

cis-Acting Sequences Located Downstream of the Human Immunodeficiency Virus Type 1 Promoter Affect Its Chromatin Structure and Transcriptional Activity

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Received 2 October 1995/Returned for modification 30 November 1995/Accepted 13 March 1996

We have examined the roles of AP-1, AP-3-like, DBF1, and Sp1 binding sites, which are located downstream of the human immunodeficiency virus type 1 (HIV-1) promoter, in regulating basal transcriptional activity directed by the integrated viral long terminal repeat (LTR). Point mutations affecting all four of these elements functionally inactivated the HIV-1 LTR when it was constrained in a chromatin configuration. Analyses of the chromatin structures of the transcriptionally active wild-type and inactive mutated HIV-1 promoters revealed several differences. In the active promoter, the 3' half of the U3 region, including the basal promoter, the enhancer, and the putative upstream regulatory sequences are situated within a nuclease-hypersensitive region. However, the far upstream U3 region appears to be packaged into a nuclease-resistant nucleosomal structure, whereas the R, U5, and *gag* leader sequences are associated with a region of altered chromatin that is sensitive to restriction endonucleases. In the inactive template, only the basal promoter and enhancer element remain sensitive to nucleases, and the adjacent upstream and downstream regions are incorporated into nuclease-resistant nucleosomal structures. Taken together, these results indicate that the chromatin structure of the integrated HIV-1 LTR plays a critical role in modulating basal transcriptional activity.

In eukaryotic cells, DNA is organized in nucleosomal arrays and is compacted into high-order chromatin structures. The packaging of DNA molecules into condensed chromatin fibers creates impediments to DNA replication and gene expression. *In vitro* and *in vivo* studies have shown that the incorporation of promoter regulatory elements into nucleosomal structures interferes with transcription initiation (for reviews, see references 12, 28, 31, 48, and 49). The intimate association of histones with DNA selectively controls the access of *cis*-acting sequences to transcriptional regulatory factors. Following gene activation, changes in chromatin structure occur that alleviate nucleosome-mediated repression and facilitate the assembly of an active transcriptional complex (12, 28, 31, 48, 49). For example, the induction of the yeast *PHO5* gene is associated with the disruption of four nucleosomes positioned over the promoter region (2, 11). Similarly, it has been proposed that repression of the mouse mammary tumor virus (MMTV) promoter by nucleosomes covering the transcriptional regulatory elements is reversed following exposure to hormones, which triggers a chromatin transition that allows binding of the NF-1 activator and assembly of the transcription initiation complex (3, 4, 39).

Because a DNA copy of the human immunodeficiency virus type 1 (HIV-1) genome becomes integrated into the chromosomal DNA of a newly infected cell, it is physiologically relevant to study the regulation of viral gene activity in the context of chromatin. The transcriptional control unit of HIV-1, which is located within the U3 region of the 5' long terminal repeat (LTR), contains a typical RNA polymerase II promoter and enhancer elements that modulate transcriptional activity as measured in transient expression assays and during a spreading

virus infection (for reviews, see references 14 and 25). The promoter includes three Sp1 binding sites, located just upstream of a canonical TATA box sequence, and an initiator-like element (14, 23, 25, 52). Adjacent to the Sp1 binding sites, tandem recognition sequences for the NF- κ B/Rel family of transcription factors constitute an activation-dependent enhancer element (36). Binding sites for USF, Ets and LEF-1 (14, 25, 42) are located further upstream and are potentially important regulatory elements for HIV-1 replication in peripheral blood lymphocytes (27).

The chromatin organization of the HIV-1 promoter in two chronically infected human cell lines, ACH2 (T cells) and U1 (promonocytic cells), has previously been analyzed by DNase I digestion and has been shown to contain four distinct DNase I-hypersensitive sites (HS) located within the 5' LTR and adjacent *gag* leader sequence (GLS) (46). The first site, HS1, which was detected only as a minor HS in the U1 cells, has been mapped to the 5' end of the integrated LTR. The HS2 and HS3 sites are situated immediately upstream of the NF- κ B binding sites and within the Sp1/TATA box sequences, respectively. The fourth site, HS4, is associated with the 3' terminus of U5 region and the GLS and contains a cluster of potential binding sites for AP-1, AP-3-like, DBF1 (downstream binding factor 1), and Sp1 proteins. HS4 was previously mapped by *in vivo* and *in vitro* footprinting procedures (10).

In the present study, we have investigated the potential role of sequences within the HS4 region (referred to as the HS4 element) in regulating basal transcription directed by the HIV-1 promoter and have analyzed chromatin structure when the promoter is transcriptionally active or inactive. To ascertain whether the HS4 element plays a role in this process, we stably transfected HeLa cells with constructs containing the HIV-1 LTR linked to a CAT reporter gene in the context of wild-type or mutagenized HS4 sequences. Point mutations affecting the HS4 element functionally inactivated the basal activity of the viral promoter when the DNA template was

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constrained in a chromatin configuration. The chromatin structures of the integrated LTRs were evaluated by micrococcal nuclease (MNase) and restriction enzyme digestion assays. The results obtained indicated that *cis*-acting sequences within the enhancer and promoter regions (i.e., encompassing the NF- κ B and Sp1 binding sites, the TATA box, and the initiator-like element) are located within constitutively open chromatin in both the active (wild-type) and the inactive (HS4 mutations) promoters. These data imply that regulatory sequences (e.g., the HS4 element) in addition to promoter sequences are required in the hierarchical control of transcriptional activity directed by the chromatin-organized HIV-1 promoter.

MATERIALS AND METHODS

Plasmid constructs. The pLCH plasmid construct (see Fig. 1A) contains the bacterial hygromycin resistance gene, driven by the herpes simplex virus thymidine kinase promoter (Tk-hygromycin) inserted into the unique *Bam*HI site of the pCAT basic plasmid (Promega Corp., Madison, Wis.), and the HIV-1 LTR and GLS (LTR/GLS) inserted upstream of the CAT gene, between the *Sph*I and *Xba*I restriction sites. The LTR/GLS DNA fragments were generated by PCR with HIV-1 pNL4-3 (1) or LAI clones (38). Nucleotide substitutions were introduced in the binding sites for the AP-1, AP-3-like, DBF1, and Sp1 factors as indicated in Fig. 1B. The mutant Del construct contains a 57-bp deletion between the *Hind*III site at position 534 (*Hind*₅₃₄) and *Bam*HI₅₉₅ (see Fig. 1B). All plasmids used in transfection experiments were prepared by alkaline lysis and purification through a Qiagen column as recommended by the manufacturer (Qiagen, Inc., Chatsworth, Calif.).

Cell culture, transfections, and CAT assays. Jurkat and Raji cells were maintained in RPMI 1640 medium, and HeLa cells were grown in Dulbecco's modified Eagle's medium. Media were supplemented with 10% fetal calf serum, 100 U of penicillin-streptomycin per ml, and 2 mM L-glutamine. Transfections of Jurkat and HeLa cells were performed with Lipofectamine (10). Raji cells were transfected by the DEAE dextran method (10). In all instances, cells were harvested 44 to 48 h posttransfection and chloramphenicol acetyltransferase (CAT) assays were performed as described previously (10). CAT conversion was assayed by thin-layer chromatography, and the acetylated and nonacetylated forms of [¹⁴C]chloramphenicol were quantitated with a Molecular Dynamics PhosphorImager. CAT activities are reported relative to the CAT activity of the wild-type construct (set at an arbitrary value of 100).

Selection of hygromycin-resistant HeLa clones. At 72 h following transfection of HeLa cells with pLCH plasmid constructs, cells were trypsinized and cultured in Dulbecco's modified Eagle's medium containing 150 to 200 μ g of hygromycin B (Calbiochem, La Jolla, Calif.) per ml. The hygromycin-containing medium was changed periodically until drug-resistant cells emerged, which usually occurred within 3 weeks. Selected individual hygromycin-resistant HeLa cell clones were propagated for 2 additional weeks, at which point DNA was prepared and analyzed by Southern blotting and hybridization. The following HeLa cell clones, containing one or two copies of the pLCH constructs, were used for CAT assays and chromatin analyses: Wt3 and Wt5 (containing the wild-type LTR/GLS); HS4mt1, HS4mt2, and HS4mt5 (containing point mutations within the HS4 element); and Del-1, Del-3, and Del-6 (containing the LTR/GLS with a 57-bp deletion between the *Hind*III and *Bam*HI sites). Pools containing more than 200 hygromycin-resistant HeLa cell clones were also propagated in selective media and were tested for CAT expression levels.

Isolation of RNA and RNase protection assay. Total cellular RNA was isolated from subconfluent cells with RNAsol B (Tel-Test Inc., Friendswood, Tex.) and was digested with DNase I prior to use in RNase protection assays. When the CAT probe was used in RNase protection assays, 50 μ g of RNA was incubated overnight at 45°C in a reaction mixture containing 20 μ l of hybridization buffer (100 mM sodium citrate, 300 mM sodium acetate [pH 6.4], 1 mM EDTA, 80% formamide) and 10⁶ cpm of a CAT RNA probe. The reaction mixtures were subsequently digested with RNase A (2.5 U/ml) and RNase T₁ (100 U/ml) for 30 min at 37°C. For RNase protection assays with a Beta-actin probe, 10 μ g of RNA was used in each reaction mixture and samples were treated as described above.

MNase and restriction enzyme digestions of nuclei. Subconfluent HeLa cell clones were scraped, washed in phosphate-buffered saline, resuspended in TSS buffer (25 mM Tris [pH 7.5], 25 mM KCl, 10 mM NaCl, 5 mM MgCl₂, 300 mM sucrose, 0.1 mM 4-2-aminoethyl-benzene-sulfonylethylfluoride hydrochloride [AEBSEF]), and incubated on ice for 10 min. To disrupt the cell membranes, an equal volume of TSS buffer, containing 0.4% Nonidet P-40, was added, and the mixture was incubated on ice for another 10 min. Nuclei were harvested by centrifugation, washed once with TSS buffer, and then resuspended (approximately 2 \times 10⁷ nuclei per ml) in the appropriate buffer for subsequent digestion with MNase or restriction enzymes.

Nuclei (~5 \times 10⁶) in 250 μ l of MNase digestion buffer (40 mM Tris [pH 8], 25 mM NaCl, 5 mM MgCl₂, 2 mM CaCl₂, 0.5 mM dithiothreitol) were incubated with various concentrations of MNase (10 to 100 U/ml) for 10 min at room temperature. For digestions of purified genomic DNA samples, DNA was sus-

pending in MNase buffer, and the suspension was incubated with MNase (5 to 40 U/ml) at room temperature for 5 min. MNase digestions were stopped by the addition of an equal volume of stop buffer (100 mM Tris [pH 7.5], 200 mM NaCl, 5 mM EDTA, 1% sodium dodecyl sulfate [SDS]) plus 200 μ g of proteinase K per ml, and samples were then incubated overnight at 55°C. DNA was purified by three phenol-chloroform extractions and ethanol precipitation.

Restriction enzyme digestions of nuclei (~5 \times 10⁶) were performed at 37°C for 30 min in 250 μ l of total sample volume with 200 U of enzyme per ml. A buffer containing 10 mM Tris (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol was used for digestions with *Sph*I, *Eco*RV, *Eae*I, *Nhe*I, *Sst*I, *Afl*III, *Hind*III, and *Xba*I. Another buffer (20 mM Tris acetate [pH 7.9], 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol) was used for digestions with *Eco*O109I, *Ava*I, *Alw*NI, *Ban*II, and *Ehe*I. The reactions were stopped and the samples were treated as described for MNase digestion. To ascertain that the activity of restriction enzymes was not impaired under the experimental conditions employed, simultaneous digestions of pLCH plasmid DNA were carried out in the presence of isolated HeLa nuclei as described above. All of the enzymes used efficiently cleaved the plasmid DNA-HeLa nuclei mixtures (data not shown).

Indirect end labeling. Purified DNA was digested to completion with the appropriate restriction enzymes, electrophoresed through 1.2 or 1.5% agarose gels in 1 \times Tris-borate-EDTA (TBE), and transferred in 0.4 M NaOH to Nylon Plus membranes (Qiagen, Inc.). The membranes were hybridized with [³²P] dCTP-labeled DNA probes for 2.5 h at 65°C in quick-hybridization buffer (Stratagene, La Jolla, Calif.), washed five times with 1 \times SSC-SDS buffer (150 mM NaCl, 15 mM sodium citrate [pH 7], 0.1% SDS) for 2.5 h at 65°C, and autoradiographed. The P2 and P3 probes used for Southern blot hybridizations were DNA fragments generated by PCR (40) with the pCAT basic plasmid as a template and the following primers: 5'-CCACCGTTGATATATCCCAATGGC and 5'-GCCCATGGTGAAAACGGGGGCG for probe P2 and 5'-GAATGG GAGCAGTGGTGAATGCC-3' and 5'-CAGGTTTCAGGGGGAGGTGTGG G-3' for probe P3.

The PCR products were purified and labeled in a second PCR (30 cycles of 94°C for 30 s, 65°C for 1.25 min, and 72°C for 2 min) with the same primers and 50 μ Ci of [³²P]dCTP in 20 μ l of total reaction volume.

Activation of the HIV-1 promoter in the Del-6 clone. In CAT assays, cells were treated with either 25 nM phorbol myristate acetate (PMA) plus 1 μ M ionomycin or 50 ng of tumor necrosis factor alpha per ml for 24 h. In analyses of chromatin structure, cells were treated with 50 nM PMA plus 1 μ M ionomycin for 4 h.

RESULTS

Functional analysis of the HS4 element in the context of chromatin. The pLCH plasmid construct used in these studies (Fig. 1A) contains the HIV-1 LTR/GLS linked to a CAT reporter gene. The bacterial hygromycin resistance gene driven by the herpes simplex virus thymidine kinase promoter (Tk-hygromycin) was inserted downstream of the CAT gene to facilitate selection of stable transfectants. The HIV-1 promoter and transcription regulatory elements that reside in the U3 region of the LTR are represented in Fig. 1B. The location of the transcription initiation site, defined by the junction of the U3 and R regions, is indicated by the arrow. Downstream of the transcription start site, a cluster of potential binding sites for AP-1, AP-3-like, DBF1, and Sp1 proteins (together constituting the HS4 element) is also indicated (Fig. 1B and C).

To ascertain whether the HS4 element plays a role in the activity of the HIV-1 promoter, point mutations that affect all of its putative recognition sites were introduced (HS4mt in Fig. 1B). The CAT activities of the wild-type and mutated constructs were initially assayed by transient transfection assays in three human cell lines (viz., HeLa, Jurkat, and Raji). Cells were harvested 48 h posttransfection, and the lysates were tested for CAT activity. Comparative analyses of CAT expression levels (Fig. 1C) revealed that point mutations affecting all of the potential binding sites within the HS4 element (HS4mt) caused an approximately fivefold reduction of CAT activity (Fig. 1C; compare Wt and HS4mt). In contrast, point mutations of individual sites (AP-1/AP-3, DBF1, or the downstream Sp1) did not significantly affect promoter activity (data not shown), suggesting that the HS4 element modulates HIV-1 promoter activity as a cluster of binding sites for several transcriptional regulatory factors.

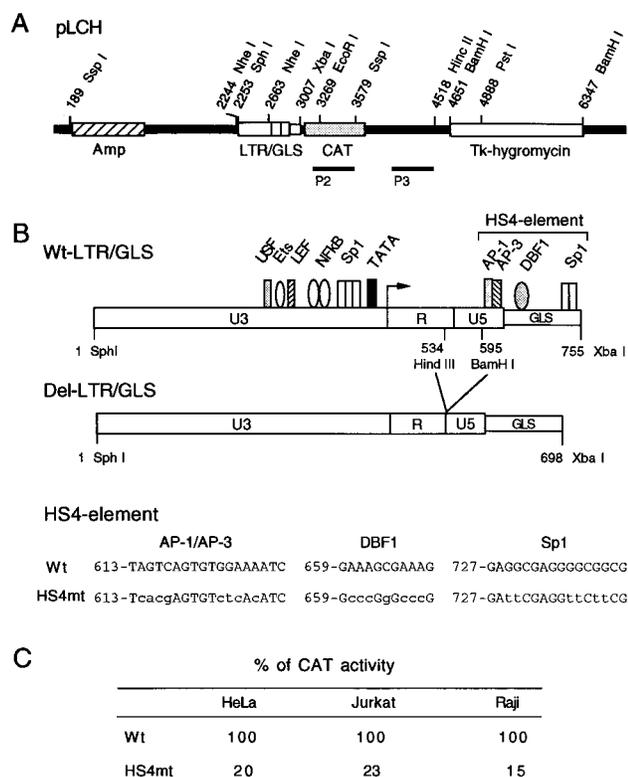


FIG. 1. Schematic representation of the pLCH plasmid construct and the HIV-1 LTR promoter. (A) Partial restriction map of the pLCH plasmid construct, containing the HIV-1 LTR and GLS linked to the CAT gene (CAT), the Tk-hygromycin gene, and the ampicillin resistance gene (Amp). (B) Open boxes, the LTR (U3, R, and U5) and GLS. The known regulatory elements, located upstream of the transcription start site (the arrow at the U3/R junction), include TATA (the TATA box), Sp1 (the three Sp1 binding sites), and NF- κ B (the two NF- κ B sites); LEF, Ets, and USF are potential binding sites for the LEF, Ets, and USF transcription factors. The LTR/GLS_{Del} construct contains a deletion of 57 bp immediately upstream of the HS4 element, from the HindIII₅₃₄ site to the BamHI₅₉₆ site. The HS4 element contains binding sites for AP-1, AP-3-like, DBF1, and Sp1 proteins. Nucleotide substitutions within the mutated HS4 element are shown in lowercase letters. Wt, wild type. (C) HeLa, Jurkat, and Raji cells were transiently transfected with pLCH plasmid constructs containing the wild-type (Wt) or mutated (HS4mt) HS4 element. CAT activities were measured in cell extracts and are presented as percentages of wild-type activity, with an arbitrary set at value of 100.

Since the putative regulatory elements under investigation were originally identified as an HS region (designated HS4) in chromatin, it seemed likely that their full activity might only be evident following integration of the HIV-1 DNA template into the host cell genome. Although we are fully aware that CD4-negative cervical carcinoma cells are not the usual target for HIV-1 in vivo, HeLa cells were used to assess the effects of chromatin structure on HIV-1 LTR-directed gene expression for several reasons. HeLa cells expressing the surface CD4 molecule are fully susceptible to HIV-1, which readily establishes a spreading infection and generates high titers of infectious cell-free progeny virions (6, 7, 29, 32). In addition, many of the transcription factors known to regulate HIV-1 promoter activity in human lymphocytes (14, 25), including NF- κ B, Sp1, initiator-like factor NF-AT, USF, LBP, UBP, AP-1, AP-3-like, and DBF1, are present in HeLa cells (5, 15, 16, 19, 20, 23–26, 30, 34, 41, 51).

The effect of the wild-type or the mutated HS4 element on expression of a stably integrated HIV-1 LTR-CAT construct was initially tested in pools of 200 hygromycin-resistant HeLa

cell clones. As shown in Fig. 2A (stable clones), the CAT activity of pooled HS4mt clones is at least 100-fold lower than that of the pool of wild-type clones. Thus, the ability of the HS4 element to influence transcription correlates with the integrity of its *cis*-acting sequences in both transient and stable transfection assays (Fig. 2A; compare Wt with HS4mt). To extend this observation, CAT expression levels of individual HeLa cell clones containing one or two copies of integrated pLCH DNA (determined by Southern blotting; data not shown) were also examined. Titrations of the CAT activity in whole-cell extracts (from 10 to 100 μ g of protein) from clones HS4mt1 and HS4mt2 indicated that each synthesized at least 100-fold less CAT activity than the wild-type clone Wt5 (Fig. 2B).

To confirm that these CAT assay results did in fact reflect differences in LTR-directed transcriptional activity, total RNA was isolated from individual cell clones and analyzed by RNase protection with a CAT RNA probe (Fig. 2C, left panel). The levels of CAT mRNA expression correlated well with the CAT enzymatic activities presented in Fig. 2B. Again, clones HS4mt1 and HS4mt2, which exhibited virtually no detectable CAT activity, also expressed negligible amounts of CAT mRNA. The difference in intensities of bands protected by Wt3 and Wt5 RNA preparations is about twofold, which is in good agreement with their respective CAT activities. RNA from nontransfected HeLa cells was not protected by the CAT probe, confirming the specificity of the protected fragment in the stable transfectants. Furthermore, as an RNA control, a probe for cellular beta-actin transcripts protected bands of similar intensity for all samples tested (right panel of Fig. 2C). These results indicate that *cis*-acting sequences within the HS4 element modulate the basal transcriptional activity of the integrated HIV-1 promoter, presumably in the context of chromatin.

Mapping the chromatin structure of the wild-type and HS4mt LTR/GLS. The chromatin structure of the HIV-1 LTR/

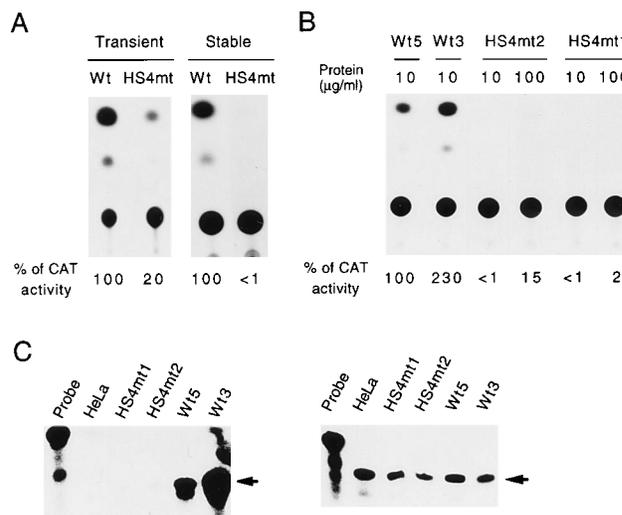


FIG. 2. Contribution of the HS4 element to the basal transcriptional activity of the HIV-1 promoter in the context of chromatin. HeLa cells were transfected with pLCH plasmid constructs containing the wild-type (Wt) or mutated (HS4mt) HS4 element. (A) CAT activities were measured in extracts of transiently transfected cells (Transient) or pools of stably transfected cells (Stable) and are presented as percentages of wild-type activity, with an arbitrary set at value of 100. (B) Titration of CAT activities in the independent HeLa cell clones Wt3 and Wt5 (both containing the wild-type LTR/GLS) and HS4mt1 and HS4mt2 (both containing the mutated HS4 element). (C) Ribonuclease protection assay showing CAT (left panel) and beta-actin (right panel) mRNA transcripts in total RNA samples from clones Wt3, Wt5, HS4mt1, and HS4mt2 and nontransfected HeLa cells (HeLa).

GLS was examined by a combination of MNase digestion and indirect end labeling (37, 50). In chromatin, MNase cuts preferentially in nucleosome-free DNA regions including the linker DNA between nucleosomes; in contrast, nucleosomal DNA is relatively resistant to digestion (28, 31). Regions that are protected in chromatin and which span about 145 to 200 bp are presumed to represent the footprint of positioned nucleosomes. Nonetheless, because of DNA sequence preferences of MNase, digestion of protein-free DNA may also generate sharp bands when cleavage is monitored by Southern blotting (8, 21). Examples of MNase digestions of chromatin and protein-free DNA are shown in Fig. 3. Isolated nuclei from the Wt3 and Wt5 clones were incubated with increasing concentrations of MNase (10 to 80 U/ml); the DNA was then purified, digested with *Ssp*I, and analyzed by Southern blotting with the P2 probe shown in Fig. 1A. Much of the reactive DNA within the pLCH construct formed a ladder of bands, differing in size by approximately 200 bp (indicated by small arrows in Fig. 3A), in agreement with repeats of DNA fragments protected by nucleosomes. However, the HIV-1 LTR/GLS was organized into a distinct chromatin structure, consisting of two MNase-protected segments (nucleotides [nt] 1 to 210 and 500/600 to 760) that flanked a large MNase-HS domain, indicated by arrowheads at positions 270, 350, 420, and 500 in Fig. 3A. This pattern is very similar to MNase digestion of protein-free DNA (Fig. 3A; compare lanes C to lanes D), except that in chromatin, the two segments of DNA between nt 1 to 210 and 600 to 760 are relatively resistant to MNase cleavage (less smearing) and additional bands between positions 600 and 640, visible in the samples of protein-free DNA, are not detected in the chromatin templates. However, in the U5/GLS region (nt 600 to 760), resistance to MNase was observed primarily at low concentrations of nuclease (Fig. 3A; compare 20- with 80-U/ml lanes); at higher concentrations of MNase (80 U/ml), the bands were less defined and smearing was observed (at position 600 and further downstream; Fig. 3A). In contrast, the nucleosomal structure at the 5' end of the U3 region (nt 1 to 210) was quite stable even when high levels of nuclease were used.

The chromatin structure of the HS4mt LTR/GLS derivative was also analyzed by MNase digestions and indirect end labeling as described above (Fig. 3B). Several differences are evident in the MNase cleavage patterns of the HS4mt and wild-type chromatin templates: (i) there is no evidence of MNase digestion between nt 1 and 290 of the HS4mt templates; (ii) fewer MNase cleavage sites are detectable within the promoter and enhancer regions of the HS4mt template (in the region encompassing nt 290 to 420, three bands are present in HS4mt templates, compared with multiple bands in the wild-type chromatin), (iii) the MNase cleavage sites at positions 500, 600, and 750 that are prominent in the wild-type template are weakly digested or undigested in the HS4mt samples. Thus, except for the promoter region (*Sp*1 sites, TATA box, and the initiator-like element) and enhancer element, most of the HS4mt-LTR/GLS template is resistant to nuclease digestion, in agreement with the incorporation of these sequences into several nucleosomes.

Accessibility of the wild-type and HS4mt LTR/GLS chromatin templates to restriction enzymes. The sensitivity of DNA in nuclei to restriction endonucleases is an alternative approach for mapping nucleosome locations and accurately quantitating the accessibility of open chromatin domains (HS sites) (22, 31). Because intact nuclei exhibit variable sensitivities to restriction enzymes, a digestion was given a score of positive when 20% or more of the chromatin substrate was cleaved. Examples of

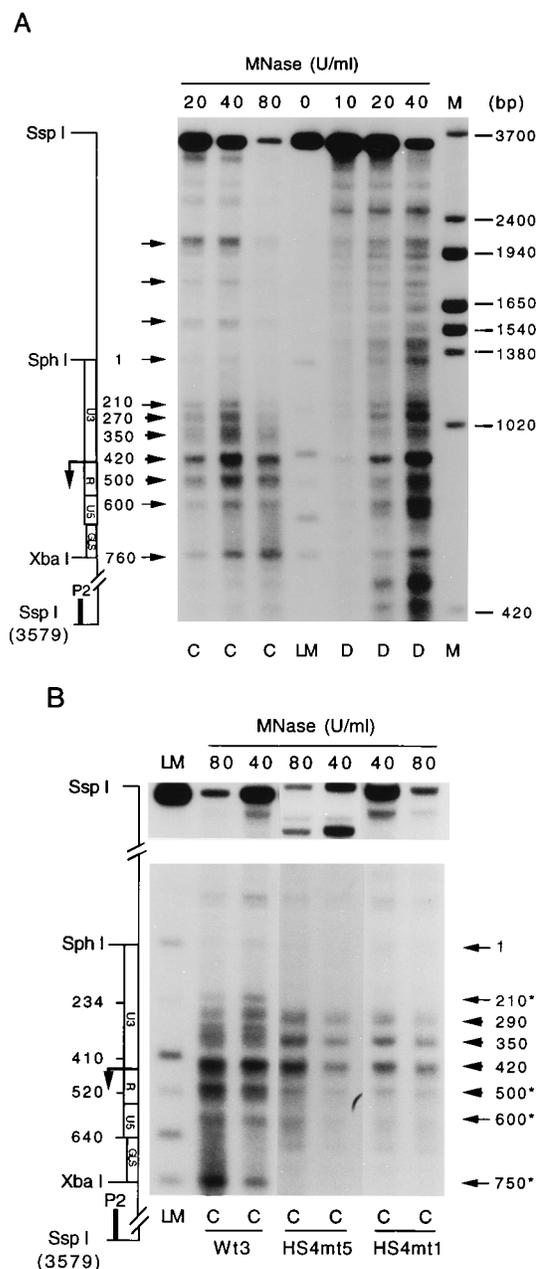


FIG. 3. Mapping of MNase cleavage sites within the HIV-1 LTR/GLS. MNase cleavage sites in chromatin (lanes C) and in protein-free DNA (lanes D) from HeLa cell clone Wt3 (A) or from clones HS4mt1, HS4mt5, and Wt3 (B) were mapped by the indirect end-labeling procedure. The P2 probe, which hybridizes to the CAT gene (solid bar labeled at the lower left of each panel), was used. The samples were treated with increasing concentrations of MNase (0 to 80 U/ml) as indicated at the top of each panel. pLCH DNA restriction digests (M) and LTR restriction markers (LM) were used as size markers. The positions of preferential MNase cleavage sites (horizontal arrows) relative to the beginning of LTR/GLS sequences are indicated. The asterisks at positions 210, 500, 600, and 750 in panel B indicate the MNase cleavage sites that are weakly cleaved or not cleaved in the HS4mt templates.

restriction enzyme digestions of nuclei from individual clones Wt3, HS4mt1, and HS4mt2 are presented in Fig. 4.

Restriction sites (*Eco*RV₁₁₅, *Ear*I₁₇₄, *Eco*O109I₂₃₅, and *Ava*I₂₉₈) that are located in the U3 region upstream of the promoter are relatively resistant to cleavage (less than 15% of

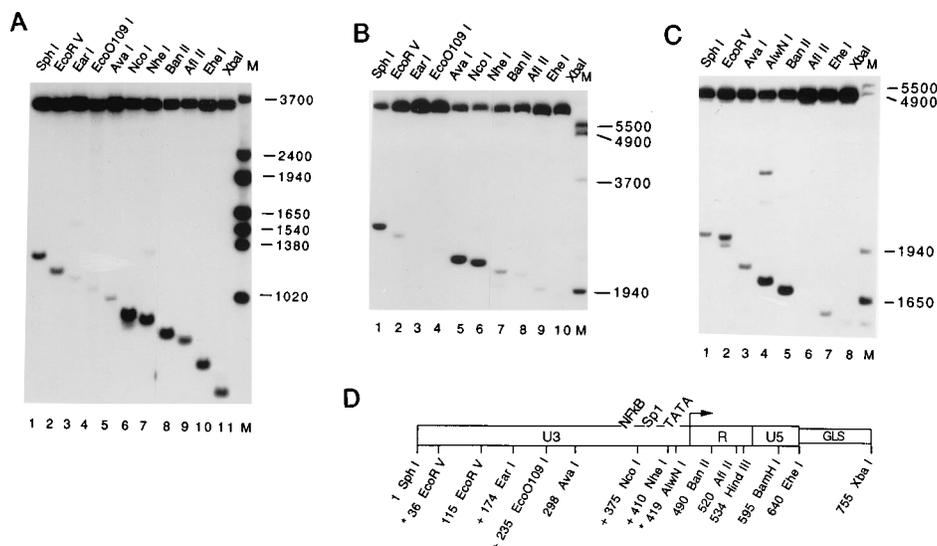


FIG. 4. Restriction enzyme accessibility of the HIV-1 LTR/GLS in chromatin. Nuclei isolated from clones Wt3 (A), HS4mt2 (B), or HS4mt1 (C) were treated with the restriction enzymes indicated at the tops of the lanes. Purified DNA was digested to completion with *Ssp*I (A), *Pst*I (B), or *Hinc*II (C) and analyzed by indirect end labeling. (D) Restriction map of the LTR/GLS. The restriction sites indicated by the asterisks are present only in the LTR derived from the HIV-1_{LAI} clone, and those indicated by plus signs are present in the LTR from the HIV-1_{NL4-3} clone.

chromatin is digested). In contrast, restriction sites that are located in the promoter region (*Nco*I₃₇₅, *Nhe*I₄₁₀, *Alw*NI₄₁₉, and *Ban*II₄₉₀) were sensitive to restriction enzymes (about 30 to 55% of DNA is digested) in both wild-type and HS4mt chromatin templates. Surprisingly, in the wild-type but not in the HS4mt templates, sequences further downstream of the transcription initiation site, including the R/U5/GLS region, are also sensitive (30 to 40% of DNA is cut) to the restriction enzymes tested (*Afl*II₅₂₀, *Ehe*I₆₄₀, and *Xba*I₇₅₅). Susceptibility to several restriction endonucleases very likely reflects an altered nucleosomal structure in this region. In the HS4mt chromatin templates (Fig. 4B and C), the R/U5/GLS region was relatively resistant to restriction endonucleases, suggesting that this DNA segment is incorporated into stable nucleosomal structures. These differences in chromatin patterns may reflect the inability of the mutated HS4 element to bind cellular factors, thereby rendering this region refractory to digestion by restriction enzymes. However, it is also possible that in the context of a wild-type template, the R/U5/GLS region assumes alternative nucleosomal configurations, depending on the functional state of the promoter.

As an internal control for restriction enzyme accessibility experiments, *Nhe*I, which cleaves the pLCH plasmid construct twice, was used (Fig. 1A). The 5' site (Fig. 4A, lane *Nhe*I), which is located upstream of the LTR and in a region resistant to MNase digestion (Fig. 3A, upper band), is not cleaved by *Nhe*I, whereas the 3' site (lane *Nhe*I, lower band), which is situated within the core promoter, is accessible to both MNase and *Nhe*I.

The MNase and restriction enzyme digestions of the wild-type and mutagenized LTR/GLS templates are summarized in Fig. 5. In the wild-type LTR, the core promoter and enhancer sequences (nt 350 to 500) are nucleosome free, and the upstream portion of the U3 region (nt 40 to 270) is packaged into a nuclease-resistant nucleosomal structure (shown as the overlapping nucleosome A [Nuc A] structures in Fig. 5A, indicating that Nuc A may be situated at multiple positions between nt 40 and 270). In agreement with *in vitro* footprinting data (15, 16), cellular factors may occupy the region between nt 282 and 303,

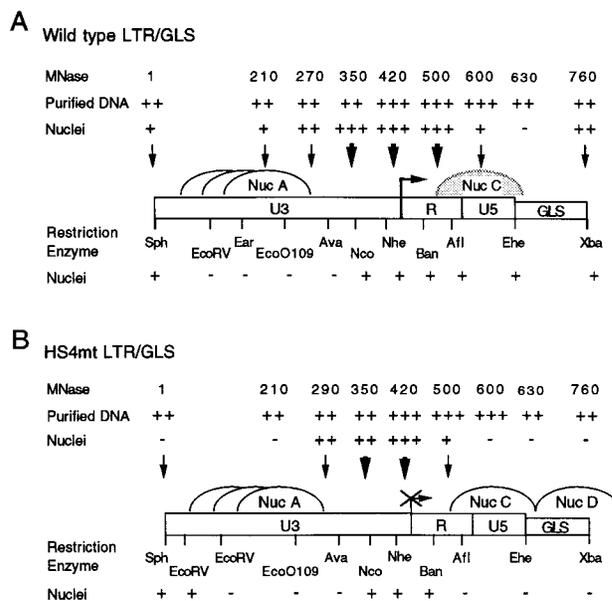


FIG. 5. Proposed nucleosomal structures of the HIV-1 promoter in transcriptionally active and inactive states. Schematic representation of nucleosomal structures of the wild-type active HIV-1 LTR promoter (A) and the inactive promoter HS4mt-LTR/GLS (B). Putative nucleosomes, designated Nuc A, Nuc C, and Nuc D, are positioned relative to MNase and restriction enzyme cleavage sites. The positions of MNase cleavage sites are indicated by arrows. The efficiency of digestion is scored as follows: +++, hypersensitivity sites; ++, readily cleaved sites; +, weakly cleaved sites; and -, protected sites. For restriction enzymes, + and - indicate digestions yielding at least 20% and less than 20%, respectively. Nuc A is shown as three adjacent and overlapping structures, indicating that this nucleosome is not precisely positioned. In the wild-type LTR/GLS, Nuc C is represented by a hatched structure to indicate that this nucleosome is unstable. In the HS4mt-LTR/GLS template, no MNase cleavage sites were detected between nt 500 and 760; this is represented by a pair of juxtapposed nucleosomes, Nuc C and Nuc D.

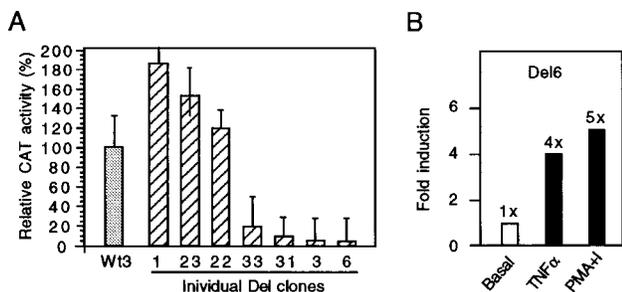


FIG. 6. Basal and induced CAT activities of stably transfected Del clones. (A) CAT activities were measured in cell extracts of individual HeLa cell clones containing the LTR/GLS_{Del} construct Del-1, Del-3, Del-6, Del-22, Del-23, Del-31, and Del-33 and were compared with the CAT activity of the wild-type clone Wt3, with an arbitrary value set at 100. (B) Basal and induced CAT activities of the Del-6 clone. Cells were untreated (Basal) or were treated with PMA plus ionomycin (PMA+I) or with tumor necrosis factor alpha (TNF α) for 24 h. CAT activities in cell extracts were measured and were reported as fold inductions relative to the basal activity, with an arbitrary value set at 1.

thereby creating a boundary for Nuc A. This might explain why the *Ava*I₂₉₈ site is not efficiently cleaved. Sequences downstream of the initiation site (nt 500 to 760) are partially resistant to MNase cleavage and sensitive to restriction endonucleases, suggesting that they reside within an altered chromatin structure (Nuc C in Fig. 5A). This pattern of MNase and restriction enzyme accessibility was observed in two independent wild-type clones (Wt3 [Fig. 3 and 4] and Wt5 [date not shown]).

In the transcriptionally inactive HS4mt template, DNA fragments resistant to nucleases encompass nt 40 to 290 within U3 and the entire region downstream of the transcription start site, between nt 500 and 760 (half of R and the U5/GLS). On the basis of the sizes of these DNA fragments, one nucleosome could be accommodated over the U3 region (Nuc A in Fig. 5B) and two nucleosomes may cover the R/U5/GLS regions (Nuc C and Nuc D in Fig. 5B). However, the positions of nucleosomes over the HS4mt-LTR/GLS cannot presently be precisely assigned because no MNase cleavage sites, in agreement with the existence of nucleosome boundaries, were detected in either region.

The effect of deleting 57 bp between the core promoter and HS4 element on chromatin structure and transcription directed by the integrated LTR/GLS. *cis*-acting DNA elements, which are capable of reacting with cellular regulatory factors, can alter chromatin structure and confer increased sensitivity to nucleases (9, 18). The hypersensitivity of the wild-type LTR/GLS chromatin template to MNase and restriction endonucleases was localized to two regions: the promoter and HS4 element (Fig. 3A and 4A). This result suggests that the interaction of *trans*-acting cellular factors with HIV-1 regulatory sequences precludes nucleosome formation in these two regions, but not in the intervening DNA segment, which can still accommodate a single nucleosome (Nuc C in Fig. 5A). Moreover, following mutagenesis of HS4, the entire downstream region (from positions 500 to 760) becomes highly resistant to nucleases, suggesting that it is incorporated into two nucleosomes (Nuc C and D in Fig. 5B). To ascertain whether the HS4 element might disrupt Nuc C if its position was shifted into the location occupied by Nuc C in the configuration of the wild-type LTR/GLS template, a deletion of 57 bp, immediately 5' to the HS4 element (from the *Hind*III₅₃₄ site to the *Bam*HI₅₉₆ site [Fig. 1B]), was introduced within the R/U5 region. The resulting template was designated LTR/GLS_{Del}.

Several independent and stable HeLa cell clones containing the LTR/GLS_{Del} template which unexpectedly exhibited a wide range of CAT activities were obtained (Fig. 6A). This variability of CAT production presumably reflected the effects of different integration sites on the basal levels of LTR-directed expression. Nonetheless, it is important to note that even clones synthesizing the lowest levels of CAT (e.g., Del-3 and Del-6) expressed at least 10-fold higher activity than any of the HS4mt HeLa cell clones (compare with Fig. 2 and 6A).

The chromatin structure of the LTR/GLS_{Del} region in the clone synthesizing the highest levels of CAT (clone Del-1) was evaluated by restriction endonuclease digestion. As shown in Fig. 7A, the cleavage pattern obtained (i.e., limited digestion of U3 restriction sites between *Sph*I and *Ava*I and substantial digestion of the downstream U3, R, and U5 sites) was nearly identical to that observed with the integrated wild-type LTR/GLS template (Fig. 4A). In contrast, restriction enzyme diges-

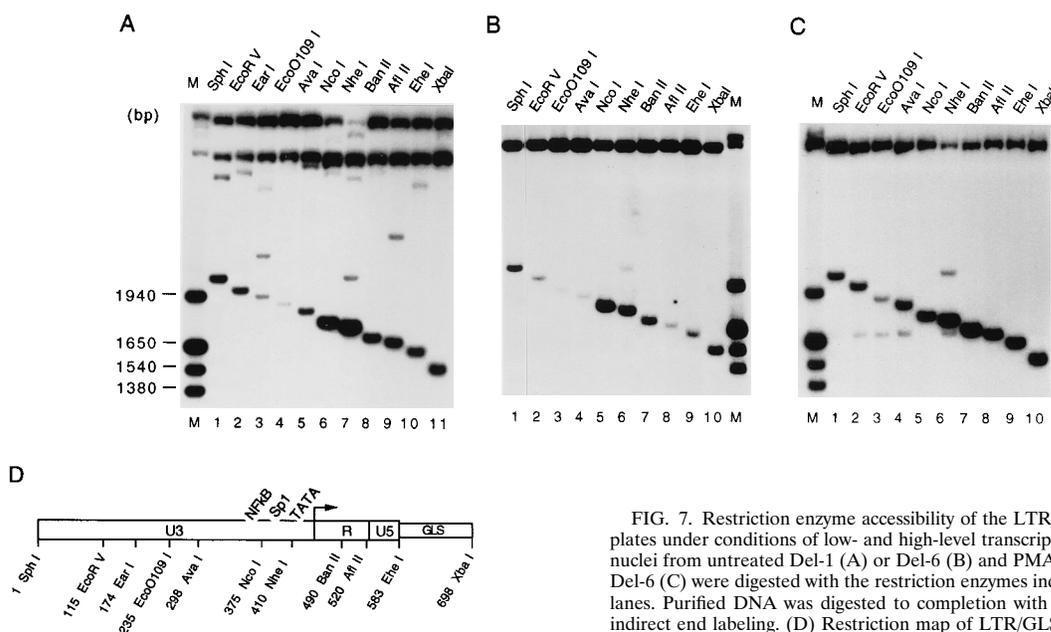


FIG. 7. Restriction enzyme accessibility of the LTR/GLS_{Del} chromatin templates under conditions of low- and high-level transcriptional activities. Isolated nuclei from untreated Del-1 (A) or Del-6 (B) and PMA-plus-ionomycin-treated Del-6 (C) were digested with the restriction enzymes indicated at the tops of the lanes. Purified DNA was digested to completion with *Hinc*II and analyzed by indirect end labeling. (D) Restriction map of LTR/GLS_{Del}.

tion of the chromatin-organized LTR/GLS_{Del} template in clone Del-6, which expressed about 30-fold less CAT than clone Del-1, revealed limited susceptibility of the *AffIII* and *EheI* sites compared with both the wild-type and clone Del-1 templates (Fig. 7B versus Fig. 7A and 4A). Taken together, these results suggest that a major determinant of LTR/GLS nucleosome organization downstream of the transcription start site is the level of basal RNA synthesis and not the repositioned HS4 element. When the viral promoter is active (Wt3 and Del-1), the R/U5 region is accessible to restriction endonucleases. In contrast, when the basal level of expression is low or markedly repressed (the Del-6 and the HS4mt clones, respectively), the R/U5 region becomes resistant to enzymatic cleavage, presumably because of the presence of a stably positioned nucleosome. Repositioning of the HS4 element per se did not alter the nucleosomal structure of the R/U5 region.

The low basal level of CAT produced by clone Del-6 provided an opportunity to monitor changes in chromatin structure following exposure to known activators of the HIV-1 LTR. As shown in Fig. 6B, CAT expression in clone Del-6 increased four- to fivefold following incubations with either tumor necrosis factor alpha or PMA plus ionomycin. Alterations in the organization and/or stability of nucleosomes associated with the integrated LTR/GLS_{Del} template following a 4-h exposure to PMA plus ionomycin was assessed with restriction endonucleases as described above. The results of this analysis (Fig. 7C) revealed greater nuclease sensitivity of the R/U5 region encompassing the *AffIII* and *EheI* sites, which was partially occluded in unstimulated clone Del-6 cells (compare Fig. 7B and C). Digestions with the other enzymes were either unchanged (*SphI* and *EcoO109I*) or slightly increased (*EcoRV*, *AvaI*, *NheI*, and *BanII*). The cleavage pattern of the PMA-induced clone Del-6 closely resembles those of the Wt3 and clone Del-1 digestions (compare Fig. 4A and 7A with 7C), suggesting that the alterations in chromatin organization within R/U5 reflect changes in basal transcriptional activity.

DISCUSSION

Contribution of the HS4 element to the transcriptional activity of the HIV-1 promoter. *cis*-acting regulatory elements are often clustered in nucleosome-free domains of eukaryotic genomes and can be identified as HS sites in chromatin (for reviews, see references 9 and 18). Recently, a cluster of binding sites for at least four distinct transcription factors (AP-1, AP-3-like, DBF1, and Sp1) have been mapped downstream of the HIV-1 transcriptional start site within the U5 and GLS in a region previously designated HS4 (10). In the present report, we have shown that point mutations in the HS4 element nearly completely inhibited HIV-1 LTR-directed expression when the template was stably integrated into human cellular DNA. These results suggest that *cis*-acting sequences within the HS4 element are required for maximal basal promoter activity in the context of chromatin.

At least two mechanisms can be entertained to explain how the HS4 element modulates HIV-1 promoter activity. First, protein-protein interactions between factors bound to the core promoter and the HS4 element could facilitate the assembly and stability of an active transcription initiation complex (a direct effect). Interactions between proteins bound at nonadjacent sites, which are located either 3' or 5' of their respective target promoters (the DNA-looping model), have been proposed to explain how enhancer elements direct the assembly of an active transcription complex (9, 12, 13, 17, 45). For example, the interaction between distal and proximal DNA-bound Sp1 proteins bridges intervening DNA sequences and augments

transcription (33, 43). In this regard, it is worth noting that binding sites for Sp1 are located both upstream and downstream (within the HS4 element) of the HIV-1 promoter. Such an Sp1-Sp1 interaction might enhance the formation of a stable preinitiation complex, thereby contributing to HIV-1 basal promoter activity. However, mutation of the downstream Sp1 sites had no effect on the transcriptional activity of the HIV-1 promoter in transient transfection assays. The consequences of these mutations on the integrated HIV-1 promoter are currently under investigation.

A second mechanism to explain the modulating effect of the HS4 element on the HIV-1 promoter involves the disruption of chromatin due to the binding of factors to the HS4 sequences, thereby blocking the assembly of nucleosomes in this region of the integrated proviral DNA (indirect effect). Since previous *in vivo* dimethyl sulfate footprinting has indicated that DBF1 and Sp1 binding sites within the HS4 element are occupied (10), it is not unreasonable to propose that binding of cellular factors to the HS4 element could cause local disruption of chromatin structure. This possibility is supported by analyses of the chromatin organization of the HS4mt template, which indicated that nucleosomes are covering this mutated HS4 element in three independent clones (Fig. 3 and 4). In contrast, MNase digestion of templates containing the wild-type HS4 element (the wild type and Del templates) generated a clearly defined band at the 3' border of the HS4 element, and the restriction enzyme *XbaI* readily cleaved DNA within the HS4 element in the Del and wild-type templates (but not the HS4mt template). These results suggest that *cis*-acting sequences in the HS4 element are involved in establishment of an open chromatin configuration within the GLS.

The chromatin structure of the HIV-1 LTR promoter determines its functional state. A number of *in vivo* and *in vitro* experiments provide strong evidence that nucleosomes are actively involved in gene regulation (for reviews, see references 12, 28, 31, 48, and 49). For example, the inactive MMTV LTR promoter is incorporated into six stably positioned nucleosomes (39). After hormone induction, a nucleosome covering the essential promoter sequences is remodeled. This change in MMTV LTR chromatin structure is believed to occur independently of the transcription activation process (3, 4, 35, 39). Transcriptional activation of the yeast PHO5 gene is also accompanied by disruption of four nucleosomes that normally cover the promoter and regulatory sequences (2, 11). However, in this system the chromatin disruption is induced by binding of the PHO4 transcription factor, which is also an activator of transcription (11). In this latter case, chromatin changes and activation of transcription are intimately linked (44).

The integrated HIV-1 promoter acquires a distinct chromatin structure which determines its functional state. First, the far upstream portion of U3 (5' of nt 210 [Fig. 3A]) is very resistant to MNase and restriction endonuclease digestions and is presumed to be packaged into a nucleosome, Nuc A (Fig. 5A). Second, the regulatory sequences controlling promoter activity (i.e., NF- κ B and Sp1 binding sites, the TATA box, and initiator-like elements) are located in a nucleosome-free region encompassing nt 350 to 500 and are readily susceptible to MNase and restriction endonucleases. Third, the R/U5/GLS sequences (spanning approximately 300 bp downstream of the transcription initiation site) are partially sensitive to MNase digestion and readily accessible to several restriction endonucleases. This last pattern of nuclease susceptibility is almost identical to that observed after MNase digestion of protein-free DNA, a result suggesting that the R/U5/GLS sequences are not associated with a canonical nucleosomal structure (Fig. 5A; Nuc C region). These results are consistent with the chro-

matin-mapping studies of the HIV-1 LTR in two chronically infected human cell lines, ACH2 (T cells) and U1 (promonocytes) (47). The proviral LTR/GLS in the ACH2 and U1 cell lines and the LTR/GLS-CAT in HeLa cells have very similar chromatin structures, except that the potential nucleosome associated with the R/U5 region (Nuc C), which we now map about 40 nt downstream of the transcription initiation site (MNase cleavage site at position 500 [Fig. 3]), had previously been positioned immediately downstream of the transcription initiation site (47). There are multiple explanations for this mapping difference, including the possibility that the positions of nucleosomes may vary depending on the functional state of the promoter. However, it is more likely that these differences reflect intrinsic technical problems associated with interpreting subtle changes in nuclease cleavage patterns. Cross-linking of histones to cellular DNA prior to the preparation of nuclei and nuclease digestion, in conjunction with high-resolution mapping of chromatin, might prove useful for determining the precise positions of nucleosomes within the HIV-1 LTR/GLS (for example, see reference 13).

The results obtained with the wild-type HIV-1 templates are to be contrasted with the changes observed in chromatin structure of the transcriptionally inactive LTR promoters in HS4mt clones. First, the MNase susceptibility of the inactive promoter appears to be confined to the enhancer and basal promoter sequences. Thus, regardless of promoter activity, sequences located between nt 350 and 500 are invariably accessible to MNase and restriction endonucleases. The open chromatin configuration at the core promoter, even in the transcriptionally inactive templates, could reflect the binding of proteins that disrupt local chromatin structure but, by themselves, are unable to initiate RNA synthesis. The enhanced susceptibility of the core promoter and immediately adjacent upstream sequences (Fig. 3B) to MNase in the active wild-type LTR might reflect its association with a transcriptionally competent RNA polymerase II complex. A second difference is that the R/U5/GLS region in the inactive template is packaged into a stable chromatin structure (Fig. 5B; Nuc C and Nuc D), which is protected from MNase digestion and relatively resistant to restriction endonucleases. In contrast, the R/U5/GLS regions in active wild-type templates are accessible to restriction endonucleases and relatively susceptible to MNase, which is evidence of either relaxed or absent DNA-histone interactions. The susceptibility of sequences located downstream of the active promoter to nucleases could reflect dynamic alterations associated with chromatin in the process of being transcribed. Thus, like the MMTV and *PHO5* promoters, the transcriptional activity of the HIV-1 promoter seems to be accompanied by changes in chromatin structure. It is not clear from these experiments whether chromatin remodeling precedes and facilitates transcription or is simply a consequence of the transcriptional process. We have also shown that the presence of the intact HS4 element in the Del-6 clone is unable to disrupt or maintain an altered chromatin structure within the R/U5 region. However, when the promoter in the Del-6 clone was activated, the R/U5 region became sensitive to endonuclease, suggesting that remodeling of the HIV-1 chromatin structure is likely to be secondary to the transcriptional process.

In conclusion, we have shown that the stable introduction of the HIV-1 LTR/GLS regions into HeLa cells results in the packaging of the DNA sequences into a distinct nucleoprotein structure that determines the functional state of the viral promoter. The active promoter is associated with two potential nucleosomal regions separated by a large open chromatin configuration (Fig. 5). The putative nucleosome located within the 3' half of the U3 is relatively stable. In contrast, the region

downstream of the transcription start site is highly accessible to restriction endonucleases and is relatively susceptible to MNase, suggesting that its chromatin structure is altered. In the inactive promoter, hypersensitivity is limited to the enhancer and basal promoter sequences and the R/U5/GLS region is incorporated into stably positioned nucleosomes. This system provides a physiologically relevant model for studying HIV-1 transcription regulation in the context of chromatin.

ACKNOWLEDGMENTS

We thank Graziella Piras for assistance in RNase protection assays and Trevor K. Archer and Graziella Piras for critical reading of the manuscript.

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