VIROLOGY 234, 42–50 (1997)
ARTICLE NO. VY978623

Transduction of the Human Immunodeficiency Virus Type 1 Promoter into Human

Piruz Nahreini[1] and Michael B. Mathews[2]

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

Received February 7, 1997; returned to author for revision March 11, 1997; accepted May 5, 1997

Transcription of the human immunodeficiency virus type 1 (HIV-1) genome takes place after integration of the provirus into human chromosomal DNA. HIV transcription is known to be modulated by viral and cellular factors but the influence of flanking chromosomal sequences on proviral gene expression has not been well defined. To investigate the activity of the integrated HIV promoter, we exploited the ability of recombinant adeno-associated virus (AAV-2) to transfer and stably integrate genes into the human genome at random or site-specifically. Chimeric AAV vectors were constructed containing an HIV-CAT reporter cassette; some vectors also contained the neomycin resistance gene to facilitate the isolation of positive clones. HeLa cells were infected with recombinant AAV, in some instances together with wild-type virus as a source of AAV rep function. We isolated 25 clones of G418-resistant cells which carried the integrated HIV-CAT cassette, generally occupying unique sites that did not correspond to the AAV-specific region of chromosome 19. The HIV promoter was transcriptionally active in most of the clones. Basal promoter activity varied substantially among the clones, and its responsivity to the HIV transactivator Tat was also variable. The integrated HIV promoter was transactivated to comparable degrees by the one-exon form and two-exon form of Tat. These findings provide evidence that the transcriptional activity of the HIV promoter can be greatly influenced by the site of proviral insertion.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) predominantly infects $CD4^+$ immune cells such as T-lymphocytes and macrophages (Staprans, 1994). The viral RNA genome is converted into a DNA intermediate that subsequently integrates into chromosomal DNA. Integration is essential for stable maintenance, gene expression, and pathogenicity of the virus (Bednarik and Folks, 1992). Continuous sporadic viral gene expression occurs in a small fraction of patients' immune cells harboring the chromosomally integrated proviral DNA (Embretson *et al.,* 1993; Pantaleo *et al.,* 1993). Such chronic viral persistence appears to dampen immune surveillance by gradual attrition of T-lymphocytes and ultimately causes immune cell depletion culminating in the onset of AIDS (Embretson *et al.,* 1993; Pantaleo *et al.,* 1993). Furthermore, the degree of HIV pathogenicity is apparently directly proportional to the level of viral gene expression from the chromosomally integrated HIV provirus (Pantaleo *et al.,* 1993).

Expression of HIV genes is influenced by numerous factors. Transcription from the viral promoter located in the integrated long terminal repeat (LTR) is activated by host cell factors such as cytokines and cellular transactivators, leading to synthesis of HIV regulatory proteins, Tat and Rev, which in turn enhance the expression of viral genes required for a productive infection (Bednarik and Folks, 1992; Nabel, 1993; Zagury *et al.,* 1986). The Tat protein interacts with its target RNA binding sequence, the transactivation response element (TAR), which is located in the 5′ untranslated region of all HIV transcripts. TAR minimally extends from residues +14 to +44 relative to the cap site at +1, forming a stem-loop structure that binds Tat as well as cellular factors (Peterlin *et al.,* 1993). These interactions among Tat, TAR, and cellular factors increase transcriptional initiation and elongation by RNA polymerase II complexes from the LTR (Kao *et al.,* 1987; Laspia *et al.,* 1990; Sheridan *et al.,* 1993). Rev, on the other hand, interacts with a separate RNA structure to facilitate the cytoplasmic accumulation of viral mRNAs that encode structural proteins (Malim *et al.,* 1989).

Circumstantial evidence suggests that the activity of the HIV promoter can also be influenced by the site of proviral integration and chromatin organization (Almouzni *et al.,* 1991; Kornberg and Lorch, 1992; Lint *et al.,* 1994; Verdin *et al.,* 1993; Wolffe, 1994). Although retroviral integration into the host cell genome occurs with little apparent target sequence specificity, HIV-1 displays a preference for sites in or near L1 and *Alu* repeats (Stevens

[1] Current address: Ribozyme Pharmaceuticals Inc., Boulder, CO 80301. E-mail: nahreip@rpi.com.

[2] To whom correspondence and reprint requests should be addressed at present address: Department of Biochemistry and Molecular Biology, UMDNJ–New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103-2714. Fax: (973) 972-5594. E-mail: mathews@umdnj.edu.

0042-6822/97 $25.00

and Griffith, 1994). In model systems, chromatin structure seems to affect site selection, and integration reportedly takes place preferentially in regions of chromosomal DNA that are being actively transcribed or replicated (Mooslehner *et al.,* 1990; Shih *et al.,* 1988; Vijaya *et al.,* 1986). Correspondingly, we and others have shown that the transcriptional activity (both basal and Tat transactivated) of the HIV promoter is stimulated by an origin of replication (Jeang *et al.,* 1993; Kessler and Mathews, 1991; Nahreini and Mathews, 1995). Conversely, replication may change the organization of chromatin in a way that enhances HIV promoter activity (Proudfoot *et al.,* 1992). Possibly as a reflection of such changes, the distribution of nucleosomes around the HIV promoter changes when cells are treated with factors that increase promoter activity (Verdin *et al.,* 1993; Lint *et al.,* 1994).

Here we test the hypothesis that the activity of the HIV promoter and its sensitivity to transactivation by Tat are influenced by the proviral integration site. To investigate the activity of HIV promoter in the context of human chromosomes, we used adeno-associated virus-2 (AAV-2) to transduce a reporter gene driven by the HIV LTR into chromosomal DNA. AAV is a small nonpathogenic human parvovirus which is dependent on a helper virus, usually adenovirus, for productive infection (Muzyczka, 1992). In the absence of helper virus, wild-type AAV DNA stably integrates into human chromosomal DNA and establishes a latent infection. The viral DNA preferentially integrates into a defined region of chromosome 19 with a frequency of up to 70% in established human cell lines such as HeLa and Detroit-60 (Kotin *et al.,* 1990, 1992; Samulski *et al.,* 1991; Linden *et al.,* 1996). Specific integration is dependent on the AAV genomic termini and its viral replication (rep) proteins (Samulski, 1993; Weitzman *et al.,* 1994; Kearns *et al.,* 1996). These features have made AAV an attractive viral vector for the transfer of foreign genes into human cells (Srivastava, 1994). We report the generation and properties of a series of cell lines containing the integrated HIV promoter linked to the chloramphenicol acetyl transferase (CAT) gene.

MATERIALS AND METHODS

Cell culture

Monolayer cultures of HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FCS) and 100 μg of penicillin and streptomycin per milliliter. Unless otherwise indicated, transfections were carried out at 60–70% confluency.

Recombinant AAV plasmids

Plasmid pWP19 (Nahreini *et al.,* 1993) contains the neomycin resistance (Neo) gene driven by the herpesvirus thymidine kinase (TK) promoter with the TK polyadenylation signal in its untranslated 3′ region. This TK.Neo cassette is flanked by AAV-termini within the pEMBL vector backbone (Nahreini *et al.,* 1993). Plasmid pWP19 was digested to completion with *Bam*HI and treated with alkaline phosphatase. The HIV-LTR-CAT reporter gene cassette was isolated from pHO (Kessler and Mathews, 1991) by digestion to completion with *Bam*HI and partial digestion with *Hin*dIII, blunt-ended with Klenow enzyme, and ligated to a *Bam*HI linker. This fragment was subcloned into the *Bam*HI site of pWP19 to generate constructs pWP30 and pWP31 (Fig. 1). Similarly, the LTR-CAT fragment was subcloned into the *Bgl*II site of plasmid pWP25 to generate pWP28 and pWP29 (Fig. 1). pWP25 is a derivative of psub201 (Samulski, 1987) which contains the full-length AAV-2 genome flanked by AAV inverted terminal repeats. Plasmid psub201 was digested to completion with *Eco*NI and partially digested with *Xba*I to remove the capsid (cap) coding region. The resulting *Eco*NI–*Xba*I fragment was blunt-ended with Klenow enzyme and ligated to *Bgl*II linkers, generating the plasmid pWP25.

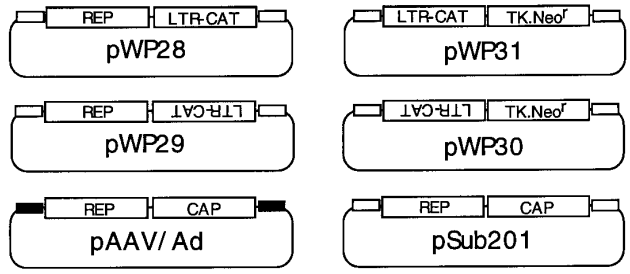

FIG. 1. Schematic drawing of recombinant AAV plasmids. Large rectangles represent the AAV replicase gene (REP), the AAV capsid gene (CAP), the HIV LTR reporter cassette (LTR-CAT), and the neomycin resistance chimera (TK.Neo^r). Small open rectangles represent AAV termini, and small closed rectangles represent adenovirus termini.

Rescue and replication assay

KB or 293 monolayer cultures were co-transfected with a recombinant AAV plasmid and the helper plasmid pAAV/Ad (Samulski *et al.,* 1989) which contains a nonrescuable AAV-2 genome in which the AAV termini have been replaced by adenovirus termini (Fig. 1). Briefly, 2 μg of pAAV/Ad and 2 μg of recombinant AAV plasmid (e.g., pWP31) were mixed with a solution containing 250 μg/ml DEAE-dextran (Pharmacia) per milliliter, in DMEM containing 0.05 *M* HEPES, pH 7.05, and applied to cells for 30 min at room temperature. Simultaneously, the cells were infected with adenovirus-2 at a multiplicity of infection (m.o.i.) of 10. Following removal of the medium, the cells were washed twice with sterile phosphate-buffered saline (PBS) and incubated in fresh medium at 37° for 48 hr. Low-molecular-weight DNA from cells was isolated essentially as described by Hirt (1967). Equal amounts of DNA were digested with *Dpn*I, resolved by electrophoresis in a 1% agarose horizontal slab gel, and transferred

to membrane (Genescreen plus, DuPont) for Southern blot hybridization as described previously (Nahreini and Mathews, 1995). As expected, the rescue and replication of pWP28 and pWP29 were independent of pAAV/Ad (data not shown). To rescue recombinant AAV proviral DNA from neomycin-resistant clones, cells were superinfected with adenovirus, and low-molecular-DNA was isolated and analyzed as described above.

Preparation of recombinant AAV

Twenty 10-cm plates of 293 or KB monolayer cultures were co-transfected with pAAV/Ad and a recombinant AAV plasmid (pWP28, 29, 30, or 31) using DEAE-dextran and simultaneously infected with adenovirus as described above, except that 10 μg of each plasmid was used per plate. Cells were harvested by gentle pipetting at 60–65 hr posttransfection/infection when a cytopathic effect was clearly visible. After three cycles of freezing and thawing, the cell lysate was incubated at 56° for 1 hr to inactivate adenovirus and then centrifuged to pellet cell debris. Supernatant containing recombinant AAV was treated with DNase (10 mg/ml) at 37° for 30 min to degrade free plasmid DNA and subsequently stored at −80°.

Generation of cell clones

HeLa monolayer culture cells at 70–80% confluency were co-infected with recombinant AAV together with wild-type AAV (m.o.i. = 2) for 1 hr at 37°. The viral inoculum was removed and the cells were rinsed with PBS and then incubated in DMEM with 10% FCS. Cells were trypsinized at 60 hr postinfection and plated at lower density in several 10-cm plates containing DMEM with 10% FCS and G418 (1 mg/ml) (Sigma Biochemicals). The medium was periodically replaced with fresh DMEM–10% FCS containing G418. Neomycin-resistant colonies appeared within 3 weeks, and individual clones were isolated as described previously (Nahreini *et al.,* 1992). Isolated clones were passaged in the presence of G418 for at least 2 weeks and then resuspended in FCS containing 10% DMSO and stored in liquid nitrogen.

Transfections and CAT assays

Monolayer cultures, which had been passaged the previous day, were transfected by the calcium phosphate precipitation protocol (Morris *et al.,* 1994; Nahreini and Mathews, 1995). Each 6-cm plate of cells received 1 or 5 μg of Tat vector, 1 μg of pON260 containing the β-galactosidase gene (Cherrington and Mocarski, 1989), and carrier salmon sperm DNA to a total of 20 μg DNA. For transient expression of episomal HIV LTR-CAT, HeLa cells were transfected with 5 μg of pWP31 in the same way. The calcium phosphate–DNA precipitate was applied to cells and transfection was carried out for 16 hr under 5% CO_2 at 37°. Cells were rinsed with PBS, and fresh medium was applied. Unless otherwise indicated, cells were harvested at 32 hr posttransfection. Transfection of cells in 10-cm plates was carried out in the same way with twice as much DNA. CAT and β-galactosidase assays were performed essentially as described previously (Morris *et al.,* 1994; Nahreini and Mathews, 1995). Cell extracts with high activity were diluted or incubated for shorter times, or both, to ensure that the assay was performed in the linear range (less than 50% acetylation of chloramphenicol). The Tat vectors were as follows: pCMV-Tat (pBC12/CMV/t2; Cullen, 1986); pGCTat (Ratnasabapathy *et al.,* 1990); pTat2ex (Jeang *et al.,* 1993); pCMV-Tat1(72R); pCMV-Tat(86R); and the empty vector pCMV (Rhim and Rice, 1994).

RESULTS

To investigate the activity of the HIV promoter when it is integrated into the human genome at various loci, including a specific site in chromosome 19, the human parvovirus AAV-2 was used to transduce a CAT reporter gene driven by the HIV LTR into chromosomal DNA. We first constructed chimeric AAV plasmids containing the HIV LTR reporter gene cassette together with the gene for AAV rep (pWP28 and 29) or for neomycin resistance (pWP30 and 31). The genes were inserted into the vector in the same (pWP28, pWP31) or opposite (pWP29, pWP30) orientations (Fig. 1). Infectious chimeric viruses generated from the constructs were used to introduce the cassettes into cells, and the resultant cell lines were characterized with regard to integration site and promoter activity.

Integration of HIV LTR-CAT sequences into human cells

First, recombinant AAV vWP28, containing the rep gene and LTR-CAT reporter cassette in the same orientation, was used to infect HeLa cells. Two days later, cells were cultured at low density to isolate independent clones which were then assayed for the presence of the LTR-CAT cassette. No CAT activity was detected in any of the 20 clones isolated, either in the presence or in the absence of a Tat-expressing plasmid (data not shown). Concluding that it is difficult to isolate cell clones containing the integrated viral genome in the absence of a selectable marker gene, we turned to the recombinant virus vWP31 in which the neomycin-resistance gene replaces the AAV rep gene. HeLa cells were infected with vWP31 together with wild-type AAV which provides rep functions and may impart site-specific integration of the recombinant virus on chromosome 19 (Kotin *et al.,* 1990, 1992; Samulski *et al.,* 1991; Linden *et al.,* 1996). Genomic DNA was prepared from 25 independent clones and analyzed by Southern blotting as shown for 14 clones in Fig.

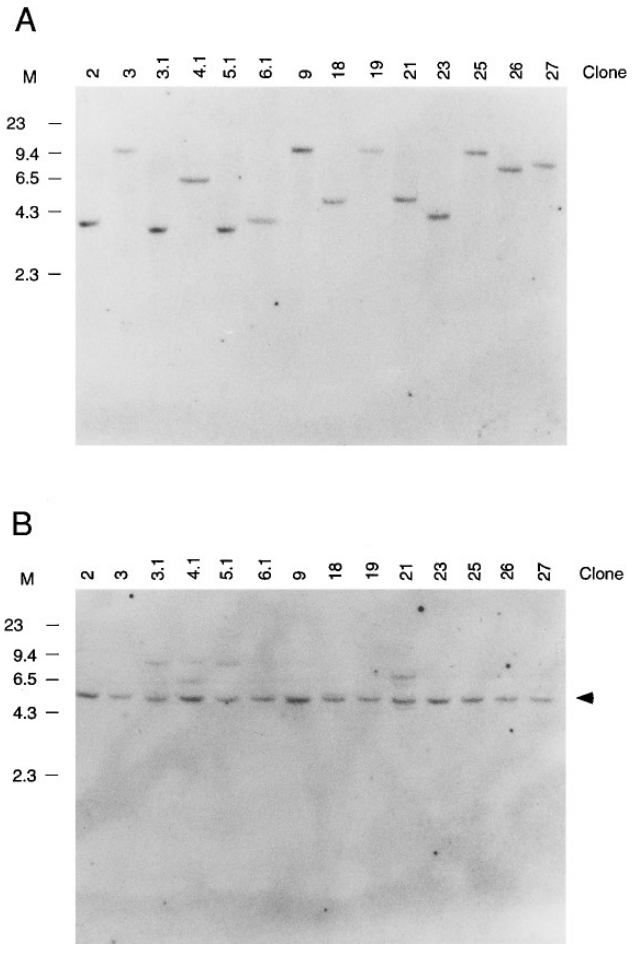

FIG. 2. Analysis of integration patterns in Neo^r clones. (A) Neo probe. Approximately 20 μg of genomic DNA from each cell line was digested with the restriction endonuclease *Hin*dIII, subjected to gel electrophoresis and blotting, and probed with a ^{32}P-labeled Neo DNA insert. Bands were detected by autoradiography. Cell clone numbers are indicated. (B) Chromosome 19 probe. The Neo probe was stripped from the membrane which was then reprobed with the ^{32}P-labeled chromosome 19-specific AAV right-junction DNA fragment (Kotin *et al.,* 1990). Sizes of *Hin*dIII-digested λ DNA fragments are indicated in kilobases. The arrowhead marks the position of the right-junction fragment in uninfected HeLa cells.

2. The DNA was digested with *Hin*dIII, which cuts at the junction between the LTR and the CAT coding sequences but not elsewhere in the recombinant viral DNA.

The blot was probed with ^{32}P-labeled Neo DNA to reveal the integration pattern of the recombinant AAV in genomic DNA from the 14 G418-resistant clones (Fig. 2A). Detection of a single band hybridizing to the Neo probe in each lane suggests that the recombinant virus integrated into a single site in chromosomal DNA in each cell line. We then investigated whether any of the recombinant viruses had targeted chromosome 19, by stripping the Neo probe from the blot and rehybridizing with a chromosome 19 probe containing sequences from the cellular AAV integration site. If the recombinant proviral DNA is integrated at the specific AAV integration site on chromosome 19, an additional band (not present in digested uninfected cell DNA) hybridizing to the chromosome 19-specific probe would be expected to appear at a new position on the blot. Furthermore, the shifted band should hybridize with both the Neo probe and the chromosome 19-specific probes unless rearrangements have taken place concomitant with integration (as frequently observed; see Samulski *et al.,* 1991; Kotin *et al.,* 1992). No shift was apparent with a majority of the clones, suggesting that this region of chromosome 19 is intact, but a shift was detected in seven cases (clones 3.1, 4.1, 5.1, and 21; data for clones 8.1, 21, and 28 not shown) as seen in Fig. 2B. When the two autoradiographs were superimposed, the shifted band appeared to cohybridize with both probes only in clone 4.1; two shifted bands were detected in this case, presumably as a result of chromosomal rearrangement, which is also common (Samulski *et al.,* 1991; Kotin *et al.,* 1992). Therefore, the recombinant AAV vWP31 integrated into a large number of sites, only some of which are located at the known site on chromosome 19.

Rescue and replication of chromosomally integrated AAV are dependent on AAV rep proteins and adenovirus helper functions (Muzyczka, 1992). To determine whether any of the vWP31-derived clones contains rescuable wild-type AAV provirus, cells were superinfected with adenovirus (Nahreini *et al.,* 1992, 1993). Low-molecular-weight DNA was extracted and digested with *Dpn*I to degrade unreplicated DNA (which is methylated) and analyzed by Southern blot probed with ^{32}P-labeled AAV DNA. Rescue and replication of the wild-type AAV provirus from four clones (5.1, 8.1, 23, and 28) were evidenced by detection of characteristic monomeric and dimeric viral DNA replicative species (Fig. 3; data for clones 23

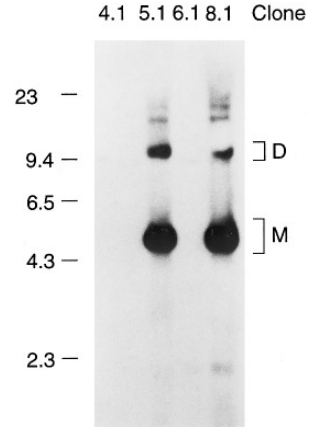

FIG. 3. Rescue and replication of the integrated wild-type AAV genome from Neo^r clones. The cell clones indicated were superinfected with adenovirus. Low-molecular-weight DNA was isolated at 48 hr postinfection/transfection, digested with *Dpn*I, separated by gel electrophoresis, blotted, and probed with ^{32}P-labeled AAV DNA. D and M denote the dimeric and monomeric replicative forms of recombinant AAV DNA, respectively. Sizes of *Hin*dIII-digested λ DNA marker fragments are indicated in kilobases.

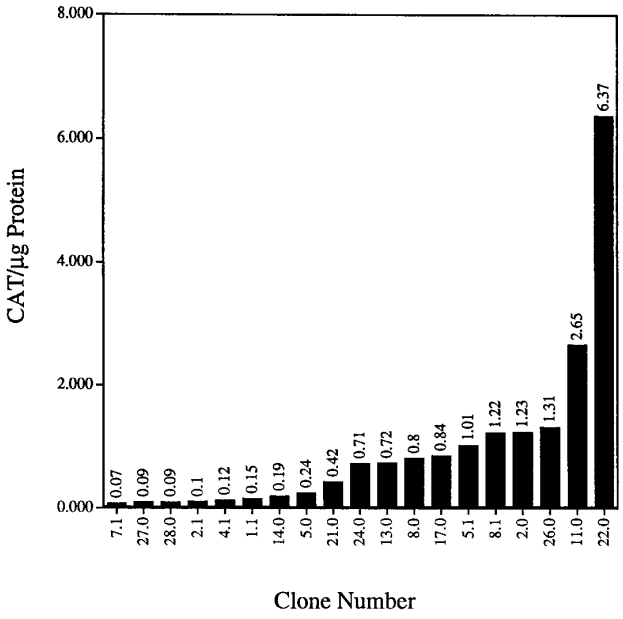

FIG. 4. Constitutive expression from the HIV LTR in cell clones. The CAT activity for each clone represents an average of four transfections, each carried out in duplicate. The measured CAT units, defined as 1% conversion of the chloramphenical form to the acetylated form in a 1-hr reaction at 37°, were normalized to equal amounts of protein.

and 28 not shown). To determine whether the remaining clones contained nonrescuable integrated wild-type AAV, genomic DNA from these clones was analyzed by Southern blotting and probed with AAV DNA (data not shown). All clones that scored negative in the rescue/replication assay were also negative for nonrescuable integrated wild-type AAV genome by this test.

Expression of the integrated LTR-CAT reporter cassette

The basal activity and Tat-stimulated activity of the chromosomally integrated HIV-LTR promoter were monitored using CAT enzyme assays. Cultures of each cell clone were transfected with salmon sperm DNA or a Tat-expressing plasmid and with an expression vector for β-galactosidase to permit normalization for transfection efficiency. Basal CAT enzyme activity varied among the clones over a wide range (Fig. 4). Six clones exhibited undetectable CAT activity (data not shown) even though the linked neomycin resistance gene was biologically active as evidenced by growth in medium containing G418 (1 mg/ml). The remaining clones can be grouped into three categories: low (clones 7.1, 27, 28, 2.1, 4.1, 1.1, 14, and 5), intermediate (clones 21, 24, 13, 8, 17, 5.1, 8.1, 2, and 26), and high (clones 11 and 22) expressors. The basal CAT activity for individual clones was stable and did not vary significantly over a 6-month period, equivalent to $\sim$30 cell culture passages. Furthermore, no significant variation in CAT expression was noted for individual clones when growth was reestablished after freezing in liquid nitrogen. These data indicate that the basal HIV-LTR promoter activity varies significantly among the lines, possibly influenced by the site of proviral integration.

To investigate the effect of the HIV Tat protein on the activity of the integrated HIV promoter, the clones were transfected with one of two different Tat expression vectors driven by the cytomegalovirus (CMV) immediate early promoter. The plasmid pCMV-Tat encodes the one-exon form of the protein, Tat72, whereas pCGTat encodes the two-exon form, Tat86. Transient expression of either Tat72 or Tat86 dramatically increased the CAT enzyme activity in the majority of clones (Fig. 5). Clones that showed no detectable basal promoter activity also failed to respond to Tat transactivation and may not contain the LTR-CAT cassette intact. The degree of Tat-induced transactivation varied significantly among the Tat-responsive clones, ranging from 2- to 260-fold. There was no correlation between the basal level of CAT activity and the degree of transactivation by Tat.

Effect of different HIV-Tat proteins

It is apparent from Fig. 5 that pCGTat, encoding Tat86, generally transactivated the integrated LTR-CAT cassette more efficiently than pCMV-Tat, encoding Tat72. This observation is reminiscent of the report that the two-exon form of Tat, Tat101, transactivated much more effectively than Tat72 when the LTR-CAT cassette was integrated into human (HeLa) or monkey (CV1) chromosomal DNA (Jeang, 1993). The two forms of Tat were equally effective when the LTR-CAT was situated on an episome, however. To determine whether Tat86 is intrinsically more effective than Tat72 at transactivating the HIV promoter, we measured their action on CAT expression in a transient expression assay. HeLa cells were co-transfected with pWP31 in the presence or absence of the Tat expression vectors. CAT activity was low in the absence of Tat, but was increased $>$100-fold in the presence of Tat (data not shown). In this assay, pCMV-Tat (encoding Tat72) consistently gave a slightly higher (1.5- to 2-fold) transactivation of the pWP31 template than pCGTat (encoding Tat 86).

Even though Tat is driven by the CMV promoter in both pCMV-Tat and pCGTat constructs, the two plasmids are not identical in other regions of the transcription units. To determine whether these differences contribute to variations in transactivation efficiency, we tested a pair of Tat expression vectors, pCMV-Tat1 (72R) and pCMV-Tat1 (86R), which differ only in the Tat coding region. Furthermore, the Tat101 vector of Jeang *et al.* (1993), pTat2ex, was tested in parallel. As compared to the empty vector (pCMV) or a transfection control lacking plasmid DNA, each of the Tat vectors dramatically increased CAT enzyme activity when transfected into three representative cell clones (Fig. 6A). The magnitude of Tat transactivation of the integrated HIV promoter varied substantially among

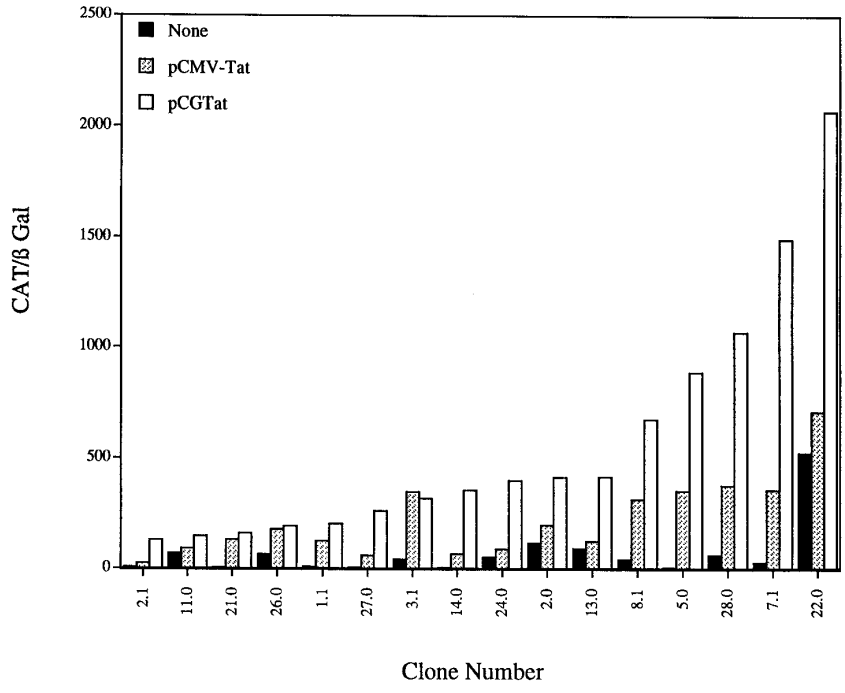

FIG. 5. Activation of the integrated HIV LTR by Tat in cell clones. Cell monolayers were transfected with either pCMV-Tat (Tat72) or pCGTat (Tat86), or without a Tat expression vector, together with pON260 (encoding β-galactosidase). The CAT activity for each clone represents an average of four experiments, each carried out in duplicate. The measured CAT units were normalized to β-galactosidase activity to control for transfection efficiency.

the three clones, ranging from 2.5- to 100-fold. Consistent with the results of Fig. 5, the degree of Tat transactivation conferred by pCMV-Tat1 (72R) was about 20% less than that given by pCMV-Tat1 (86R); pTat2ex was about 20% less effective still (Fig. 6A). When tested in a HeLa cell transient expression assay with pWP31 DNA, all three of these Tat expression vectors elicited a large stimulation of CAT activity (averaging 300-fold). In this co-transfection assay, pCMV-Tat1 (72R) was about 10% more effective than pCMV-Tat1 (86R) and about 20% more effective than pTat2ex (Fig. 6B). In sum, Tat72 was somewhat more effective than Tat86 in transactivating the HIV promoter in co-transfection assays but somewhat less effective on the integrated HIV promoter; Tat101 was not dramatically more effective in either assay.

DISCUSSION

Stable integration of the HIV genome into chromosomal DNA is important for viral gene expression and propagation (Bednarik and Folks, 1992; Stevenson *et al.,* 1990; Zack *et al.,* 1990). In the absence of proviral integration viral gene expression can be detected, but no productive infection ensues (Stevenson *et al.,* 1990; Zack *et al.,* 1990). Therefore, it is essential to examine the activity of the HIV promoter in the context of the neighboring chromosomal DNA. To this end, we used recombinant AAV vectors to transduce a reporter gene driven by the HIV LTR into human chromosomes at random or into a specific site. The recombinant virus appeared to have integrated into a single chromosomal site in each clone. The sites varied from clone to clone but integration into the AAV-specific site on chromosome 19 was relatively rare, probably because of a shortage of the AAV rep protein which was supplied by coinfection with limiting amounts of wild-type AAV.

Basal promoter expression varied significantly among the isolated cell clones, consistent with the view that the level of HIV gene expression is dictated by the site of proviral integration *inter alia.* When HIV Tat was transiently expressed in these clones, the transcriptional activity of the promoter increased dramatically, indicating that the responsivity of TAR to Tat was maintained in the chromosomal environment. Quantitatively, the degree of Tat-induced transactivation of the integrated HIV promoter differed significantly among the clones, implying that the site of integration also influences the sensitivity of the promoter to its viral transactivator. Evidently, the integration site can influence both the activity of the HIV promoter and its responsivity to activators. Such effects may have profound consequences in the course of an infection. It is also possible that deletions and (or) rearrangements of the HIV promoter contribute to the differences in expression of the integrated CAT gene among the clones. Such alterations are most likely to result in complete loss of CAT expression, however, and could account for the absence of activity (even in the presence of Tat) from 24% of the clones.

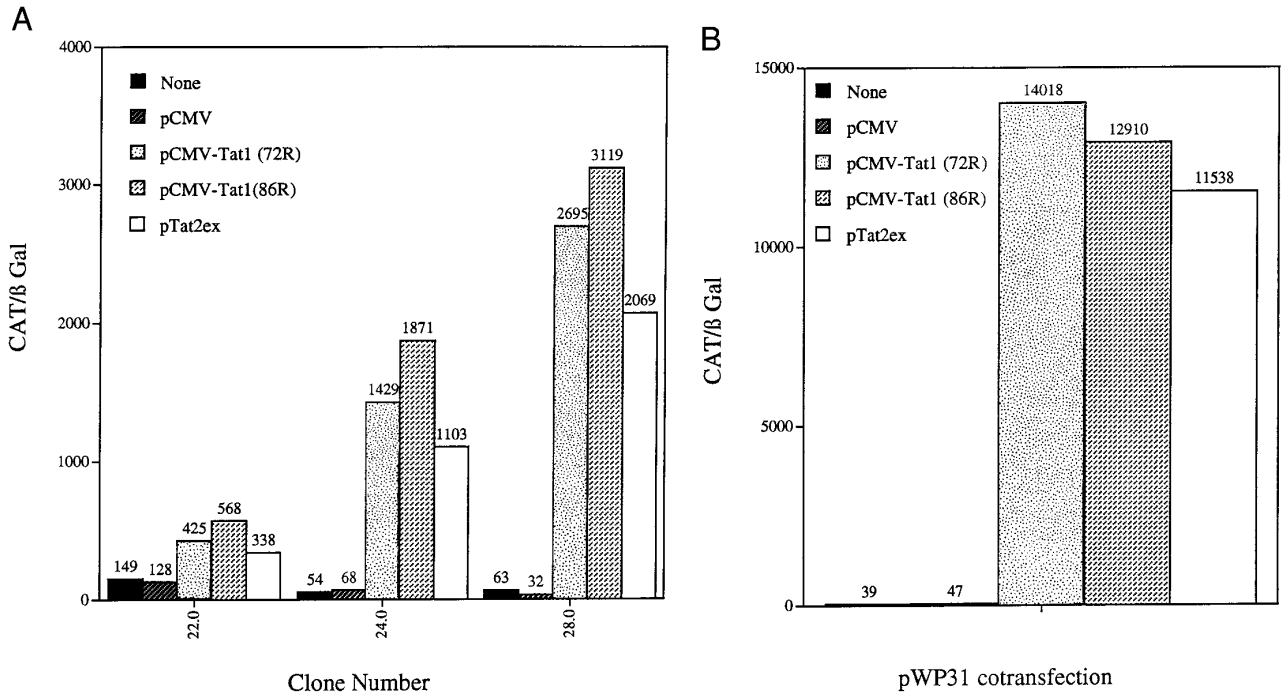

FIG. 6. Transactivation of the HIV LTR by different forms of Tat. (A) Activation of the integrated HIV LTR-CAT cassette was measured in three cell clones as in Fig. 5. (B) Activation of episomal HIV LTR-CAT expression was measured in HeLa cells transfected with 5 μg of pWP31 together with pON260 and the Tat vector indicated. The CAT activities represents averages of duplicate transfections. The Tat expression vectors were pCMV-Tat1(72R), pCMV-Tat1(86R), and pTat2ex (Tat101). Control transfections were carried out with or without the empty vector (pCMV).

The expression of eukaryotic genes can vary dramatically depending on their integration site or the activity of neighboring chromosomal DNA (Navas *et al.,* 1995; Moser *et al.,* 1996). How might these position effects be brought about? Precedents for both positive and negative effects of chromosomal components on the activity of the different promoters have been documented in a variety of systems (Adams and Workman, 1993; Almouzni *et al.,* 1991; Wolffe, 1994). Nucleosome positioning and modification can allow transcription factors to gain access to a chromosomal template by countering the repressive effect of histone–DNA interactions on promoter activity (Almouzni *et al.,* 1991). Nucleosomes can also function as scaffold structures allowing transcription factors to communicate more effectively (Verdin *et al.,* 1993). Such effects have been demonstrated for the mouse mammary tumor virus LTR (Archer *et al.,* 1992) and for cellular promoters (Jackson and Benyajati, 1993; Schild *et al.,* 1993). Furthermore, cellular factors such as the SWI–SNF complex in yeast and its human counterparts are involved in chromatin remodeling, which in turn can facilitate the effect of transactivators (Peterson and Tamkun, 1995). Hypermethylated regions can repress promoter activity and conversely hypomethylation can stimulate promoter activity (Bird, 1992). Correspondingly, the integrated HIV promoter is transcriptionally inactive in the monocytic cell line THP-1 unless the cells are treated with 5-azacytidine (Mikovits *et al.,* 1990). Both basal and Tat-activated transcription from the HIV promoter are stimulated by the presence of an SV40 origin of replication (Kessler and Mathews, 1991; Nahreini and Mathews, 1995). Although it is tempting to ascribe the effects reported here to integration in the proximity of cellular origins, we do not currently have any data bearing on this hypothesis.

Jeang and co-workers reported that Tat72 and Tat101 transactivate the HIV-LTR promoter comparably when the LTR is situated on an episome, but that Tat101 is a 10- to 20-fold more efficient transactivator than Tat72 when the promoter is integrated into chromosomal DNA (Jeang *et al.,* 1993). They therefore postulated a transcriptional role for exon 2 of HIV Tat, specifically in regard to the transactivation of the integrated promoter. We were unable to reproduce these findings with our cell lines although we noted some relatively small differences among the forms of Tat. In our experiments, Tat86 was slightly more effective than Tat72 on the chromosomally integrated promoter and slightly less effective on the episomal promoter; Tat101 was less effective in both assays. Thus, our data argue against the notion that the two-exon form of Tat is significantly more capable of transactivating the integrated HIV promoter than the one-exon form.

Finally, our experiments emphasize the utility of AAV as a suitable vector for the stable transduction of foreign genes into mammalian cells for long-term expression. Further examination of the cell lines described here should throw more light on the processes governing expression of the HIV promoter in the biologically relevant chromosomally integrated state.

ACKNOWLEDGMENTS

We thank Drs. Shobha Gunnery, Gilbert Morris, Michael Laspia, and Frank Ma for advice and help and Christina Yordan, Patricia Wendel, Brigitte Hofmann, and Lisa Manche for excellent technical assistance. We are grateful to Drs. Robert Kotin, Andrew Rice, Kuan-Teh Jeang, Arun Srivastava, Nouria Hernandez, and Bryan Cullen for the generous gift of plasmids. This work was supported by National Institutes of Health Grant AI31802 from NIAID.

REFERENCES

Adams, C. C., and Workman, J. L. (1993). Nucleosome displacement in transcription. *Cell* 72, 305–308.

Almouzni, G., Mechali, M., and Wolffe, A. (1991). Transcription complex disruption caused by a transition in chromatin structure. *Mol. Cell. Biol.* 11, 655–665.

Archer, T. K., Lefebvre, P., Wolford, R. G., and Gager, G. L. (1992). Transcription factor loading on the MMTV promoter: A bimodal mechanism for promoter activation. *Science* 255, 1573–1576.

Bednarik, D. P., and Folks, T. M. (1992). Mechanisms of HIV-1 latency. *AIDS* 6, 3–16.

Bird, A. (1992). The essentials of DNA methylation. *Cell* 70, 5–8.

Cherrington, J. M., and Mocarski, E. S. (1989). Human cytomegalovirus iel transactivates the alpha promoter-enhancer via an 18-base-pair repeat element. *J. Virol.* 63, 1435–1440.

Cullen, B. R. (1986). Trans-activation of human immunodeficiency virus occurs via a bimodel mechanism. *Cell* 46, 973–982.

Embretson, J. M., Zupancic, Ribas, J. L., Burke, A., Racz, P., Tenner-Racz, K., and Haase, A. T. (1993). Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* 362, 359–362.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26, 365–369.

Jackson, J. R., and Benyajati, C. (1993). DNA–histone interactions are sufficient to position a single nucleosome juxtaposing *Drosophila* Adh adult enhancer and distal promoter. *Nucleic Acids Res.* 21, 957–967.

Jeang, K.-T., Berkhout, B., and Dropulic, B. (1993). Effects of integration and replication on transcription of the HIV-1 long terminal repeat. *J. Biol. Chem.* 268, 24940–24949.

Kao, S. Y., Calman, A. F., Luciw, A. P., and Peterlin, B. M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature (London)* 330, 489–493.

Kearns, W. G., Afione, S. A., Fulmer, S. B., Pang, M. G., Erikson, D., Egan, M., Landrum, M. J., Flotte, T. R., and Cutting, G. R. (1996). Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. *Gene Therapy* 3, 748–755.

Kessler, M., and Mathews, M. B. (1991). Tat transactivation of the human immunodeficiency virus type 1 promoter is influenced by basal promoter activity and the simian virus 40 origin of DNA replication. *Proc. Natl. Acad. Sci. USA* 88, 10018–10022.

Kornberg, R. D., and Lorch, Y. (1992). Chromatin structure and transcription. *Annu. Rev. Cell Biol.* 8, 563–587.

Kotin, R. M., Siniscalco, M., Samulski, R. J., Zhu, X., Hunter, L., Laughlin, C. A., McLaughlin, S., Muzyczka, N., Rocchi, M., and Berns, K. I. (1990). Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* 87, 2211–2215.

Kotin, R. M., Linden, R. M., and Berns, K. I. (1992). Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination. *EMBO J.* 11, 5071–5078.

Laspia, M. F., Rice, A. P., and Mathews, M. B. (1990). HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* 59, 283–292.

Linden, R. M., Ward, P., Giraud, C., Winocour, E., and Berns, K. I. (1996). Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* 93, 11288–11294.

Lint, C. V., Ghysdael, J., Paras, P., Jr., Burny, A., and Verdin, E. (1994). A transcriptional regulatory element is associated with a nuclease-hypersensitive site in the pol gene of human immunodeficiency virus type 1. *J. Virol.* 68, 2632–2648.

Malim, M. H., Hauber, J., Le, S.-H., Maizel, J. V., and Cullen, B. R. (1989). The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature (London)* 338, 254–257.

Mikovits, J. A., Raziuddin, Gonda, M., Ruta, M., Lohrey, N. C., Kung, H. F., and Ruscetti, F. W. (1990). Negative regulation of human immune deficiency virus replication in monocytes. Distinction between restricted and latent expression in THP-1 cells. *J. Exp. Med.* 171, 1705–1720.

Mooslehner, K., Karls, U., and Harbers, K. (1990). Retroviral integration site in transgenic Mov mice frequently map in the vicinity of transcribed DNA regions. *J. Virol.* 64, 3056–3058.

Morris, G. F., Labrie, C., and Mathews, M. B. (1994). Modulation of transcriptional activation of the proliferating cell nuclear antigen promoter by the adenovirus E1A 243-residue oncoprotein depends on proximal activators. *Mol. Cell. Biol.* 14, 543–553.

Moser, T., Harbers, K., and Kratochwil, K. (1996). Tissue- and stage-specific activation of an endogenous provirus after transcription through its integration site in the opposite orientation. *Mol. Cell. Biol.* 16, 384–389.

Muzyczka, N. (1992). Use of adeno-associated virus as a general transduction vector for mammalian cells. *Curr. Top. Microbiol. Immunol.* 158, 97–129.

Nabel, G. J. (1993). The role of cellular transcription factors in the regulation of human immunodeficiency virus gene expression. *In* "Human Retroviruses" (B. Cullen, Ed.), pp. 49–73. IRL Press, Oxford.

Nahreini, P., Larsen, S. H., and Srivastava, A. (1992). Cloning and integration of DNA fragments in human cells via the inverted terminal repeats of the adeno-associated virus 2 genome. *Gene* 119, 265–272.

Nahreini, P., Woody, M. J., Zhou, S. Z., Srivastava, A. (1993). Versatile adeno-associated virus 2-based vectors for constructing recombinant virions. *Gene* 124, 257–262.

Nahreini, P., and Mathews, M. B. (1995). Effects of the simian virus 40 origin of replication on transcription from the human immunodeficiency virus type 1 promoter. *J. Virol.* 69, 1296–1301.

Navas, P. A., Josephson, B., Furukawa, T., Stamatoyannopoulos, G., and Li, Q. (1995). The position of integration affects expression of the a.γ-globin-encoding gene linked to HS3 in transgenic mice. *Gene* 160, 165–171.

Pantaleo, G., Graziosi, C., Demarest, J. F., Butini, L., Montroni, M., Fox, C. H., Orenstein, J. M., Kotler, D. P., and Fauci, A. S. (1993). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* 362, 355–358.

Peterlin, B. M., Adams, Alonso, A., Baur, A., Ghosh, S., Lu, X., and Luo, Y. (1993). Tat trans-activator. *In* "Human Retroviruses" (B. Cullen, Ed.), pp. 75–100. IRL Press, Oxford.

Peterson, C. L., and Tamkun, J. W. (1995). The SWI-SNF complex: A chromatin remodeling machine? *Trends Biochem. Sci.* 20, 143–146.

Proudfoot, N. J., Lee, B. A., and Monks, J. (1992). Multiple SP1 binding sites confer enhancer-independent, replication-activated transcription of HIV-1 and globin gene promoters. *New Biology* 4, 369–381.

Ratnasabapathy, R., Sheldon, M., Johal, L., and Hernandez, N. (1990). The HIV-1 long terminal repeat contains an unusual element that induces the synthesis of short RNAS from various mRNA and snRNA promoters. *Genes Dev.* 4, 2061–2074.

Rhim, H., and Rice, A. P. (1994). Exon2 of HIV-2 Tat contributes to transactivation of the HIV-2 LTR by increasing binding affinity to HIV-2 TAR RNA. *Nucleic Acids Res.* 22, 4405–4413.

Samulski, R. J., Chang, L.-S., and Shank, T. (1987). A recombinant plasmid from which an infectious adeno-associated virus genome can

be excised in vitro and its use to study viral replication. *J. Virol.* 61, 3096–3101.

Samulski, R. J., Chang, L.-S., and Shenk, T. (1989). Helper-free stocks of recombinant adeno-associated viruses: Normal integration does not require viral gene expression. *J. Virol.* 63, 3822–3828.

Samulski, R. J., Zhu, X., Xiao, X., Brook, J. D., Housman, D. E., Epstein, N., and Hunter, L. A. (1991). Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* 10, 3941–3950.

Samulski, R. J. (1993). Adeno-associated virus: Integration at a specific chromosomal locus. *Curr. Opin. Genet. Dev.* 3, 74–80.

Scherdin, U., Rhodes, K., and Breindel, M. (1990). Transcriptionally active genome regions are preferred targets for retrovirus integration. *J. Virol.* 64, 907–912.

Schild, C., Claret, F.-X., Wahli, W., and Wolffe, A. P. (1993). A nucleosome-dependent static loop potentiates estrogen-regulated transcription from the Xenopus vitellogenin B1 promoter in vitro. *EMBO J.* 12, 423–433.

Sheridan, P. L., Sheline, C. T., Milocco, L. H., and Jones, K. A. (1993). Tat and the HIV-1 promoter: A model for RNA-mediated regulation of transcription. *Semin. Virol.* 4, 69–80.

Shih, C.-C., Stoye, J. P., and Coffin, J. M. (1988). Highly preferred targets for retrovirus integration. *Cell* 53, 531–537.

Srivastava, A. (1994). Parvovirus-based vectors for human gene therapy. *Blood Cells* 20, 531–538.

Staprans, S. I., and Feinberg, M. B. (1994). Natural history and immunopathogenesis of HIV-1 disease. *In* "The AIDS Knowledge Base" (P. T. Cohen, Ed.), Chapter 3.4, pp. 1–24. Little, Brown, Boston, MA.

Stevens, S. W., and Griffith, J. D. (1994). Human immunodeficiency virus type 1 may preferentially integrate into chromatin occupied by L1Hs repetitive elements. *Proc. Natl. Acad. Sci. USA* 91, 5557–5561.

Stevenson, M., Stanwick, T. L., Dempsey, M. P., and Lamonica, C. A. (1990). HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO J.* 9, 1551–1560.

Verdin, E., Paras, P., Jr., and Lint, C. V. (1993). Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J.* 12, 3249–3259.

Vijaya, S., Steffen, D. L., and Robinson, H. L. (1986). Acceptor sites for retroviral integrations map near DNase I-hypersensitive sites in chromatin, *J. Virol.* 60, 683–692.

Weitzman, M. D., Kyostio, S. R. M., Kotin, R. M., and Owens, R. A. (1994). Adeno-associated virus (AAV) rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc. Natl. Acad. Sci. USA* 91, 5805–5812.

Wolffe, A. P. (1994). Nucleosome positioning and modification: Chromatin structures that potentiate transcription. *Trends Biochem. Sci.* 19, 240–244.

Zack, J. A., Arrigo, S. J., Weitsman, S. R., Go, A. S., Haislip, A., and Chen, I. S. Y. (1990). HIV-1 entry into quiescent primary lymphocytes: Molecular analysis reveals a labile, latent viral structure. *Cell* 61, 213–222.

Zagury, D., Leonard, B. J., Cheynier, R., Feldman, R., Sarin, M. P. S., and Gallo, R. C. (1986). Long-term cultures of HTLV-III-infected T cells: A model of cytopathology of T-cell depletion in AIDS. *Science* 231, 850–853.