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Effects of the Simian Virus 40 Origin of Replication on Transcription from the Human Immunodeficiency Virus Type 1 Promoter

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Positive and negative effects of DNA replication on gene transcription have been documented in a variety of systems. We examined the effects of the simian virus 40 (SV40) origin of replication on transcription from the human immunodeficiency virus type 1 (HIV-1) promoter, using a transient expression assay in COS-1 cells. The basal activity and Tat transactivation of the HIV promoter were greatly stimulated by the SV40 origin of replication independent of its position relative to the long terminal repeat. These effects were abolished by mutational inactivation of the SV40 origin and were reduced by a DNA replication inhibitor. The magnitude of promoter activation exceeded the increment expected from the increase in template number resulting from DNA replication. The SV40 T-antigen-induced DNA replication augmented the generation of both processive and nonprocessive HIV long terminal repeat-directed transcripts, and Tat primarily enhanced the initiation of those transcripts that were destined to be efficiently elongated. Our data suggest that the HIV promoter displays greater transcriptional activity on replicative DNA templates. This property may influence the activity of integrated HIV provirus and its transition from latency to productive infection.

One of the critical requirements of the human immunodeficiency virus (HIV) life cycle, essential for stable maintenance, gene expression, and subsequent pathogenicity, is its integration into chromosomal DNA (4). Activation of HIV transcription, which is dependent on the HIV Tat protein and its target RNA binding sequence, TAR, is required for the virus to exit from latency and initiate a productive infection (2, 4). TAR is present in the 5′ untranslated region of all HIV transcripts and 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 (33). These interactions between TAR, Tat, and cellular factors increase transcriptional initiation and elongation by RNA polymerase II complexes from the long terminal repeat (LTR) (22, 23, 38).

Transcription from the integrated HIV LTR is activated by host cell factors such as cytokines (e.g., interleukin-2) and cellular transactivators (e.g., NF-κB), leading to synthesis of Tat, which in turn amplifies the expression of viral genes needed to commence a productive infection (4, 31, 52). Numerous viral and host cell factors have been implicated in initial activation of the HIV promoter (31), but little is known concerning the role of the flanking chromosomal DNA on proviral gene expression and exit from latency. Retroviral integration occurs primarily in dividing cells and is blocked in quiescent cells (41, 51), implying a link between the replicative state of the cell and the integration process. Consistent with this, retroviral integration sites frequently overlap with the DNase I-hypersensitive regions in chromosomes, which are a hallmark of regions of chromosomal DNA that are actively transcribed or replicated (29, 36, 39, 47).

Replication has been implicated in the induction of transcription independent of an increase in DNA template number (13, 16, 28, 34, 44–46, 50). These observations taken together suggested that the proximity of the integrated HIV provirus to a DNA replication origin may affect the viral promoter activity and its subsequent pathogenicity. The effect of the simian virus 40 (SV40) origin of replication on the activity of the HIV promoter was investigated in COS cells by Kessler and Mathews (20). This study concluded that the transcriptional activity of the HIV promoter is influenced by the SV40 DNA replication origin and that the magnitude and nature of the effect are dependent on the position of the origin sequence with respect to the LTR promoter. To explore the mechanism of this effect, the LTR-chloramphenicol acetyltransferase (CAT) cassette from pU3RIII was subcloned together with the SV40 origin into the pBluescript SK(+) bacterial vector to facilitate further cloning manipulations. During the course of this work, we discovered that the presence of the SV40 origin in the plasmid, but not its position relative to the LTR, is the determinative feature. Upon reexamination of the previous constructs in the pH series (20), pH1, which was supposed to contain SV40 origin sequence upstream of the LTR, was found devoid of it. Authentic pH1 was constructed as depicted in Fig. 1. Here, we examine the effect of the position of the SV40 origin and the mechanism of its action on gene expression. Since very similar results were obtained with constructs which were based on either the pUC19 (pH series) or pBluescript SK(+) (pWP series) vector, only data regarding the pWP series of constructs are presented.

Effects of origin on basal expression and Tat transactivation. The plasmids depicted in Fig. 1A carry a minimal 200-bp SV40 origin either upstream or downstream of the reporter cassette, LTR-CAT. These constructs were transfected into COS cells, which constitutively express the SV40 T antigen, in the presence or absence of a Tat expression vector (pCMV-Tat), and CAT expression relative to a β-galactosidase control was quantified at 32 h posttransfection (30). In the absence of Tat, CAT expression was detectable at low levels with the construct lacking an SV40 origin, pWP0 (Fig. 2A). In the presence of Tat, a large (up to 500-fold) transactivation was observed. When transfected without pCMV-Tat, the origin-containing plasmids pWP1 and pWP2 generally gave 30- to

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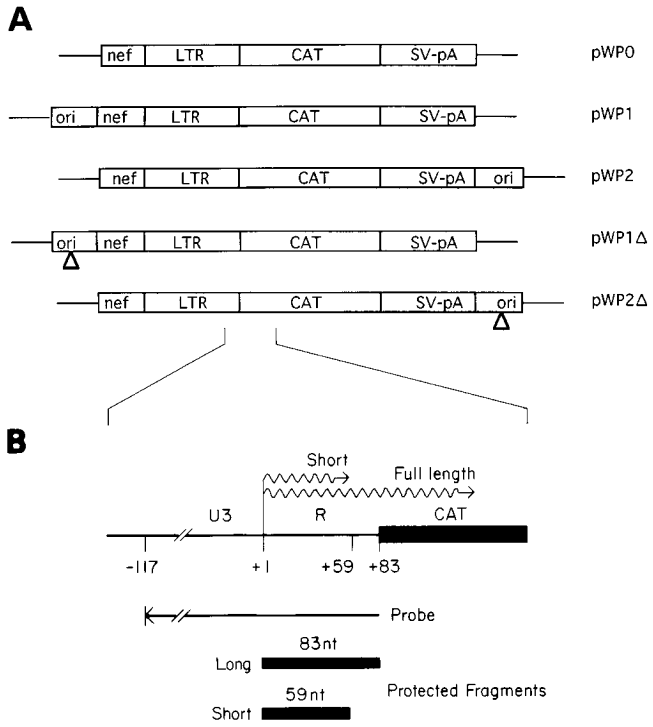

FIG. 1. Schematic diagram of HIV CAT expression constructs and transcripts. (A) Constructs. The LTR-CAT cassette of pU3RIII was cloned into the polylinker site of pBluescript SK(+) to create pWP0. The reporter cassette contains the portion of the *nef* gene lying upstream of the LTR and SV40 splicing and poly(A) signals (SV-pA). The SV40 origin of replication (SV40 *Hin*dIII-*Sph*I fragment, nt 5171 to 128; ori) was inserted immediately either upstream or downstream of the reporter cassette in pWP0, and the resulting construct was designated pWP1 or pWP2, respectively. Plasmids pWP1Δ and pWP2Δ were derived from pWP1 and pWP2, respectively, by deletion of the *Sfi*I restriction site (triangles) within the SV40 core origin. (B) Portion of the LTR-CAT cassette, depicting short and long transcripts, protecting fragments of 59 and 83 nt, respectively, of the 200-nt-long antisense riboprobe generated from plasmid pGEM23 (22). Diagram is not drawn to scale.

40-fold-higher CAT expression than their counterpart devoid of the SV40 origin, pWP0. Tat also greatly increased expression from the LTR in the presence of the SV40 origin. Although the relative increases were lower, the absolute magnitude of the increase in CAT expression due to Tat was greater than in the absence of the origin. In multiple repeats of this experiment, differences between plasmids containing the origin upstream (pWP1) and those with it downstream (pWP2) were negligible. Thus, the SV40 origin contributes to the enhancement of basal as well as transactivated levels of CAT expression from the HIV promoter.

The HIV LTR gives rise to two classes of transcripts, a class of short nonpolyadenylated RNAs and a class of full-length poly(A)$^+$ transcripts (mRNA), whose formation is favored by HIV Tat (19–22). To investigate the effect of the SV40 origin and its position on the formation of short and long transcripts, cytoplasmic RNA isolated from transfected COS cells was examined by RNase protection analysis (22). The cellular RNA was annealed to a complementary RNA probe of 200 nucleotides (nt), which spans positions −117 to +83 of the HIV promoter (Fig. 1B). A series of short RNAs (55 to 59 nt), corresponding to the short transcripts, was detected together with longer RNA (83 nt) derived from the full-length transcripts. In the absence of Tat, pWP0 gave no visible protection of either short or long fragments (Fig. 2B, lane 1), but when a larger amount of cytoplasmic RNA was analyzed, protection of transcripts of both size classes was detected by scanning of the dried gel. This finding correlates well with the low CAT expression observed with pWP0 alone. In the absence of Tat, pWP1 or pWP2 gave rise to protected fragments of both size classes (lanes 3 and 7). Therefore, the SV40 origin of replication apparently augments an intrinsic property of the HIV promoter that favors the formation of short over long transcripts in the absence of Tat.

In the presence of Tat, pWP0 produced long transcripts almost exclusively (Fig. 2B, lane 2). Both pWP1 and pWP2 gave rise to roughly equivalent amounts of long and short transcripts (lanes 4 and 8). Much greater amounts of long transcripts, as well as somewhat greater amounts of short transcripts, were produced by the origin-containing plasmids in the presence of Tat compared with its absence. This finding suggests that Tat mainly stimulates the production of RNA polymerase II complexes that are destined to be elongated efficiently and is not involved in the conversion of short to long transcripts. Previous reports have suggested the formation of two different RNA polymerase II complexes on the HIV LTR promoter, one of which is sensitive to Tat and gives rise to long transcripts, whereas the other is minimally affected by Tat and generates short transcripts (22, 35, 37).

Mutation of the SV40 replication origin. Numerous regulatory signals are present in the vicinity of the SV40 replication origin (14). We therefore next sought to determine whether the positive effect of the inserted origin-containing sequence on the HIV promoter is due to replication, SV40 T-antigen binding, or auxiliary *cis*-acting elements. The SV40 origin insert in these constructs comprises the core origin (67 bp, containing T-antigen binding site II), three binding sites for transcription factor Sp1, and T-antigen binding site I (14). The central domain of binding site II is a 27-bp palindrome which contains two pairs of T-antigen binding sites (GAGGC) in opposite orientations. These sites are critical for assembly of the T-antigen complex and for the initiation of SV40 replication (10). Deletion of the *Sfi*I site which overlaps one of the SV40 binding sites within the core origin eliminates its replication activity (11). Two new constructs, pWP1Δ and pWP2Δ (Fig. 1A), containing this deletion were prepared, and their expression was assayed (Fig. 2).

In the absence of Tat, pWP1Δ and pWP2Δ expressed approximately 40-fold-less CAT activity than their counterparts containing the intact SV40 origin, essentially equivalent to pWP0 (Fig. 2A). Similarly, in the presence of Tat, CAT expression from pWP1Δ and pWP2Δ was approximately eightfold less than from pWP1 and pWP2 and again equivalent to that from pWP0. The reduction in Tat transactivation seen with pWP1 and pWP2 may be due to the increased basal promoter activity of the origin-containing plasmids as reported previously (27). The CAT expression data suggest that SV40-induced replication or some closely related characteristic of T-antigen binding imparts positive effects on LTR-directed gene expression. Comparable results were obtained by RNase protection analysis (Fig. 2B). In the absence or presence of HIV Tat, both pWP1Δ and pWP2Δ gave rise to patterns of protected transcripts similar to that from pWP0 (compare lanes 6 and 10 with lane 2). The lower protection of long transcripts in the case of pWP2Δ is due to poor transfection efficiency, as inferred from the lower protection of α-globin mRNA, used as an internal control in these assays (lane 10). Since the mutation that specifically abolishes the replication function of the origin abrogated the transcriptional effects, it seemed that increased DNA copy number may fully account for the increased expression of the HIV-CAT gene.

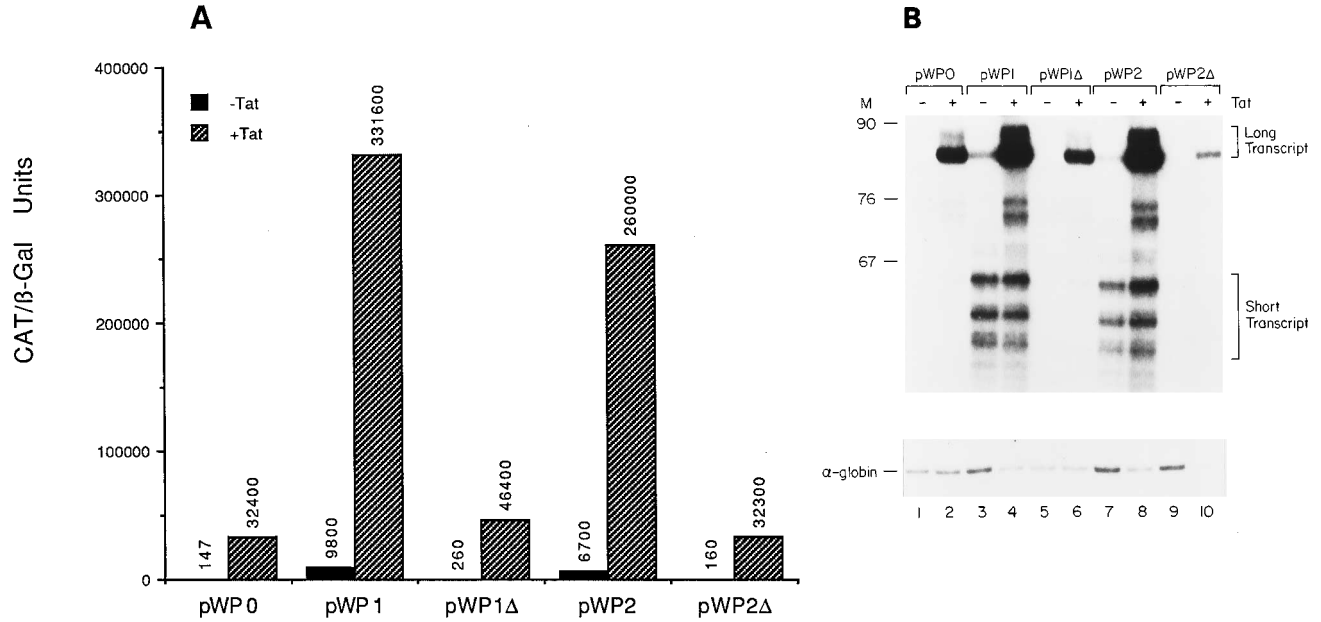

FIG. 2. Activation of HIV LTR-CAT expression by the SV40 origin of replication. The indicated constructs were transfected into COS-1 cell monolayers (5 μg/6-cm-diameter dish, 50% confluent) in the presence or absence of pCMV-Tat (9) expression vector (5 μg) for 16 h. (A) The indicated constructs were transfected into COS-1 cells with pON260 (1 μg/6-cm-diameter dish) to allow for normalization for transfection efficiency by measurement of β-galactosidase (β-Gal) activity (7). CAT assays were performed at 32 h posttransfection. The CAT activity for each construct represents an average of duplicate transfections. The CAT units, defined as 1% conversion of the chloramphenicol to the acetylated form in a 1-h reaction at 37°C, were divided by the β-galactosidase activity. (B) COS-1 cells were transfected with the indicated pWP constructs together with a human α-globin expression vector (43), pαA+C (1 μg/6-cm-diameter dish), to normalize for transfection efficiency. Cytoplasmic RNA (10 μg) was analyzed by RNase protection assay (22), using the antisense riboprobe (10^6 cpm) depicted in Fig. 1B. Plasmid pα132 was used to generate an RNA probe complementary to the α-globin gene (43). After annealing and RNase A and T_1 digestion, products were resolved by electrophoresis in 8% polyacrylamide–7 M urea denaturing gels, which were then fixed, dried, and subjected to autoradiography with an intensifying screen. Protected fragments were quantified by scanning the dried gel on a bioimaging analyzer (Fuji). + and − denote the presence and absence of cotransfected pCMV-Tat; the locations of the long and short transcripts are bracketed. M, pBR322/*Hpa*II marker (sizes are indicated in nucleotides).

To determine whether these origin-containing plasmids underwent one or more rounds of DNA replication in COS cells, low-molecular-weight DNA isolated from transfected cells was linearized by digestion with *Hin*dIII, and its replication status was tested with two methylation-sensitive restriction enzymes, *Dpn*I and *Mbo*I (Fig. 3). *Dpn*I cuts the input DNA, which is methylated, but not DNA replicated in mammalian cells, which is unmethylated; conversely, *Mbo*I digests replicated but not unreplicated DNA. As expected, pWP0, pWP1Δ, and pWP2Δ were fully sensitive to *Dpn*I but not *Mbo*I digestion, whereas a substantial fraction of pWP1 and pWP2 DNA was digested by *Mbo*I but not by *Dpn*I. When the ratio of replicated and unreplicated DNA was quantified by scanning the blot, the DNA template number of the origin-containing plasmids was determined to increase on average two- to threefold. The same increase was also measured by slot blot hybridization of the low-molecular-weight DNA from cells harvested at 32 h posttransfection (data not shown).

The stimulation of LTR promoter activity by the SV40 origin is much greater than the two- to threefold elevation of DNA template number, as reported previously (34, 49). This discrepancy can be resolved in several ways. First, it is possible that a fraction of the input DNA template is not available as a substrate for the replication and transcription machineries, because it is not located in the nucleus, for example. In this case, the two- to threefold increase in DNA copy number would correspond to a much greater increase in the relevant DNA fraction, which might fully account for the observed levels of HIV LTR-CAT expression. In the experiment shown in Fig. 3, about half of the pWP1 or pWP2 DNA was replicated, so the approximately 40-fold transactivation (measured as an increase in CAT expression relative to pWP0) could be explained if only about 1% of the DNA reached the nucleus. However, when the DNA from purified nuclei was examined by *Dpn*I and *Mbo*I digestion, very similar results were obtained (e.g., Fig. 4B), suggesting that sequestration of the transfected DNA is not the correct explanation. Second, the binding of T antigen to the origin may exert positive effects on the transcriptional activity of the HIV promoter through localized conformational changes or protein-protein interactions. We addressed this possibility by using hydroxyurea (HU) to inhibit replication without altering T-antigen binding sites.

Effects of DNA replication block. To distinguish effects on replication per se from effects on T-antigen binding, we examined the influence of a DNA synthesis inhibitor on the transcriptional activity of the LTR promoter in the constructs containing the wild-type origin. HU inhibits the cellular enzyme ribonucleotide reductase and slows DNA replication by reducing the deoxyribonucleotide pool. As a result, HU causes nascent DNA chains to terminate, leading to abortive replication, but it does not interfere with the initiation of DNA replication (25). COS cells were transfected with pWP0, pWP1, or pWP2 in the presence of 10 mM HU, which was maintained during the entire course of the experiment. For comparison, plasmids pWP0, pWP1, and pWP1Δ were included in the same experiment without HU treatment of COS cells.

Analysis of cytoplasmic RNA by RNase protection showed that the pattern of transcripts from pWP1- and pWP2-transfected cells maintained in the presence of 10 mM HU resembled that from cells transfected with origin-negative plasmids pWP0 and pWP1Δ rather than that from uninhibited pWP1 (Fig. 4A). This finding indicates that T-antigen binding is not

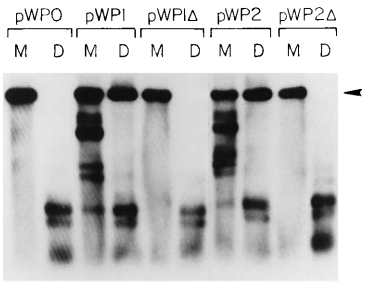

FIG. 3. DNA replication assay on whole-cell Hirt extract. The indicated pWP constructs were transfected into COS-1 cells as described in the legend to Fig. 2. Low-molecular-weight DNA was isolated by the Hirt procedure (18) at 32 h posttransfection, linearized with *Hin*dIII, and digested with either *Dpn*I (D) or *Mbo*I (M) methylation-sensitive restriction enzyme. After agarose gel electrophoresis, the blot was probed with a ^{32}P-labeled CAT insert labeled by the random primer labeling technique as described previously (32). The arrowhead indicates the linearized full-length plasmid.

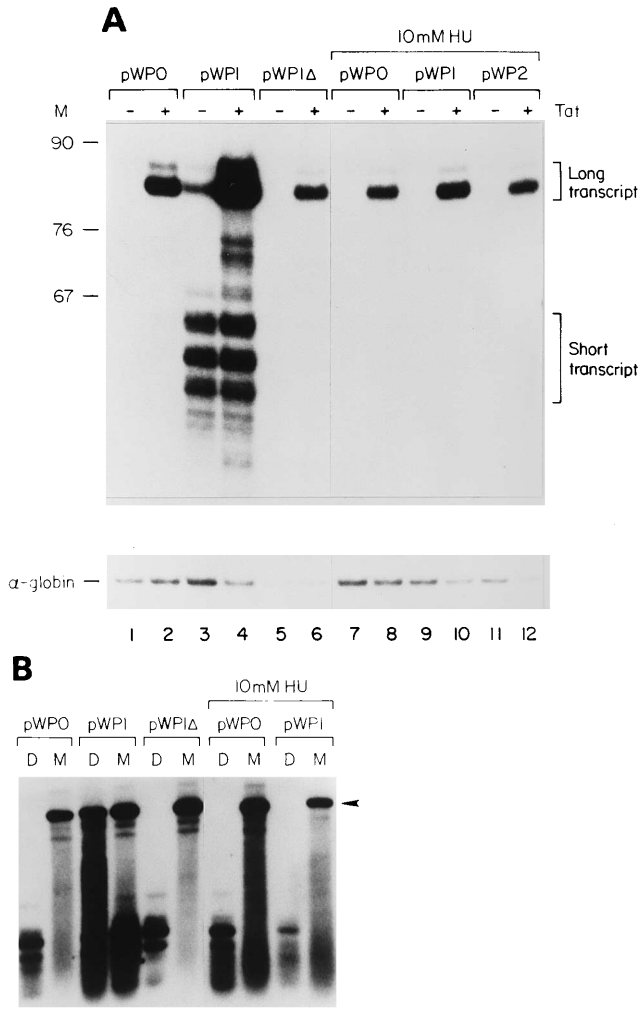

FIG. 4. Effect of HU on HIV LTR-directed RNA transcripts and replication. Transfection protocol and RNase protection assays were as for Fig. 2 except that 10 mM HU (Sigma) was present in the media of the indicated cultures throughout the experiment. (A) RNase protection assay, using the indicated constructs and HU as shown. M, size markers (sizes are indicated in nucleotides). (B) DNA replication assay on nuclei (48) from transfected COS cells. Isolated DNA (10 μg) from the nuclei was digested with either *Hin*dIII and *Dpn*I (D) or *Hin*dIII and *Mbo*I (M) and examined as in Fig. 3. The arrowhead indicates the linearized full-length plasmid.

sufficient to elicit the transcriptional effects. However, when differences in transfection efficiency were corrected by normalization to the α-globin control signal, it was apparent that pWP1 and pWP2 both gave rise to two- to fourfold-greater amounts of long transcripts than pWP0 in the presence of Tat (compare lanes 10 and 12 with lane 8). Similar results were obtained in several repeats of this experiment. Moreover, when the experiment was conducted with a lower concentration of HU (2.5 mM), the effect was increased: pWP1 and pWP2 gave rise to four- to sixfold-greater protection of long transcripts than pWP0 in the presence of Tat (data not shown).

These results could be explained if some degree of replication occurred even in the presence of high concentrations of HU. To examine the replication status of the plasmids in the presence or absence of HU, DNA was isolated from the nuclei of the transfected COS cells and tested for its sensitivity to *Dpn*I and *Mbo*I digestion. At 10 mM, HU abolished DNA replication, as demonstrated by the complete sensitivity of the recovered pWP1 DNA to *Dpn*I and its insensitivity to *Mbo*I (Fig. 4B). Similar results were obtained with origin-minus plasmids pWP0 and pWP1Δ (Fig. 4B) and at 2.5 mM HU (data not shown), whereas in the absence of HU, pWP1 replication was clearly evident. These findings rule out extensive replication of the DNA, implying that increased copy number is not the whole explanation for the origin dependence. Limited replication, which is not sufficiently extensive to be detected by the methylation sensitivity assay, might still occur, however, and the formation of a replication bubble could contribute to the stimulation of LTR expression observed in the presence of HU.

Replication and gene expression. Positive and negative effects of DNA replication on gene expression have been documented in animal viruses and in eukaryotic cells (12, 42). For example, adenovirus DNA replication is essential for the early-to-late switch in gene expression, which is independent of template copy number or early gene products known to activate late gene expression (28, 44, 46). Similarly, the early-to-late switch in SV40 gene expression is dependent on viral DNA replication per se (8). In contrast, SV40 early gene expression occurs in human 293 cells when DNA replication is blocked (24, 26). Also, the silencing effect of DNA replication on the expression of mating locus (*MAT*) in the yeast *Saccharomyces cerevisiae* has recently been reported (5, 15). These observations underscore the functional link between DNA replication and gene expression, but at present no single mechanism of action which could account for all of the observed effects of DNA replication on transcription is known.

In this study, the transcriptional activity of the HIV promoter is stimulated by the origin of SV40 DNA replication. Mutation of the T-antigen binding site within the core origin abolished both plasmid DNA replication and the positive transcriptional effects of the SV40 origin on the HIV LTR promoter. The transcriptional effects on the HIV promoter were greatly reduced but not eliminated by HU, suggesting that DNA replication per se is required for the observed transcriptional effects. Since both pWP1Δ and pWP2Δ contain at least two intact SV40 T-antigen binding sites in the core origin, T-antigen binding is not sufficient to elicit the observed transcriptional effects, nor are any other *cis*-acting elements or proteins that bind within the origin fragment. At least two mechanisms could contribute to the induction of LTR promoter by T-antigen-induced DNA replication: an increase in DNA template number as a result of plasmid DNA replication,

and T-antigen-induced changes in other properties of the template such as its conformation and protein associations.

The SV40 T-antigen-induced replication led to a 2- to 3-fold increase in promoter template number, but reporter gene expression increased 40-fold. This finding suggests that the activity of the HIV promoter is augmented on a replicative DNA template. This is reminiscent of the effect of DNA replication on the switch from early to late gene expression in adenovirus- and SV40-infected cells. It is conceivable that DNA replication changes chromatin structure in ways that allow access to either transcription activators or repressors of a promoter (1, 3). Our data are also compatible with the observation of Proudfoot et al. (34) that replication-mediated high levels of transcriptional activity of HIV LTR can be achieved independent of Tat transactivation. The three Sp1 binding sites in the proximity of the LTR TATA box were sufficient to confer enhancer-independent activation of the HIV and heterologous promoters situated on the replicative DNA template. These workers hypothesized that DNA replication modifies chromatin structure, thereby allowing Sp1 to stimulate promoter activity. Similarly, by examining the effect of replication on the activity of several promoters in different cell types, Wilson and Patient (49) concluded that DNA replication modifies chromatin structure and facilitates the interaction between an enhancer and its promoter. On the other hand, it is conceivable that only a small fraction of the input DNA which reaches the nucleus has access to the DNA synthesis and transcription machinery, in which case increased DNA copy number alone could fully account for the observed transcriptional effects. This possibility is difficult to test experimentally. We speculate that T-antigen-induced unwinding of DNA duplex and the subsequent formation of a replication bubble bestow a positive effect on the HIV promoter. Alternatively, the binding of T antigen may lead to changes in the position of the SV40 DNA in the nucleus or its associations with nuclear components without any conformational change.

HIV Tat protein stimulates the expression of the HIV-1 gene expression predominantly at the level of transcription (33), although it has also been implicated in posttranscriptional regulation (6). Tat activates transcription of the LTR promoter by increasing the transcriptional initiation rate as well as increasing the efficiency of transcriptional elongation (22, 23). In our experiments, Tat transactivation appeared to be independent of the basal level of LTR-directed transcription. Whether the basal promoter activity was low (with the constructs devoid of the SV40 origin) or high (with origin-containing constructs), Tat increases the initiation of those transcription complexes that elongate processively. Similarly, in a natural latent HIV infection of T cells, short transcripts were primarily detected, and significant amounts of long transcripts were generated subsequent to the synthesis of Tat (2). Jeang et al. (17) have reported that SV40-induced replication solely induces the generation of short transcripts without affecting the amounts of long transcript in either the presence or absence of the HIV Tat. In contrast, our data indicate that replication augmented the generation of short and, to a somewhat lesser extent, long transcripts. Moreover, SV40 T-antigen-induced replication enhanced Tat transactivation severalfold when the HIV promoter was situated on a replicative template. We cannot explain this discrepancy.

How might DNA replication influence HIV pathobiology in a natural infection of human cells? It is clear that the replicative state of cells is essential for stable integration of the HIV DNA intermediate (4), and it is reasonable to speculate that integration occurs in regions of chromosomal DNA which are actively transcribed or replicated (29, 36, 39, 47). Numerous cellular factors are thought to play a role in activation of proviral gene expression (31), but little is known concerning the flanking chromosomal DNA. In view of these observations, an origin of replication might be an important influence in the transition from latency to a productive infection via effects on HIV LTR promoter activity.

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