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Minireview

Nucleosomes and regulation of gene expression. Structure of the HIV-1 5′LTR*°

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Packaging of DNA into chromatin adds complexity to the problem of regulation of gene expression. Nucleosomes affect the accessibility of transcription factors to occupy their binding sites in chromatin of eukaryotic cells. The disruption of nucleosome structure within the enhancer/promoter region of the integrated HIV-1 proviral genome is an instructive example of a chromatin remodeling process during transcriptional activation. To investigate the mechanism responsible for generating nuclease hypersensitive sites that exist *in vivo* in the promoter/enhancer region of the 5′LTR (long terminal repeat) of integrated HIV-1 we have utilized an *in vitro* chromatin assembly system with *Xenopus* oocyte extracts. Chromatin assembly in the presence of Sp1 and NFκB transcription factors induces DNase I hypersensitive sites on either side of their binding sites and positions the adjacent nucleosomes. This structure can also be formed in a factor-induced, ATP-dependent chromatin remodeling process and closely resembles the *in vivo* chromatin structure. The DNase I hypersensitive sites that form within the HIV LTR are probably histone-free and remain after removal of transcription factors.

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Abbreviations: LTR, long terminal repeats; MNase, micrococcal nuclease; NURF, nucleosome remodeling factor; RSC, remodeling the structure of chromatin; SWI/SNF, switching the mating type/sucrose non-fermenting.

THE PACKAGING OF DNA INTO CHROMATIN REPRESSES GENE ACTIVITY

In the nucleus of an eukaryotic cell DNA is packaged into a nucleoprotein complex termed chromatin. This packaging of the template provides the compaction and organization of DNA for transcription, replication, repair and recombination processes. The fundamental structural unit of chromatin is the nucleosome. Within each nucleosome about two turns of left-handed superhelical DNA (146 bp) are wrapped around an octamer of core histones to make a disk of about 11 nm in diameter and 5.6 nm in height (core particle). In addition the nucleosome consists of a linker region of variable length (generally less than 50 bp), which interacts with linker histone (e.g. histone H1) and/or other non-histone proteins. The polynucleosomal chain is further looped and folded into various higher order structures (e.g. 30 nm chromatin fibers and chromatin loops); this leads to structural and functional organization of the genome in the interphase nucleus (reviewed in [1–3] and references therein).

A nucleosome can be placed in a unique position with respect to DNA sequence, so a particular sequence might face either toward or outward from the histone octamer (rotational positioning). A particular sequence can also be specifically located within nucleosomal core or linker DNA (translational positioning). Such specific histone-DNA contacts seem to be common features of the regulatory regions of genes. Nucleosomes positioned in these regions often hinder the accessibility of transcription factors to occupy their binding sites. On the other hand, nucleosome formation might bring together binding sites that would be separated if DNA was a straight linear molecule (termed juxtaposition), and modulate the action of transcription factors. In addition, transcriptional elongation is attenuated by the formation of the polynucleosomal chain, leading to further regulatory opportunities. It is also suggested that formation of specific chromatin structures leads to transcriptional repression of chromatin regions and domains (e.g. interactions between histones and Sir3/Sir4 proteins required for silencing at telomeres and mating loci in yeast). Although it seems clear that the packaging of DNA into chromatin creates new possible mechanisms of transcriptional regulation *in vivo*, we are now only beginning to understand these processes (reviewed in: [4–8] and references therein).

HISTONE ACETYLATION AND ATP-DRIVEN CHROMATIN PROCESSORS DEREPRESS CHROMATIN DOMAINS AND FACILITATE TRANSCRIPTION FACTOR BINDING

Sites in chromatin that have already bound transcription factors, or that will allow their binding, are experimentally detected as nuclease hypersensitive sites and are usually indicators of regulatory regions of potentially active or active genes (reviewed in [9]). It is theoretically possible that DNA replication might be required for transcription factors to invade nucleosomes at the time of chromatin reassembly, but no experimental evidence for this mechanism has been obtained yet. Rather, replication-independent mechanisms seem to have evolved to allow access of transcription factors to DNA complexed to histone octamers during transcriptional activation. At least two different mechanisms that act on the level of chromatin to activate transcription are known: non-covalent remodeling of nucleosome structure and covalent post-translational modification of histones. At least three different (yet related) protein com-

plexes have been identified so far that remodel chromatin to facilitate loading of transcription factors onto regulatory sequences originally packaged as nucleosomes: (i) the SWI/SNF complex (term originated from switching the mating type/sucrose nonfermenting) in yeast and other eukaryotes, (ii) NURF (nucleosome remodeling factor) in *Drosophila* and (iii) RSC complex (remodeling the structure of chromatin) in yeast. Although all these complexes utilize ATP, the mechanisms of their action are still under investigation. It is also unclear whether transcription factor binding results in loss of histones or in the formation of a ternary complex that contains histones (reviewed in [8, 10–12] and references therein). The most common post-translational modification of the core histones is acetylation and evidence continues to accumulate that histone acetylation/deacetylation plays an important role in chromatin repression/derepression and transcriptional regulation. Histone acetyltransferases transfer acetyl groups onto conserved lysine residues in the amino-terminal domains of the core histones (histone tails). Neutralization of highly charged histone tails leads to weakening of the histone-DNA interactions and changes interactions between histones and non-histone proteins, "opening up" the chromatin. It has been recently found that histone acetyltransferases and deacetylases are brought to the specific sites on the chromatin due to their interactions with transcriptional activators and repressors (reviewed in [13–15]). At the moment, it is generally accepted that structural transitions in chromatin caused by histone acetylation and/or "chromatin remodeling machines" facilitate the binding of regulatory proteins to specific DNA sequences in promoters, which in turn allows assembly of RNA polymerase complex to activate a gene.

ACTIVATION OF THE INTEGRATED HIV-1 GENOME LEADS TO DISRUPTION OF A NUCLEOSOME POSITIONED WITHIN ITS PROMOTER REGION

HIV-1 is a highly pathogenic retrovirus that causes AIDS. Like other retroviruses HIV-1 contains two long terminal repeats (LTR) that flank three structural genes *gag*, *pol* and *env*, and also a number of specific regulatory genes. The replication rate of the integrated HIV-1 is primarily controlled at the level of transcription. *Cis*-acting elements necessary for transcription initiation are localized within the 5'LTR while elements responsible for polyadenylation of the viral transcripts are present within 3'LTR. Once the virus integrates into the host genome, its gene expression is regulated by cellular transcription factors. Binding sites for several human transcription factors have been described within the 5'LTR (see Fig. 1A). In addition to these cellular factors, the activity of the HIV-1 promoter strongly depends on the viral transactivator *tat* (reviewed in [16–18]). *In vivo* analysis of HIV-1 infected T-cells has demonstrated that nucleosomes are positioned in the 5'LTR of the integrated HIV-1 approximately between −415 and −255 bp (nuc-0) and between −5 and +155 (nuc-1). Two DNase I hypersensitive sites, termed HS2 and HS3, are observed between nuc-0 and nuc-1. An additional DNase I hypersensitive site (HS4) is positioned downstream nuc-1 (approximately +200 to +265 bp) (see Fig. 1B). Activation of infected T-cells results in disruption of nuc-1 (that originally covered the transcription initiation site) and leads to a subsequent increase in HIV-1 transcription [19, 20]. The hypersensitive sites HS2, HS3 and HS4 contain binding sites for a battery of transcription factors. Some of these factors (e.g. Sp1, NFκB, LEF1 and ETS1) are known

to be activators of viral gene transcription either *in vivo* or *in vitro* from chromatin but not naked DNA templates [21, 22]. Thus transcription factor binding is involved in chromatin remodeling and derepression of the proviral genes.

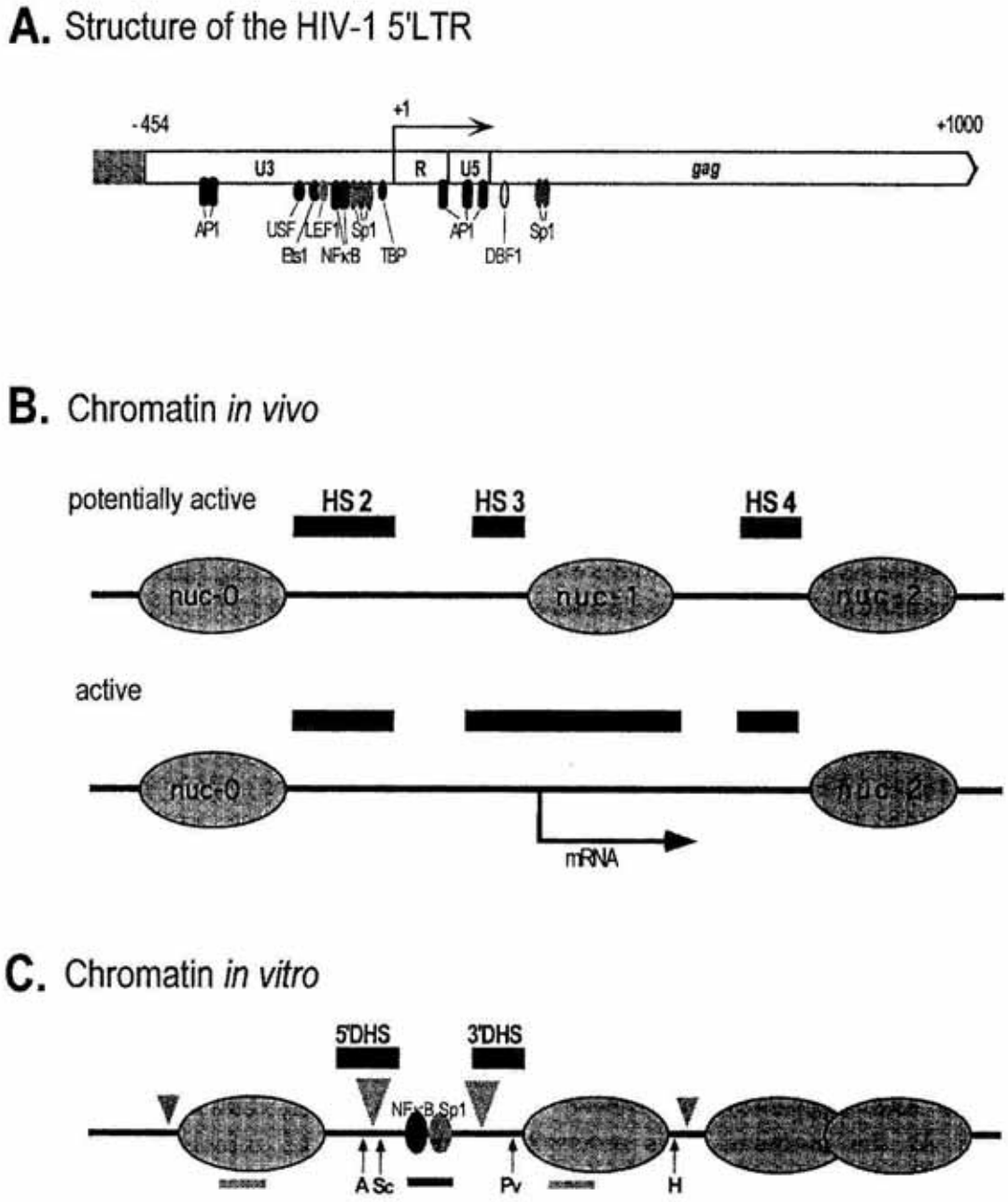

Figure 1. Structure of the HIV-1 5'LTR.

Panels: **(A)** Segment of the HIV-1 genome used in this study, showing the transcription initiation site and positions of transcription factor binding sites; **(B)** Schematic model for chromatin structure of the 5'LTR of proviral DNA integrated into the human genome, either in its potentially active or active states (adapted from Verdin [19, 20]); **(C)** Structure of the 5'LTR assembled into chromatin using *Xenopus* oocyte extracts in the presence of Sp1 and NFκB transcription factors ([25] and this study). Symbols indicate the positions of nucleosomes (large ovals), bound transcription factors (small ovals), DNase I hypersensitive sites (HS or DHS; black bars above), MNase hypersensitive sites (gray arrowheads), MNase footprints (gray lines below) and DNase I footprint (black line below), and restriction sites exhibiting enhanced cleavage (small arrows; A, $AvaI_{158}$; Sc, $ScaI_{140}$; Pv, $PvuII_{21}$; H, $HinfI_{+124}$).

XENOPUS OOCYTE EXTRACTS ASSEMBLE *BONA FIDE* CHROMATIN ON THE HIV-1 5'LTR TEMPLATES

To study the mechanism by which transcription factors establish hypersensitive sites in the chromatin of the HIV-1 5'LTR we utilized an *in vitro* chromatin assembly system with *Xenopus* oocyte extracts and the direct end-labeling technique pioneered by Worcel and coworkers [23, 24]. DNA circles 4.2 kbp in length that contained about 1.5 kbp of the HIV-1 proviral DNA (5'LTR and 5' fragment of the *gag* gene) were linearized at a specific restriction site, end-labeled with polynucleotide kinase and ligated to reclose the circles. Radioactive circles were incubated with *Xenopus* oocyte extracts in the presence of an ATP-regenerating system. To characterize the kinetics and efficiency of chromatin assembly we assayed for the production of negative DNA supercoils and investigated the micrococcal nuclease (MNase) generated ladder of the fully assembled minichromosomes. The analyses indicated that the *Xenopus* oocyte system assembled regular polynucleosomal structures with nucleosome repeat lengths of about 175 bp ([25] and data not shown). Transcription factors were added either prior to or after chromatin assembly, thereby allowing potentially to distinguish between replication-dependent and independent mechanisms for hypersensitive site formation. The resulting chromatin structures were studied by nuclease digestion, DNA was purified and the molecules were cleaved at a restriction site immediately adjacent to the original site that was ^{32}P-labeled, thereby transferring the label to one specific end of all molecules. The samples were separated by gel electrophoresis, gels were dried and exposed to PhosphorImager screens. The advantages of this approach are to eliminate a time-consuming conventional Southern blot-hybridization and reduce the amounts needed of costly transcription factors.

SP1 AND NFκB BINDING INDUCES NUCLEASE HYPERSENSITIVE SITES AND POSITIONS ADJACENT NUCLEOSOMES IN THE HIV-1 5'LTR

We determined that Sp1 and NFκB transcription factors added individually or together before or after chromatin assembly with *Xenopus* oocyte extracts created DNase I hypersensitive sites within the 5'LTR. When added individually, each factor generated hypersensitive sites apparently flanking both sides of their DNA binding sites, and when added together the hypersensitive sites (centered at positions -40 and -160 bp) exhibited a broadening intervening footprint. All of these sites were chromatin specific and did not appear when the factors were complexed with naked DNA. Significantly, when chromatin was assembled in the absence of Sp1 and NFκB, the later addition of these factors induced the DNase I hypersensitive sites in an ATP-dependent fashion. This suggests the involvement of a SWI/SNF- or NURF-related complex in chromatin remodeling in the *Xenopus* system ([25] and Fig. 2A). To determine whether nucleosome positions within the LTR are modulated by Sp1 and NFκB binding we mapped MNase cutting sites within minichromosomes. In the absence of the factors all MNase cutting sites were common between the naked DNA and chromatin, while binding of Sp1 and NFκB to chromatin generated a pair of major MNase hypersensitive sites in positions essentially identical to the DNase I hypersensitive sites. In addition, minor MNase hypersensitive sites and footprinted regions were observed, that indicated that binding of Sp1 and NFκB positioned adjacent nucleosomes, one upstream of NFκB-binding site and the other downstream of Sp1-binding site ([25] and Fig. 2B). The factor-induced formation of hypersensitive sites within the 5'LTR was also confirmed by assaying for a local disruption of the nucleosomal ladder (Fig. 2C). To more accurately deter-

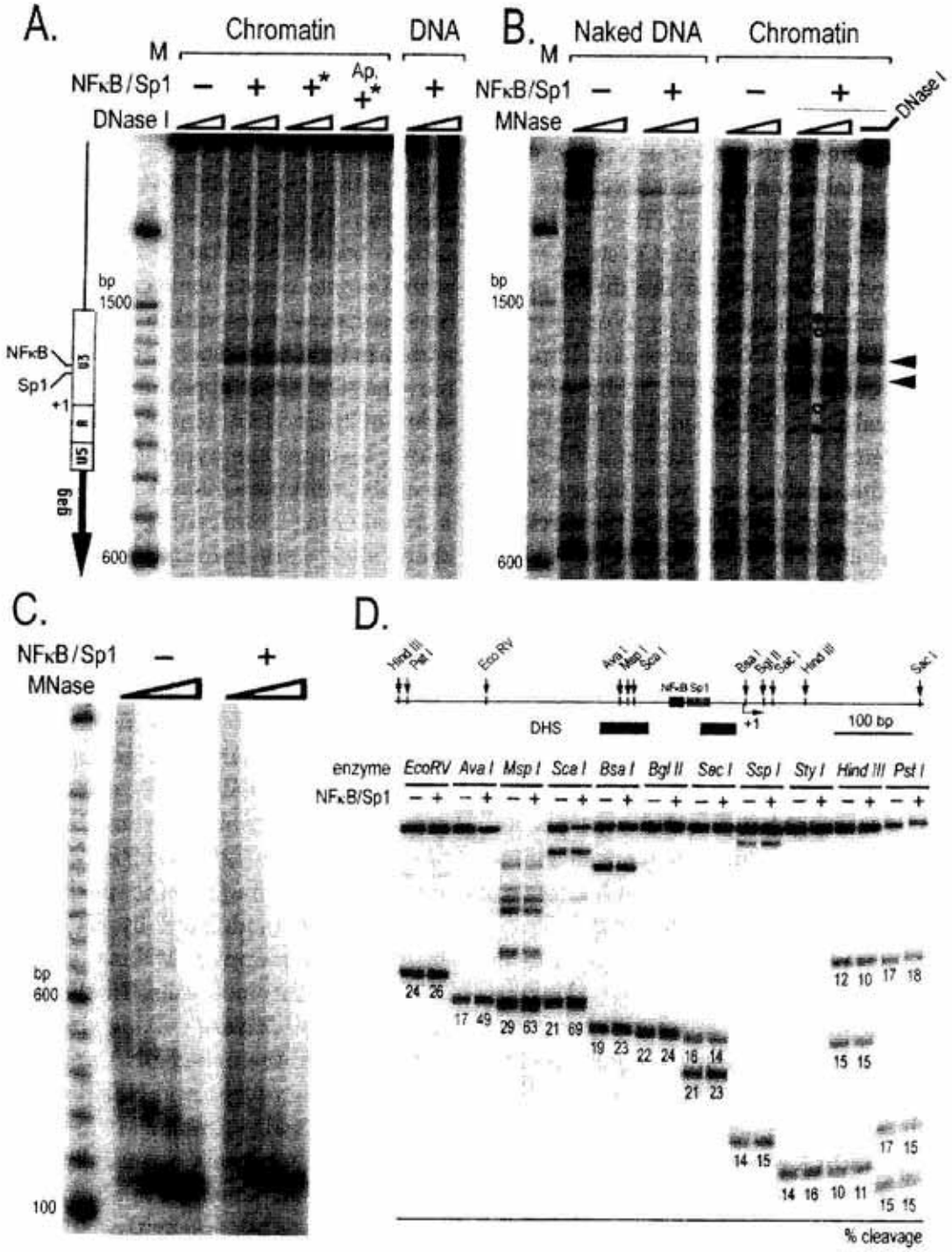

Figure 2. Sp1 and NFκB induce nuclease hypersensitive sites and adjacent positioned nucleosomes in the HIV-1 5'LTR.

Panels: **(A)** Formation of DNase I hypersensitive sites in the presence of transcription factors. The location of factor binding sites is indicated alongside the diagram. Lane M represents a marker 100 bp-ladder. Factors added after assembly are indicated with asterisks. To remove ATP the assembly mixture was treated with apyrase (Ap.; 0.01u/μl, for 15 min) before transcription factors were added to minichromosomes. **(B)** Pattern of MNase digestion of DNA and reconstituted chromatin. Indicated are major (large arrowheads) and minor (closed circles) MNase hypersensitive sites and footprinted regions (open circles). **(C)** Disruption of the nucleosomal ladder by transcription factors binding. Minichromosomes were labeled at the *Ava*I site located at −158 bp. **(D)** Digestion of reconstituted chromatin with restriction enzymes. The positions of key restriction sites within the 5'LTR (above) and the effects of transcription factor binding upon accessibility of restriction sites (bottom) are shown. The percentages of cleavage upon reaching complete digestion are shown below the indicated restriction fragments.

mine the change in nuclease accessibility generated by Sp1 and NFκB we digested minichromosomes to completion with several different restriction enzymes and determined the cleavage efficiencies. Binding of the factors increased the accessibility of the *Ava*I, *Msp*I, *Sca*I restriction sites located upstream of the NFκB-binding site (and the *Pvu*II site located downstream of the Sp1-binding site [25]), while numerous other sites revealed no increase in accessibility upon factor binding (Fig. 2D). The *Xenopus* oocyte extracts used in our chromatin assembly experiments contain only an embryonic form of a linker histone termed B4. We aimed to determine whether histone H1 association with nucleosomes would inhibit chromatin remodeling induced by Sp1 and NFκB. We found that histone H1 binding increased the nucleosome repeat length, however the histone H1 containing chromatin still could be remodeled whether the factors were added before or after assembly (not shown).

HYPERSENSITIVE SITES WITHIN THE HIV-1 5'LTR ARE PROBABLY HISTONE-FREE AND PERSIST AFTER REMOVAL OF SP1 AND NFκB

An interesting question is whether histones still remain bound within a hypersensitive chromatin region after ATP-dependent nucleosome disruption [10]. In a model *in vitro* system Workman and coworkers [26] have shown that the SWI/SNF complex can displace histones from a GAL4-occupied hypersensitive site, and the hypersensitivity remained even after removal of bound transcription factors by oligonucleotide competition. However, a similar study on the HIV LTR revealed that histones remain bound [27]. We attempted related experiments in the *Xenopus* system with the HIV LTR, where we performed gel mobility analysis on the hypersensitive region after its excision by restriction enzymes from minichromosomes. Minichromosomes that were assembled either in the absence or presence of Sp1 and NFκB were digested with *Bgl*II and *Ava*I to partially liberate a 178 bp fragment containing Sp1 and NFκB-binding sites (3' fragment) or with *Eco*RV and *Ava*I to generate an immediately upstream 183 bp fragment (5' fragment) (Fig. 3A, upper diagram). In addition, corresponding naked DNA fragments were also complexed with Sp1 and NFκB, and then all samples were analyzed by native gel electrophoresis (Fig. 3A). After binding of the transcription factors the excised chromatin fragments of the 3' (but not the 5') sequence exhibited a gel mobility indistinguishable from that of histone-free DNA molecules associated only with Sp1 and NFκB (Fig. 3A, lanes 4 and 8, closed circles). To determine whether the excised hypersensitive site was histone-free we analyzed its electrophoretic mobility after removal of bound transcription factors by oligonucleotide competition. Minichromosomes were digested with *Sca*I and *Bgl*II to liberate 158 bp fragments that contained Sp1 and NFκB-binding sites and their gel mobility was determined in the presence of an excess competitor oligonucleotide (Fig. 3B). The Sp1 and NFκB binding competing with that of the oligonucleotides resulted in removal of chromatin complexes that contained Sp1 and NFκB (closed circle), and part of the released material was recovered as naked DNA (open circle). We suspect that only partial recovery of the naked 158 bp fragment was due to its "artificial" binding to other proteins that were present in oocyte extracts (even after purification of minichromosomes by sucrose gradient centrifugation). Assuming that oligonucleotide competition could displace transcription factors from their binding sites we aimed to examine if the hypersensitive sites generated within the HIV LTR persisted after Sp1 and NFκB removal (Fig. 3C and 3D). The DNase I hypersensitive sites (Fig. 3C) and positioned nucleosomes adjacent to DNase I and MNase hypersensitive sites (Fig. 3D) were still present in the LTR after oligonucleotide competi-

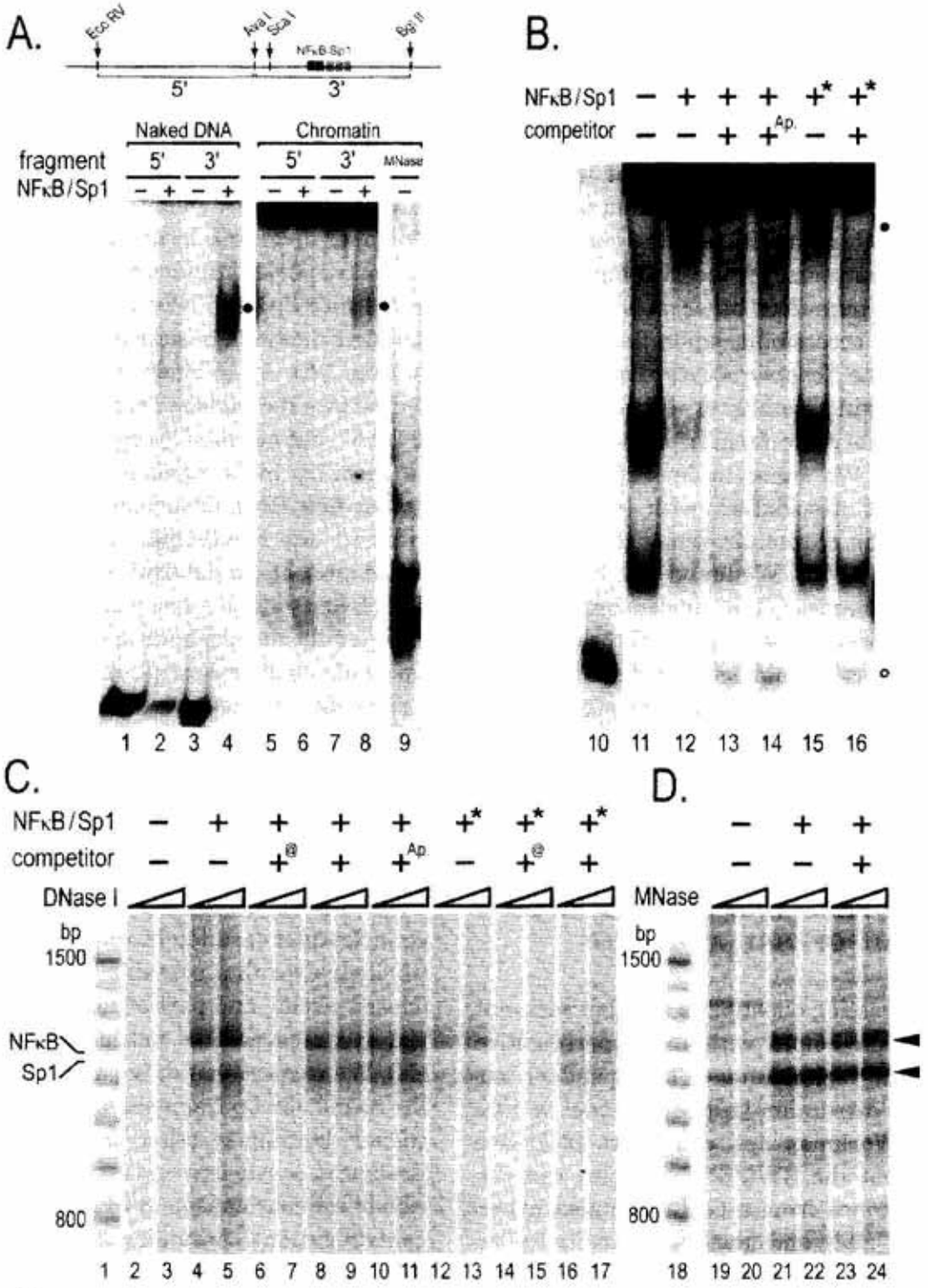

Figure 3. Hypersensitive sites within the 5'LTR are probably histone-free and remain after Sp1 and NFκB binding competing with oligonucleotides.

Panels: **(A)** Gel mobility shift analysis of chromatin fragments released from minichromosomes with restriction enzymes and complexes formed between transcription factors and corresponding naked DNA restriction fragments. Minichromosomes were labeled at the *Ava*I site and digested with *Ava*I and *Eco*RV or *Ava*I and *Bgl*II restriction enzymes (positions of restriction sites are shown above). Naked DNA (lanes 1–4) and chromatin fragments (lanes 5–8) were run on a 2.5% native polyacrylamide gel. The positions of complexes containing Sp1 and NFκB are marked with closed circles. Chromatin digest with MNase (lane 9) is shown as a mononucleosomal standard.

tion of Sp1 and NFκB binding. The oligonucleotides prevented Sp1 and NFκB from inducing hypersensitivity when added before the factors (lanes 6, 7 and 14, 15) and removed the footprint between hypersensitive sites when added after the factors (lanes 8–11 and 16, 17), confirming efficient competition. In summary, our data strongly suggest that hypersensitive sites induced in the HIV-1 promoter/enhancer region in response to Sp1 and NFκB are histone-free and persist after removal of bound transcription factors. This is in contrast to the observations of Steger & Workman [27] who used a more refined *in vitro* system, apparently lacking components that mediate histone displacement.

CHROMATIN STRUCTURE INDUCED *IN VITRO* BY SP1 AND NFκB BINDING RESEMBLES AN *IN VIVO* STATE OF THE HIV-1 PROMOTER/ENHANCER REGION

Our results on how the *Xenopus* system remodels the chromatin structure of the HIV-1 promoter/enhancer region in response to human Sp1 and NFκB are schematically summarized in Fig. 1C. In the absence of transcription factors, the nucleosomes along the HIV-1 5'LTR are randomly positioned. Binding of Sp1 and NFκB to chromatin creates hypersensitive nuclease cutting sites on either side of their binding sites and positions adjacent nucleosomes. This local *in vitro* chromatin structure is similar to the potentially active state of the proviral promoter/enhancer *in vivo*. It is likely that the binding of other transcription factors to this regulatory region (see Fig. 1A) is responsible for broadening of these hypersensitive sites *in vivo* (compare HS2 in Fig. 1B with 5'DHS in Fig. 1C) and creation of the downstream HS4 site [28]. Our *in vitro* results are in agreement with the work of Kadonaga, Jones and others [22] who used *Drosophila* extracts for chromatin assembly experiments. The fact that such evolutionarily diverse systems yield very similar results with heterologous constructs and transcription factors argues for their importance in studies on structure-function relationships in chromatin. Activation of HIV-1 infected T-cells leading to subsequent increases in HIV-1 transcription results in nuc-1 displacement (Fig. 1B). In our *in vitro* model we have not observed disruption of the related nucleosome, thus further analysis will be needed to determine the mechanism of nuc-1 rearrangement.

Experimental data presented in this paper were completed during a stay of P.W. at the University of Texas, Southwestern Medical Center at Dallas.

Figure 3 (continued)

(B) Gel mobility shift analysis of chromatin fragments after competing Sp1 and NFκB binding. Minichromosomes were labeled at the *Bgl*II site and digested with *Sca*I and *Bgl*II restriction enzymes (see scheme in panel A). Transcription factors were added either before chromatin reconstitution or after it (marked with asterisks). After transcription factors were bound chromatin was incubated in the presence of excess competitor oligonucleotides (NFκB; −108 to −76 bp, final conc. 1.25 μM and Sp1; −76 to −44 bp, final conc. 0.25 μM). Some preparations were incubated with apyrase (Ap.) before adding oligonucleotides. After incubation with oligonucleotides minichromosomes were digested with restriction enzymes and run on a 4% native polyacrylamide gel (lanes 11–16) together with *Sca*I/*Bgl*II purified restriction fragment (lane 10). The positions of complexes that contained Sp1 and NFκB are marked with a closed circle, the position of recovered naked DNA (lanes 13, 14, 16) is marked with an open circle. Effects of competing Sp1 and NFκB binding with oligonucleotides on the persistence of the DNase I **(panel C)** and MNase hypersensitive sites **(panel D)**. Chromatin treatment was as described for panel B. Competitor oligonucleotides added to the mixture before transcription factors are marked with @. The location of factor binding sites, and DNase I and MNase hypersensitive sites (arrowheads) are indicated alongside the diagram.

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