds to one of these sites at the given protein concentration, since the major ternary complex migrates more slowly than one homodimer of NF- κ B1 bound to DNA, but faster than two DNA-bound homodimers. USF, LEF-1 and ETS-1 each yield a predominant gel shift complex, when bound to the HIV-1 nucleosome cores, which migrates slightly more slowly than their respective factor-DNA complexes. As expected, all of these factors display a greater affinity for DNA versus nucleosomal DNA. This effect is magnified for those proteins having binding sites buried deep within the nucleosome, as all factors tested to date have weaker affinities for sites positioned near the dyad axis of the nucleosome compared with sites located at the nucleosome edge.

Having established that Sp1, NF- κ B1, LEF-1, ETS-1 and USF can bind to HIV-1 nucleosome core particles, we wanted to determine whether each of these factors interacts specifically with its recognition element, and if multiple proteins can bind simultaneously to the core particles. Therefore, DNase I protection analysis was

Fig. 1. Transcriptional activators specifically bind to HIV-1 nucleosome core particles. **(A)** Schematic of the DNA fragment used for nucleosome core reconstitution. The fragment spans base pairs -199 to -49 in the HIV-1 5' LTR, and the locations, relative to the nearest DNA end, of the center of each factor-binding site are indicated. **(B)** SDS-PAGE analysis of purified recombinant transcription factors and HeLa nucleosome cores. NF-κB1 (i.e. p50 subunit, 2 μg), LEF-1 (1 μg), ETS-1 (3 μg) and USF (2 μg) were run on a 12% polyacrylamide gel; HeLa nucleosome cores (2.5 μg) were loaded onto a 15% gel. Proteins were visualized by Coomassie staining. Molecular size standards (M) are indicated. **(C)** EMSA of transcriptional regulators bound to HIV-1 nucleosome core particles. The concentrations of the various proteins used to bind the mock-reconstituted DNA (DNA) and nucleosome-assembled DNA (nucl.) are given. **(D)** DNase I protection analysis of proteins bound to HIV-1 mononucleosomes. Binding conditions are identical to **(C)** except for the amounts of transcriptional activators added to the reactions as indicated. DNase I digestion of mock-reconstituted DNA and nucleosomal DNA in the absence of activators is labeled as control. G+A indicates the Maxam-Gilbert purine sequencing reaction for the probe DNA. Factor-binding sites are designated by solid lines on the right.

performed with binding reactions comprised of the various transcription factors and HIV-1 mononucleosomes (Figure 1D). As a control, DNase I footprinting was also conducted with HIV-1 DNA, to demonstrate that each of the activators recognizes its defined binding site (lanes 3–7), and that all five proteins are able simultaneously to occupy their sites on DNA (lane 8).

DNase I treatment of the HIV-1 mononucleosomes yields a dramatically different pattern of digestion from that generated with HIV-1 DNA (compare lanes 9 and 10). DNase I cut throughout the DNA to produce a relatively uniform ladder of fragments. In contrast, discrete sites of DNase I cleavage, surrounded by regions of protection, are apparent in the nucleosomal DNA ladder. Ten base pairs separates many of these cleavage sites, which is characteristic of the DNase I digestion profile of rotationally phased nucleosomal DNA (for example, see Côté *et al.*, 1995). Addition of NF-κB1 to the HIV-1 nucleosome core particles, followed by nuclease treatment, revealed DNase I protection of the NF-κB-binding sites

in nucleosomal DNA (lane 11). However, unlike NF-κB1 binding to HIV-1 DNA, where the protein exhibits little or no preference for either recognition element, binding to nucleosome cores is affected by the translational position of the underlying histone octamer. At a concentration of 300 nM, NF-κB1 fully protects its external site from DNase I. In contrast, complete binding of the internal recognition element is not achieved at this protein concentration, since DNase I is still able to access this DNA region. Interestingly, the binding of NF-κB1 to the HIV-1 mononucleosomes severely disrupts the DNase I digestion profile throughout the entire length of the nucleosomal DNA, which includes the production of strong DNase I cleavage sites directly flanking the NF-κB1 elements. The downstream hypersensitive site, positioned within the Sp1-binding sites, is specifically footprinted upon the addition of Sp1 to binding reactions containing NF-κB1 (lane 12). Further addition of either LEF-1 or ETS-1, to reactions containing both NF-κB1 and Sp1, reveals detectable protection from DNase I cleavage within the LEF-1-

and ETS-1-binding sites, respectively (lanes 13 and 14). Finally, incubation of USF with NF- κ B1, Sp1, LEF-1 and ETS-1 generates a pattern of footprints quite similar to that produced by all of these factors bound to naked DNA (compare lane 15 with lane 8). We conclude that these proteins bind to the HIV-1 nucleosomes in a sequence-specific manner, and that they can occupy their sites on the same templates simultaneously.

Factor-bound HIV-1 nucleosome core particles resist histone displacement by the chromatin remodeling activity, yeast SWI-SNF complex, and the histone-binding protein, nucleoplasmin

The binding of five GAL4-AH dimers to nucleosome core particles, in concert with the chromatin remodeling activity, yeast SWI-SNF complex, or the histone chaperone, nucleoplasmin, has been demonstrated *in vitro* to displace histones from the DNA (Walter *et al.*, 1995; Owen-Hughes *et al.*, 1996). Given these interesting findings, we investigated whether the binding of transcriptional activators to the HIV-1 mononucleosomes could also destabilize the underlying histones adequately enough to enable histone displacement. For these experiments, the HIV-1 nucleosome cores were incubated with amounts of activators sufficient to achieve complete binding, either in the presence or absence of activities mediating histone dissociation. The transcriptional activators subsequently were removed from the complexes by oligonucleotide competition, and the resulting products subjected to gel electrophoresis. This resolves whether histones dissociated from the DNA, since DNA and histones cannot re-assemble efficiently into nucleosomes in this purified system under the physiological salt conditions used for the binding reactions. Thus, the appearance of free DNA after oligonucleotide challenge, rather than nucleosome core particles, indicates that histone displacement occurred.

Figure 2 displays the results of an experiment investigating the ability of SWI-SNF to displace histones from factor-bound HIV-1 mononucleosomes. Gel shift analysis with Sp1 and NF- κ B1 reveals complete binding of the HIV-1 core particles by these proteins, with the majority of the complexes remaining in the well (lane 2). Removal of the bound factors by oligonucleotide competition regenerates the nucleosome cores (lane 3), indicating that the combined actions of Sp1 and NF- κ B1 do not dissociate underlying histones. Importantly, addition of SWI-SNF to binding reactions containing these proteins, followed by oligonucleotide competition, also give back the HIV-1 nucleosome cores (lane 4). These data suggest that the binding of Sp1 and NF- κ B1 to the HIV-1 mononucleosomes does not disrupt histone-DNA contacts sufficiently to allow SWI-SNF-mediated histone displacement. Moreover, this result can be extended to include LEF-1, ETS-1 and USF, since the HIV-1 cores withstand histone displacement by SWI-SNF upon binding these proteins alone (lanes 8–10), or in combination with Sp1 and NF- κ B1 (lanes 5–7). To demonstrate that SWI-SNF was active in these studies, control reactions were performed displaying SWI-SNF-mediated displacement of histones from GAL4-AH-bound mononucleosomes (lanes 11–14).

Because nucleoplasmin also dissociates histones *in vitro* from GAL4-AH-bound nucleosomes, we included this

Fig. 2. Factor-bound HIV-1 mononucleosome cores resist histone displacement by yeast SWI-SNF. Combinations of Sp1 (23 nM), NF- κ B1 (150 nM), LEF-1 (50 nM), ETS-1 (100 nM) and USF (150 nM) were added to the HIV-1 nucleosome core particles with and without highly purified SWI-SNF (10 nM) as indicated. Upon completion of the binding reactions, the designated samples were passed through an oligonucleotide competition step to remove bound factors from the core particles. All samples subsequently were subjected to EMSA. Binding reactions composed of GAL4-AH (45 nM) and the 5-GAL4-site mononucleosomes were conducted under conditions identical to those for the HIV-1 reactions, with the exception that a GAL4 oligonucleotide was substituted for the HIV-1 oligonucleotide mix at the competition step.

protein in the analysis of histone displacement from factor-bound HIV-1 nucleosome cores. In the presence of nucleoplasmin, different combinations of Sp1, NF- κ B1, LEF-1, ETS-1 and USF produce discrete gel shift complexes (Figure 3). Similarly to the experiment with SWI-SNF, however, removal of the bound factors by oligonucleotide challenge reveals intact nucleosome cores. Since control reactions display nucleoplasmin-mediated histone dissociation from GAL4-AH-bound mononucleosomes, we conclude that the HIV-1 nucleosome core particles withstand either nucleoplasmin- or SWI-SNF-mediated histone displacement when bound by numerous transcriptional activators.

Multiple factors specifically remodel an HIV-1 nucleosome located within an array of positioned nucleosomes

The simplicity of biochemical studies utilizing mononucleosome core particles allows direct determination of the effects of transcription factor binding on nucleosome stability. However, remodeled chromatin is usually identified as being hypersensitive to nucleases relative to the neighboring chromatin. Therefore, to examine *in vitro* potential roles played by transcriptional activators in reconfiguring HIV-1 chromatin, a template composed of arrays of nucleosomes was employed.

Nucleosome reconstitution by histone octamer transfer onto a 1400 bp DNA fragment, including the entire HIV-1 5' LTR, does not yield positioned nucleosomes resembling those observed *in vivo* (data not shown). Therefore, we constructed a DNA template which places tandem repeats of sea urchin 5S rDNA on both sides of the HIV-1 promoter sequence presumed to be part of the

Fig. 3. Factor-bound HIV-1 mononucleosomes withstand histone displacement by nucleoplamin. Different combinations of Sp1 (35 nM), NF-κB1 (200 nM), LEF-1 (100 nM), ETS-1 (200 nM) and USF (200 nM) were incubated with the HIV-1 nucleosome core particles in the presence of highly purified nucleoplamin (67 nM). Upon completion of the binding reactions, the indicated samples were passed through an oligonucleotide competition step to remove bound factors from the core particles. All samples subsequently were subjected to EMSA. Binding reactions containing GAL4-AH (90 nM) and the 5-GAL4-site mononucleosomes were performed under conditions identical to those used for the HIV-1 reactions, with the exception that a GAL4 oligonucleotide was substituted for the HIV-1 oligonucleotide mix at the competition step.

disrupted or displaced nucleosome between nuc-0 and nuc-1. Simpson *et al.* (1985) have demonstrated that nucleosome reconstitution of direct repeats of 5S rDNA yields a continuous array of positioned nucleosomes. As a result, the HIV-5S DNA fragment schematically represented in Figure 4A is designed to adopt positioned nucleosomes on the 5S rDNA repeats upon reconstitution, which subsequently encourage the positioning of a nucleosome on the HIV-1 sequence in-phase with the neighboring nucleosomes.

MNase digestion of the nucleosome-reconstituted HIV-5S array fragment, followed by agarose gel electrophoresis, generates 10 discrete cut sites spaced evenly apart by regions apparently inaccessible to enzymatic activity (Figure 4B, lane 1). Because MNase preferentially cleaves chromatin in the linker DNA between nucleosomes, this alternating pattern of cutting and protection is consistent with 11 uniformly positioned nucleosomes occupying the DNA. Titration of NF-κB1 with the HIV-5S nucleosomal array increases the level of MNase digestion between the sixth and seventh nucleosomes, given that the nucleosomes are numbered 1–11 starting from the top of the gel (lanes 3–5). DNase I treatment of the polynucleosomal template in the absence of transcription factors gives a digestion pattern identical to that of MNase (lane 2). However, in contrast to MNase, NF-κB1 stimulates DNase I cutting not only in the linker region between nucleosomes 6 and 7, but also within nucleosome 6 (lanes 6–8).

To determine the level of reconstitution for the nucleosomal array, the histone-free mock-reconstituted HIV-5S

DNA fragment and nucleosome-assembled template were digested with DNase I, and the cleavage patterns compared (Figure 4C). Digestion of the naked DNA produces a smear of bands (lanes 1–3), confirming that the distinct ladder of cut sites observed for the reconstituted array results from the association of histones with DNA to form nucleosomes. For the experiments reported in Figures 4B and 5, the DNase I digestion conditions are identical to those in Figure 4C, lane 6. At DNase I digestion conditions ~3-fold less than this, the majority of the mock-reconstituted DNA is reduced to small fragments <200 bp in length (lane 3), illustrating that the DNase I digestion profile of the reconstituted array is produced from nucleosomal DNA. Finally, limited digestion of the HIV-5S DNA fragment by *EcoRI*, which cleaves at the very end of each 5S rDNA repeat, reveals that nucleosomes adopt their predicted positions on the 5S repeats (compare lane 4 with lanes 5–7). Thus, the sixth nucleosome within the array is comprised of HIV-1 sequence, and the binding of NF-κB1 increases access to the DNA for both MNase and DNase I specifically at this nucleosome.

To investigate whether the other transcriptional activators can also alter nuclease sensitivity within the HIV-1 nucleosome, combinations of Sp1, NF-κB1, LEF-1, ETS-1 and USF were incubated with the HIV-5S nucleosomal array, and subsequently treated with DNase I. Figure 5 reveals that Sp1 increases DNase I cutting in the linker region directly adjacent to its binding sites, and, to a lesser extent, within the HIV-1 nucleosome (lane 3). As shown above, NF-κB1 binding yields distinct DNase I cleavage sites which appear to flank the NF-κB recognition sequences directly on both sides (lane 4). Interestingly, the downstream NF-κB1-mediated cleavage site is reduced in magnitude upon addition of Sp1 to the reaction mix (lane 5). This effect is consistent with the binding of NF-κB1 and Sp1 to the HIV-1 mononucleosomes, since Sp1 also footprinted NF-κB1-mediated DNase I hypersensitivity in that system (Figure 1D above). Given these results, we believe the remodeling effects produced by NF-κB1 and Sp1 in the nucleosomal array are caused by sequence-specific binding of these factors to their defined sites. Indeed, the pattern of increased DNase I sensitivity observed within the array upon binding of Sp1 and NF-κB1 is strikingly similar to that observed on the uninduced integrated HIV-1 provirus (Verdin *et al.*, 1993, also see discussion below). The only major difference between the *in vitro* digestion pattern of the Sp1/NF-κB1-bound HIV-5S nucleosomal array and the *in vivo* profile for the integrated virus is the clear definition of linker DNA in the HIV-5S array due to the positioned 5S nucleosomes. Incubation of the HIV-5S array with LEF-1, ETS-1 and USF (lane 7), or USF alone (lane 8), produces only minor changes in the DNase I digestion pattern. Furthermore, addition of all five proteins to the nucleosomal array diminishes the DNase I hypersensitivity conferred by Sp1 and NF-κB1 (lane 6), suggesting that the binding of LEF-1, ETS-1 and/or USF footprints the upstream NF-κB1-mediated DNase I cleavage site.

Enhanced sensitivity to DNase I within the HIV-1 nucleosome upon factor binding occurs without histone displacement

Although factor-bound HIV-1 mononucleosomes resist histone displacement, the disrupted DNase I digestion

Fig. 4. Nucleosome assembly of the HIV-5S DNA template produces an array of positioned nucleosomes. (A) Schematic of the HIV-5S DNA fragment used to reconstitute a nucleosomal array by histone octamer transfer. The 210 bp HIV-1 fragment is flanked on both sides by five direct repeats of a 208 bp 5S rDNA fragment. Note that the nucleosome positioning sequence is not centered within the 208 bp 5S rDNA fragment, and that *EcoRI* sites are present at the junction between each 5S repeat. (B) Nuclease digestion of the nucleosome-reconstituted array. The end-labeled nucleosomal array was treated with either MNase or DNase I, and the digestion products were resolved by agarose gel electrophoresis. Incubation of increasing amounts of NF-κB1 with the array prior to digestion is indicated. Nucleosome positions on the HIV-5S DNA fragment are represented schematically on the right. (C) Comparison of the DNase I digestion profiles for histone-free and nucleosome-assembled HIV-5S DNA. The chemical components of reactions containing mock-reconstituted DNA (mock recon.) are identical to those in reactions containing nucleosome-reconstituted DNA (nucl. recon.); however, DNA in the mock-reconstituted samples is not bound by histones. The amount of DNase I and time of digestion are indicated for each lane. *EcoRI* digestion of the HIV-5S DNA fragment under limiting conditions is shown in lane 4.

profile within the HIV-5S nucleosomal array suggested that factor binding, in this case, may destabilize the underlying histones sufficiently to allow dissociation from the DNA by chromatin remodeling activities. To test this idea, different combinations of Sp1, NF-κB1, LEF-1, ETS-1 and USF were incubated with the nucleosomal array in the presence of nucleoplasmin. In addition, prior to DNase I treatment, the factors were competed off the DNA by oligonucleotide challenge. If histones were displaced from the combined actions of nucleoplasmin and activators, we expected the entire HIV-1 sequence to become hypersensitive to DNase I, since neither histones nor transcription factors would be bound to this region (for example, see Owen-Hughes and Workman, 1996).

Figure 6 illustrates that the nucleosomal array structure is not affected by nucleoplasmin and the oligonucleotide competition conditions (lanes 2 and 3). Moreover, nucleoplasmin does not alter the manner in which the various proteins reconfigure the HIV-1 nucleosome and linker regions (lanes 4, 6, 8 and 10). Interestingly, removal of the bound transcription factors by oligonucleotide challenge did not produce a large domain of DNase I hypersensitivity (lanes 5, 7, 9 and 11). Rather, for each combination of bound activators, the degree of cutting by DNase I following oligonucleotide competition reverts back to that observed for the initial state without factors, indicating that histones are not displaced from the factor-bound HIV-1 nucleosome. We conclude, therefore, that the sites displaying altered sensitivity to DNase I within the HIV-5S nucleosomal array result from ternary complexes formed by the binding of transcriptional activators directly to the HIV-1 nucleosome.

Discussion

In this study, we have analyzed the effects of transcription factor binding to DNA sequences present on a single HIV-1 nucleosome core, and to this nucleosome placed within the more physiological context of an array of nucleosomes. The affinities of Sp1, NF-κB1, LEF-1, ETS-1 and USF for sites positioned within isolated core particles do not appear to be significantly different from those for sequences located in a polynucleosomal template. Interaction of NF-κB1 with the HIV-1 mononucleosomes enhances DNase I sensitivity in the regions directly flanking its recognition elements, and this effect is reproduced upon NF-κB1 binding to the HIV-5S array of nucleosomes. In addition, the changes in DNase I accessibility resulting from transcription factor binding to either the HIV-1 core particles or the nucleosomal array are both derived from the formation of ternary complexes composed of transcriptional activators, histones and DNA. Thus, the results obtained from these binding studies utilizing the HIV-1 mononucleosome core particles are consistent with those obtained with the HIV-5S nucleosomal array, illustrating the utility of mononucleosomes as an experimental system.

The binding of Sp1, NF-κB1, LEF-1, ETS-1 and/or USF to the HIV-1 nucleosome generates ternary complexes which are stable *in vitro*. Surprisingly, the HIV-1 transcription factor-nucleosome complexes resist the loss of histones by the action of the histone-binding protein, nucleoplasmin, or the ATP-driven SWI-SNF complex. This suggests that the binding of these factors to this particular nucleosome adopts a conformation favoring the

Fig. 5. Multiple proteins specifically remodel the HIV-1 nucleosome located within an array of nucleosomes. Combinations of Sp1 (47 nM), NF-κB1 (200 nM), LEF-1 (50 nM), ETS-1 (100 nM) and USF (150 nM) were incubated with the array prior to DNase I treatment as indicated, and the digestion products resolved by agarose gel electrophoresis. Lane 1 displays partial digestion of the HIV-5S DNA fragment by *EcoRI*. Nucleosome positions and the orientation of the HIV-1 fragment within the HIV-5S template are illustrated on the right.

retention of the histone proteins. In this regard, it is intriguing that NF-κB1 binding dramatically alters the DNase I digestion profile of the HIV-1 mononucleosomes at sequences located more than one superhelical turn away from the site of protein-DNA interaction (see Figure 1D). In addition, the binding of Sp1 or USF also changes the DNase I cleavage pattern of the HIV-1 core particles throughout the entire nucleosome. Thus, the interaction of each of these factors alters histone-DNA interactions without forcing the eviction of histone proteins from the DNA sequences.

When analyzed within the context of an array of nucleosomes, Sp1 and NF-κB1 remodel the HIV-1 nucleosome in the absence of LEF-1, ETS-1 and USF, which is consistent with *in vivo* footprinting of the HIV-1 5' LTR revealing occupancy of only the two upstream Sp1 elements and the downstream NF-κB site in the enhancer/promoter domain (Demarchi *et al.*, 1993). Furthermore, binding by Sp1 and NF-κB1 *in vitro* specifically increases MNase and DNase I accessibility at the HIV-1 nucleosome to produce hypersensitive sites strikingly similar to those detected *in vivo* for the integrated HIV-1 provirus. HIV-1 proviral DNA harbors three predominant DHSs in the 5' LTR that are constitutively present (Verdin, 1991; Verdin *et al.*, 1993). Two of these sites, DHSs 2 and 3, are present within the disrupted nucleosome between nuc-0 and nuc-1 (i.e. the sequences used in this study). The 3' border of DHS 2 falls within the LEF-1 recognition sequence and

Fig. 6. Remodeling of the HIV-1 nucleosome occurs without dissociation of the underlying histones. Different combinations of Sp1 (47 nM), NF-κB1 (200 nM), LEF-1 (50 nM), ETS-1 (100 nM) and USF (150 nM) were incubated with the array in the presence of nucleoplasmin (67 nM). After completion of the binding reactions, the designated samples were subjected to oligonucleotide competition to remove bound factors from the nucleosomal array. All samples subsequently were treated with DNase I, and the digestion products resolved by agarose gel electrophoresis. Partial digestion of the HIV-5S DNA fragment by *EcoRI* is shown in lane 1. Nucleosome positions on the HIV-5S template are illustrated on the right.

extends upstream of the ETS-1 and USF binding elements, whereas the 5' edge of DHS 3 falls within the second Sp1-binding site and stretches downstream to position -6. As a result, these DHSs essentially flank the upstream Sp1-binding site, as well as the two NF-κB sites. Importantly, the binding of Sp1 and NF-κB1 to the HIV-5S nucleosomal array causes these same regions to become hypersensitive to DNase I digestion. Furthermore, MNase accessibility in cellular chromatin is pronounced, compared with the neighboring linker regions, at sites directly adjacent to the 3' and 5' boundaries of nuc-0 and nuc-1, respectively (Verdin *et al.*, 1993). In agreement with this *in vivo* observation, NF-κB1 binding to the HIV-5S array increases the level of MNase digestion within the linker region directly downstream of the HIV-1 nucleosome, which is the location in the array theoretically representing the site adjacent to the 5' border of nuc-1. Thus, given the similarities between the nuclease digestion profiles for HIV-1 nucleosomal DNA *in vivo* and *in vitro*, these data provide evidence that the HIV-1 proviral chromatin organization may include a nucleosome positioned between nuc-0 and nuc-1, which is bound by Sp1 and NF-κB. Indeed, the possibility that a nucleosome resides in this DNase I-sensitive region in proviral chromatin is consistent with the fact that this region contains a nucleosome-length area of MNase resistance (Verdin *et al.*, 1993). This is similar to the MMTV promoter where

factor-bound nucleosome B is DNase I sensitive but MNase resistant (Truss *et al.*, 1995).

The results described here are in general agreement with an earlier *in vitro* study implicating Sp1 and NF- κ B in the modeling of chromatin at the HIV-1 LTR (Pazin *et al.*, 1996). That study used a *Drosophila* embryo extract to assemble nucleosomes on plasmids containing the HIV-1 5' LTR. Our studies utilizing purified systems provide two important advancements relative to the earlier work. First, Pazin and colleagues found that Sp1 and NF- κ B alter the local chromatin structure at the HIV-1 promoter when these factors are pre-bound to the DNA before nucleosome assembly in the extract. We show here that these factors can in fact access their sites in pre-formed mononucleosomes and pre-formed nucleosomal arrays containing the HIV-1 sequences. Second, based on DNase I sensitivities, the authors of the earlier study suggested that the pre-binding of Sp1 and NF- κ B changes the translational positioning of adjacent nucleosomes on the DNA to resemble the native structure. However, the predicted position for nuc-0 in the *Drosophila* embryo extracts (Pazin *et al.*, 1996) appears to be at least 100 bp downstream of its *in vivo* location (Verdin *et al.*, 1993). In contrast, we show here that DNase I hypersensitivities which closely resemble the *in vivo* structure are formed upon binding of Sp1 and NF- κ B1 to HIV-1 5' LTR sequences within a pre-formed nucleosomal array without requiring repositioning of the adjacent nucleosomes. In addition, these enhanced DNase I sensitivities are generated by the formation of a stable ternary complex containing both transcription factors and histones. Indeed, the presence of a ternary complex between nuc-1 and nuc-0, rather than a nucleosome-free gap generated by an alteration of nucleosome positioning, is more consistent with the *in vivo* resistance of these DHSs to MNase digestion (Verdin *et al.*, 1993).

The issue of when the regulatory regions of remodeled genes in cellular chromatin are free of histones is unclear. Indeed, the possibility that DHSs vary in histone composition from site to site *in vivo* is now supported by biochemical studies. This work suggests that transcriptional activators can co-occupy DNA sites with histones to form ternary complexes yielding increased accessibility to DNase I. In contrast, a histone-free DHS is produced *in vitro*, through the *trans* displacement of histones from the DNA, by the binding of GAL4-AH to a nucleosomal array in the presence of accessory remodeling activities (Owen-Hughes and Workman, 1996; Owen-Hughes *et al.*, 1996). It is interesting to speculate that the rearrangement of HIV-1 chromatin for transcription may produce DHSs consisting of both ternary complexes and histone-free regions. HIV-1 native chromatin between nuc-0 and nuc-1 is resistant to MNase digestion, yet sensitive to DNase I activity (Verdin *et al.*, 1993). Our data indicate that this could result from the constitutive binding of Sp1 and NF- κ B to a nucleosome. However, prior to transcriptional activation, additional remodeling events lead to increased accessibility by both MNase and DNase I within the region encompassed by nuc-1 (Verdin *et al.*, 1993), suggesting that histones may be removed from that region. As nuc-1 is positioned over the transcription start site, its displacement may be necessary for the binding and engaging of RNA polymerase II (Kornberg and Lorch, 1995).

Materials and methods

Plasmid construction

To construct the HIV-5S array plasmid, p208-5@RVrev (Owen-Hughes and Workman, 1996) was digested with either *Xho*I and *Sma*I, or *Xho*I and *Pvu*II, which generates two fragments each containing five direct repeats of the sea urchin 5S rDNA nucleosome positioning sequence. The *Xho*I-*Sma*I fragment was subcloned into pIC20R (Marsh *et al.*, 1984) digested with *Xho*I and *Eco*RV to produce pIC208-5@E.X. The *Xho*I-*Pvu*II fragment was placed into pIC20R digested with *Sa*II and *Nru*I to generate pIC208-5@S.N. To make pIC-2085S, a plasmid which has five tandem repeats of 5S rDNA sequence flanking both sides of a short linker region, pIC208-5@S.N was treated sequentially with *Xba*I, Klenow and *Bgl*II, the 5S rDNA fragment isolated, and subcloned into pIC208-5@E.X sequentially treated with *Asp*718, Klenow and *Bam*HI. The HIV-5S array plasmid, p2085S/HIV, was constructed by subcloning an HIV-1 5' LTR PCR product from -204 to -34 into pIC-2085S sequentially treated with *Xho*I under limiting conditions, *Xba*I and Klenow. p2085S/HIV contains the HIV-5S array fragment illustrated in Figure 4A.

Protein purification

Bacterial expression vectors for human NF- κ B1 (i.e. p50 subunit) (Kretzschmar *et al.*, 1992) and human USF (Pognonec and Roeder, 1991) were transformed into BL21(DE3)pLysS (Studier *et al.*, 1990), and the respective proteins prepared as described in Adams and Workman (1995). To isolate LEF-1, 500 ml of BL21(DE3)pLysS cells transformed with a human LEF-1 expression plasmid (Waterman *et al.*, 1991) were grown to a density of OD₆₀₀ = 0.25, then treated with 0.5 mM IPTG for 2.5 h to induce expression of the protein. Cells were harvested and lysed in lysis buffer [20 mM Tris-HCl pH 7.8, 500 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine] with one cycle of freeze-thawing. After a 30 min spin at 40 000 g, the supernatant was brought up to 10% glycerol and dialyzed to 100 mM salt with buffer T (20 mM Tris-HCl pH 7.8, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 10% glycerol). The dialyzed bacterial extract was loaded onto a 0.8 ml heparin-Sepharose column equilibrated in buffer T. Following a column wash with buffer T-200 mM KCl, LEF-1 was eluted with buffer T-400 mM KCl. To isolate ETS-1, BL21(DE3)pLysS cells transformed with a human ETS-1 expression plasmid (Fisher *et al.*, 1994) were treated as described for LEF-1 bacterial expression and cell harvest. After a 30 min spin at 40 000 g, proteins in the cell pellet were resolubilized in 1 ml of buffer A (6 M guanidine-HCl, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0) by gently mixing at 4°C for 30 min. After a 30 min spin at 40 000 g, the 6×His-tagged ETS-1 protein was purified by passing the supernatant over a Ni-NTA spin column (Qiagen), and eluting bound protein according to the manufacturer's specifications. The purified ETS-1 protein was renatured slowly by stepwise dialysis in buffer T with successively decreasing concentrations of guanidine-HCl. Affinity-purified human Sp1 was obtained commercially from Promega. H1-depleted oligonucleosomes were prepared from HeLa nuclear pellets as described by Côté *et al.* (1995). Preparation of nucleoplasmin (Walter *et al.*, 1995) and SWI-SNF (Côté *et al.*, 1994) were as described previously. GAL4-AH was prepared from bacterial strains as described by Lin *et al.* (1988).

Preparation of probe DNA and nucleosome reconstitution

The nucleosome-length HIV-1 fragment from -199 to -49 was produced by PCR amplification of 5' LTR DNA with primers spanning nucleotides -199 to -180 and -49 to -68. To radioactively label only one DNA end, the downstream primer was phosphorylated with [γ -³²P]ATP by T4 polynucleotide kinase prior to the PCR. After the PCR, the HIV-1 DNA fragment was purified by electrophoresis through a 2% low melting point agarose gel, and isolated by β -agarase I treatment. To prepare the end-labeled HIV-5S nucleosomal array fragment, p2085S/HIV was digested with *Asp*718, treated with Klenow in the presence of [α -³²P]dATP, and digested further by *Pvu*II and *Ssp*I. The HIV-5S DNA fragment was gel purified as described above for the nucleosome-length PCR product.

Nucleosome reconstitution by histone octamer transfer was performed essentially as described by Côté *et al.* (1995). In short, 20 and 40 pmol of HeLa nucleosomes were mixed at 37°C with ~0.5 pmol of the 151 bp HIV-1 probe DNA and ~0.2 pmol of the HIV-5S array fragment, respectively, in a final volume of 10 μ l containing 50 mM HEPES pH 7.5, 1 M NaCl, 1 mM EDTA, 5 mM DTT and 0.5 mM PMSF. These

were serially diluted by adding 1.8, 3.5, 4.7 and 13 μ l of 50 mM HEPES pH 7.5, 1 mM EDTA, 5 mM DTT and 0.5 mM PMSF, with 30 min incubations at 30°C for each dilution step. Transfer reactions were brought to 0.1 M NaCl by adding 67 μ l of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% NP-40, 0.5 mM PMSF, 20% glycerol and 100 μ g/ml bovine serum albumin (BSA), and incubated for 30 min at 30°C. Reconstitutions were aliquoted and stored at either -80°C for the mononucleosomes or 4°C for the nucleosomal array. For a mock reconstitution, the probe DNA was added to the transfer reaction after the salt had been brought down to 100 mM NaCl, rather than at the initial high-salt step.

Binding reactions

Binding reactions were performed in 20 μ l with 10 mM HEPES pH 7.8, 50 mM KCl, 5 mM DTT, 0.5 mM PMSF, 0.25 mg/ml BSA and 5% glycerol. Typical binding reactions contained ~1–2 fmol of reconstituted probe, along with donor nucleosomes, to give a total of 200 fmol of nucleosomes. For the experiments in Figures 2 and 3, the binding reactions contained 5- and 4-fold less probe, respectively, than that present in a typical reaction. Also, reactions in Figure 2 contained 1 mM ATP and 4 mM MgCl₂ as required for SWI-SNF activity. All protein dilutions of stocks were made in binding reaction conditions, except for SWI-SNF and Sp1. These proteins were added directly to the binding reactions without dilution, and, therefore, the appropriate amounts of SWI-SNF and/or Sp1 buffers were added to reactions not receiving these proteins, so that the buffer conditions within an experiment were identical from sample to sample. Binding reactions were performed at 30°C for 30 min. For the HIV-1 nucleosome-binding reactions passing through the oligonucleotide competition step, 1 μ l of an HIV-1 competition mix (50 μ M each double-stranded oligonucleotide representing binding sites for NF- κ B1, LEF-1, ETS-1 and USF, 5 μ M double-stranded oligonucleotide representing a Sp1-binding site, 2 M NaCl) was added and the reactions incubated at 37°C for 60 min. Listing the top strands only, the Sp1-binding site oligonucleotide is from the human metallothionein-IIa gene: 5'-GGACGGCCGGGGCGGGCTTTTG-CAC-3'; the NF- κ B-binding site oligonucleotide is from the mouse immunoglobulin κ light chain gene: 5'-CAACAGAGGGGACTTCCG-AGAGGCC-3'; the LEF-1-binding site oligonucleotide is from the human T-cell receptor α gene: 5'-GATCGGCACCTTTGAAGCTCATG-3'; the ETS-1-binding site oligonucleotide is from the murine sarcoma virus LTR: 5'-GATCGGAGAGCGGAAGCGCGCATAG-3'; and the USF-binding site oligonucleotide is a synthetic optimal sequence: 5'-GAT-CTTAGTCACGTGGCATGATCG-3'. To remove GAL4-AH from bound complexes, 1 μ l of a GAL4-AH competition mix (300 μ M double-stranded oligonucleotide representing a GAL4-binding site, 2 M NaCl, 50 mM MgCl₂) was added to reactions and incubated at 37°C for 60 min as described previously (Owen-Hughes and Workman, 1996).

EMSA, MNase and DNase I protection analysis

Binding reactions were either subjected to analysis by native gel shift or nuclease digestion. EMSA was performed in 4% polyacrylamide (29:1 acrylamide:bisacrylamide), 0.5 $\times$ TBE gels. Mononucleosome-binding reactions were digested with DNase I by adding 2 μ l of 0.5 U/ μ l DNase I in 50 mM MgCl₂, and incubating for 1 min at room temperature. Reactions containing mock-reconstituted DNA were treated with 10-fold less DNase I (i.e. 2 μ l of 0.05 U/ μ l DNase I in 50 mM MgCl₂) for 1 min at room temperature. DNase I activity was terminated by adding 20 μ l of stop mix (20 mM Tris-HCl pH 7.5, 50 mM EDTA, 1% SDS, 0.25 mg/ml tRNA, 0.2 mg/ml proteinase K), and the proteins degraded by incubating at 50°C for at least 1 h. The DNA subsequently was ethanol precipitated, and loaded onto 8% polyacrylamide (19:1 acrylamide:bisacrylamide), 8 M urea, 1 $\times$ TBE sequencing gels. Unless indicated in the figure legend, binding reactions containing the HIV-5S nucleosomal array were treated with either 2 μ l of 0.01 mU/ μ l MNase in 30 mM CaCl₂ for 1 min at room temperature, 2 μ l of 0.125 U/ μ l DNase I in 50 mM MgCl₂ for 1 min at room temperature or, for samples passing through the oligonucleotide competition step, 2 μ l of 0.25 U/ μ l DNase I in 50 mM MgCl₂ for 5 min at room temperature. The nuclease activity was terminated and the proteins degraded as described above for the nucleosome core particles. Following ethanol precipitation, the DNA was electrophoresed through 2% agarose gels. Gels subsequently were fixed by soaking in a solution composed of 10% acetic acid and 10% methanol, and then dried. Probe DNA for EMSA and MNase/DNase I protection analysis was detected by autoradiography.

Acknowledgements

We are grateful to Marian Waterman and Takis Papas for providing the hLEF-1 and hETS-1 bacterial expression vectors, respectively. We kindly

thank the members of the Workman laboratory for helpful advice and many interesting discussions. This work was supported by grants from the NIH and NSF to J.L.W. D.J.S. is a recipient of a postdoctoral fellowship from the Cancer Research Institute. J.L.W. is a Leukemia Society Scholar.

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Received on November 22, 1996; revised on January 16, 1997