DNA-dependent protein kinase interacts functionally with the RNA polymerase II complex recruited at the human immunodeficiency virus (HIV) long terminal repeat and plays an important role in HIV gene expression

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INTRODUCTION

Phosphorylation is the most common and one of the most important mechanisms of acute and reversible regulation of protein function (Hill & Treisman, 1995; Hunter, 1995). Specifically, human immunodeficiency virus (HIV) transcriptional elongation, which is the main functional activity of the Tat protein, is totally dependent on the association of Tat with kinases able to phosphorylate the carboxy-terminal domain (CTD) of human RNA polymerase II (RNAP II). In the absence of Tat, the RNAP II complexes present at the HIV promoter are dysfunctional and elongate poorly. However, once Tat is synthesized, it recruits positive transcription elongation factor b (P-TEFb) at the HIV LTR and enhances the processivity of RNAP II, thus fostering increased levels of full-length transcripts (Feinberg et al., 1991; Kao et al., 1987; Karn, 1999). The recruitment of P-TEFb (a complex of cyclin T1/2 and CDK9) through its interactions with Tat and the transactivation response region (TAR) results in activation of the CDK9 kinase and the consequent hyperphosphorylation of the CTD of RNAP II at serine 2.

The CTD of mammalian RNAP II contains 52 tandemly arranged heptapeptide repeats of the Tyr-Ser-Pro-Thr-Ser-Pro-Ser (YSPTSPS) motif. Phosphorylation of serine residues Ser-5 and Ser-2 of these repeats is mainly correlated with transcriptional initiation and elongation capacity of RNAP II, respectively (Dahmus, 1995; Orphanides et al., 1996). It is well-established that paused RNAP II contains predominantly hypophosphorylated CTDs, whereas progressively elongating polymerase possesses hyperphosphorylated
CTDs (O’Brien et al., 1994). These findings clearly demonstrate the critical role of cellular protein kinases in HIV-1 transcription. More than half a dozen kinases are known to phosphorylate CTD of RNAP II, and the involvement of two kinases, namely CDK9 and CDK7, in CTD phosphorylation during HIV gene expression has already been well established (Dahmus, 1996; Isel & Karn, 1999; reviewed by Majello & Napolitano, 2001). One of the kinases that is known to phosphorylate the RNAP II CTD is the DNA-dependent protein kinase (DNA-PK), a nuclear kinase that specifically requires association with DNA for its activity (Anderson & Carter, 1996; Carter et al., 1990; Dvir et al., 1993; Lees-Miller et al., 1990). Human DNA-PK comprises two components: a 450 kDa catalytic subunit (DNA-PKcs) (Hartley et al., 1995), which is a serine/threonine kinase, and a regulatory component known as Ku autoantigen (Gottlieb & Jackson, 1993). Ku is a heterodimer composed of subunits of 70 kDa (Reeves & Sthoeger, 1989) and 80 kDa (Yaneva et al., 1989). The 70 kDa subunit possesses ATPase and DNA helicase activities (Tuteja et al., 1994). The critical role of DNA-PK in the non-homologous end joining (NHEJ) DNA-repair pathway is well-established (Lees-Miller et al., 1995; Taccioli et al., 1994). The NHEJ pathway is essential for the maintenance of chromosomal integrity in developing lymphocytes and to repair DNA double-strand breaks that occur during V(D)J recombination. Impaired V(D)J recombination results in severe combined immunodeficiency disease (SCID), which is represented by undeveloped B and T lymphocytes (Blunt et al., 1995; Meek et al., 2004; Nussenweig et al., 1996; Taccioli et al., 1994). Curiously, whilst some studies have suggested an important role for DNA-PK during HIV integration into the host-cell genome (Daniel et al., 1999; Jeanson et al., 2002; Nunnari et al., 2005), others have indicated that the kinase is dispensable (Ariumi et al., 2005; Baekelandt et al., 2000; Kilzer et al., 2003; Li et al., 2001). Thus, the exact role of DNA-PK during HIV integration into the host-cell genome has not received a universal consensus. The DNA-binding component of DNA-PK is the heterodimeric Ku factor, one of the most abundant DNA-end-binding proteins in human cells (de Vries et al., 1989). Ku binds mainly to the dsDNA ends of a variety of DNA structures; however, it does not bind to closed circular DNA (Dyan & Yoo, 1998; Yoo & Dyan, 1998), underscoring the absolute need for free DNA ends for its binding activity.

There have been several published critical observations that DNA-PK can phosphorylate the RNAP II CTD. DNA-PKcs and Ku are components of the RNAP II holoenzyme (Maldonado et al., 1996). Ku specifically recognizes stem-loop structures and, in particular, binds to HIV-1 TAR RNA (Kaczmarski & Khan, 1993). Moreover, Tat interacts in vitro with DNA-PK (Chun et al., 1998). Recent work from the Rice group (Liu et al., 2010) has reported the interaction of Ku70 with the minor 55 kDa isoform of CDK9, and this interaction was suggested to play a role in the post-integration repair and/or circularization of viral LTR circles. Furthermore, An et al. (2011) have also reported an interaction between DNA-PK and cyclin T2, another subunit of the P-TEFb complex, thus strengthening the notion of a possible role for DNA-PK in HIV transcription. All of these findings prompted us to investigate the role of DNA-PK in HIV transcription and to determine whether the HIV-1 Tat protein itself could be a substrate for DNA-PK phosphorylation.

RESULTS

Recruitment of DNA-PK at the HIV LTR

As it is known that DNA-PK phosphorylates the CTD of RNAP II (Dvir et al., 1993; Trigon et al., 1998), we performed chromatin immunoprecipitation (ChIP) assays to investigate the possible presence of this enzyme at the HIV LTR. These assays were performed by using a Jurkat T-cell-based clone, the E4 clone, which carries latent HIV provirus in its genome (Pearson et al., 2008; Tyagi & Karn, 2007) (Fig. 1a). Assays were performed before and after activating latent provirus with tumour necrosis factor alpha (TNF-α). The immunoprecipitated DNA was analysed by using two primer sets, one amplifying the promoter region of the LTR (−116 to +4 with respect to the transcription start site), which provides a measure of the factors involved in transcriptional initiation. The second primer set used amplifies the nucleosome-2 region of the LTR (+286 to +390 with respect to the transcription start site), which quantifies those factors involved in the elongation phase of HIV transcription.

As shown in Fig. 1(b), latent proviruses have low but detectable levels of RNAP II at the promoter region of the LTR (−116 to +4). However, following induction with TNF-α, RNAP II levels increase rapidly at the promoter, reaching at least fivefold higher than the pre-induction levels. Parallel to RNAP II recruitment, we also found a proportional jump in the levels of DNA-PK at the promoter, clearly suggesting a significant role for DNA-PK in HIV transcription. As a control, we used P80, a subunit of the transcription factor (TF) IIH complex, which plays an important role during the initial phase of HIV transcription (Kim et al., 2006). Nuclear factor kappa B (NF-κB), upon activation by TNF-α, induces the recruitment of TFIIH at the HIV promoter and enhances the phosphorylation of the RNAP II CTD through CDK7 kinase, another component of the TFIIH complex (Kim et al., 2006). Consistent with that observation, we detected the enrichment of P80 at the promoter region of LTR after TNF-α stimulation, thus confirming the validity of our assay conditions.

Analogous to the observations at the promoter region, at the nucleosome-2 region we found parallel enrichment in the levels of RNAP II and DNA-PK upon LTR transactivation by TNF-α. However, enhanced recruitment of P80 following TNF-α activation was not observed at the nucleosome-2 region, confirming our previous results that
TFIIH does not play any role during the elongation phase of transcription (Kim et al., 2006). Thus, these results provide direct evidence for the recruitment of DNA-PK at the HIV LTR during HIV transactivation.

**DNA-PK is a component of the RNAP II complex involved in HIV transcription**

In order to confirm the association between RNAP II and DNA-PK at the HIV provirus, we performed high-resolution ChIP assays to measure the distribution of both DNA-PK and RNAP II along the HIV provirus (Fig. 1c). In the absence of TNF-α stimulation, the latent proviruses carry both DNA-PK and RNAP II at extremely low levels. However, TNF-α activation for 3 h results in a sixfold increase in the amount of RNAP II at the promoter. The major part of the transcription complex was shifted to the downstream regions, as we detected even higher levels of RNAP II at the nucleosome-1 region of the LTR. The nucleosome-2 region, which is a marker of the elongation phase of transcription, also exhibits a fourfold increase in RNAP II levels. We also analysed several other downstream regions of the provirus, such as the 5′ and 3′ regions of the env gene, as well as a reporter GFP gene (approx. 4.5 kb downstream). We found notably higher levels of DNA-PK and RNAP II up to the 5′ region of the env gene (approx. 1.7 kb downstream), but lower levels further into the downstream regions. However, levels of both proteins remained significantly higher than on their latent counterparts, demonstrating ongoing transcriptional elongation. The parallel distribution pattern of both proteins along the HIV provirus clearly demonstrates the alliance between RNAP II and DNA-PK, thus validating our hypothesis that DNA-PK is part of a larger RNAP II transcription complex involved in HIV gene expression.

Furthermore, these results provide direct evidence for the enrichment of DNA-PK at the LTR during HIV transactivation. Thus, the parallel recruitment of DNA-PK and RNAP II at the HIV LTR clearly suggests an important role for DNA-PK in HIV gene expression.

**DNA-PK phosphorylates all three serine residues of CTD**

Phosphorylation of residue Ser-5 of the RNAP II CTD is linked with the initiation phase of HIV transcription (Kim et al., 2006).
et al., 2002; Yamamoto et al., 2001), whilst phosphorylation of Ser-2 of the CTD is found to be correlated with the elongation phase of HIV gene expression (Kim et al., 2006; Price, 2000; Saunders et al., 2006). Earlier reports that DNA-PK is a CTD kinase (Dvir et al., 1993; Trigon et al., 1998) and our finding that DNA-PK is present at both the promoter and downstream regions of the LTR (Fig. 1) clearly suggest its role during both the initiation and the elongation phases of transcription. We performed phosphorylation studies using synthetic peptides with various amounts of purified DNA-PK in order to establish the role of this protein complex during specific phases of HIV gene expression and to determine the exact target site(s) of DNA-PK phosphorylation. We synthesized three peptides containing two hepta repeats of CTD with double mutations of serine residues to alanine at positions 5 and 7 (Fig. 2a), positions 2 and 7 (Fig. 2b) or positions 2 and 5 (Fig. 2c). Phosphorylation reactions were performed; the phosphorylated peptides were separated by electrophoresis and quantified (see Methods).

Interestingly, DNA-PK was able to phosphorylate all three peptides. However, consistent with previous studies (Arias et al., 1991; Dvir et al., 1993; Trigon et al., 1998), serine residues at positions 2 (Fig. 2a) and 7 (Fig. 2c) of the hepta peptide were better substrates than serine 5 (Fig. 2b). These results suggest clearly that DNA-PK could be involved in both the initiation and elongation phases of transcription, albeit more significantly during elongation. The amount of CTD phosphorylation was directly proportional to the amounts of DNA-PK used. Utilizing DNA-PK purchased from Promega, we also observed some faint bands in control reactions without DNA, probably indicating a less pure enzyme preparation. Nevertheless, specific DNA-dependent CTD phosphorylation by DNA-PK is quite evident, with a clear dose-dependent outline. Taken together, our results demonstrate that DNA-PK interacts dynamically with the HIV LTR and suggest its role during both phases of transcription.

**DNA-PK plays a significant role in HIV gene expression**

In order to confirm the functional significance of the recruitment of DNA-PK to the HIV LTR and to understand its role in HIV gene expression, we performed knockdown experiments by using small hairpin RNA (shRNA) to knock down endogenous DNA-PK specifically (Fig. 3). Jurkat T cells were infected with lentiviral vectors carrying luciferase reporter genes either under the control of the HIV LTR promoter (pHR’P-Luc) or under an internal human cytomegalovirus (CMV) immediate-early promoter (pHR’P-SIN18-CMV-Luc), as negative control. These cells were then superinfected with lentiviral vectors expressing either shRNA sequences against DNA-PK or control scrambled shRNA sequences (Fig. 3a). In Fig. 3(b), in addition to lentiviral vectors expressing shRNAs, we also included lentiviral vectors expressing HIV Tat from the CMV promoter. Upon specific knockdown with shRNA against DNA-PK, the endogenous DNA-PK levels were reduced by >85% in all instances (Fig. 3a, b) and led to remarkable repression of HIV transcription.

By doing luciferase assays, we detected a clear dose-dependent inhibition of the HIV LTR promoter (Fig. 3a, lanes 2 and 3), whereas luciferase expression under the

![Fig. 2. DNA-PK phosphorylates all three serine residues of CTD. In vitro kinase assays using purified HeLa cell DNA-PK, purchased from Promega, were done in the absence (lane 1) or presence (lanes 2–4) of DNA. Reactions were performed using three peptides, each carrying two hepta repeats of CTD with mutations in serine residues as follows: (a) mutated in Ser-5 and -7; (b) mutated in Ser-2 and -7; (c) mutated in Ser-2 and -5. Reactions were performed in the presence of 1 μCi [γ-32P]ATP. The phosphorylated peptides were separated by electrophoresis using phosphate buffer–polyacrylamide gel at pH 6.0 (Trigon et al., 1998) and quantified by densitometry.](http://vir.sgmjournals.org)
CMV promoter was inhibited only weakly upon DNA-PK knockdown (lanes 5 and 6). Similar results were obtained when assays were done in the presence of Tat (Fig. 3b). As Tat is able to recruit CDK9 (another CTD kinase), in the presence of Tat we obtained a comparatively lower, but still significant impact of DNA-PK knockdown on HIV transcription (Fig. 3b). Considering that the CMV promoter was not affected due to reduced DNA-PK levels, whilst HIV gene expression was heavily reduced, these results clearly demonstrate the specific role of DNA-PK in HIV gene expression. Furthermore, this analysis also demonstrates that cellular levels of DNA-PK are high enough to contribute significantly to facilitating HIV transcription.

DNA-PK is required for efficient HIV replication

In order to demonstrate the requirement of DNA-PK for HIV replication, latently infected Jurkat J1.1 cells, which carry replication-competent HIV, were superinfected with lentiviral vectors carrying either shRNA sequences for DNA-PK, to deplete this kinase, or scrambled shRNA sequences as control. Thirty-six hours after this superinfection, cells were stimulated by treatment with TNF-α. Subsequently, after 51 h, the Gag p24 antigen was analysed in the supernatant by ELISA (Fig. 3c). We could detect fairly reduced levels of p24 in those cells expressing shRNA against DNA-PK. These data substantiate that loss of DNA-PK appreciably reduces HIV replication, and validate the importance of DNA-PK not only in HIV gene expression, but also in HIV replication.

Tat is a substrate for DNA-PK phosphorylation

As DNA-PK is known to interact directly with HIV Tat (Chun et al., 1998), we investigated whether the Tat protein is a substrate for DNA-PK and the potential effects of its phosphorylation state on HIV transcription. We analysed the amino acid sequence of Tat (HXB2, HIV-1 strain) and discovered three putative target sites of DNA-PK phosphorylation (Fig. 4a). In fact, each of the serine residues at positions 16, 62 and 75 is found to be followed by glutamine, a typical amino acid configuration of the most common target sites of DNA-PK (Bannister et al., 1993).

Considering that DNA-PK specifically requires DNA to phosphorylate its substrates, we employed DNase- and RNase-treated recombinant glutathione S-transferase (GST)–Tat protein, to eliminate possible nucleic acid contamination, in our phosphorylation assays (Ochem et al., 2008). As shown in Fig. 4(b), Tat is an efficient substrate for DNA-PK in the presence of DNA, both when

**Fig. 3.** Knockdown of endogenous DNA-PK restricts HIV gene expression and replication in Jurkat T cells. (a) Jurkat cells were infected with the indicated m.o.i. of lentiviral vectors expressing shRNA from the Pol III promoter, either against DNA-PK (lanes 2, 3, 5 and 6) or control scrambled shRNA (lanes 1 and 4). Twenty-four hours later, cells were superinfected with lentiviral vectors expressing a luciferase gene under the control of either the LTR promoter (lanes 1–3) or the CMV promoter (lanes 4–6). (b) Jurkat cells were coinfected with lentiviruses expressing Tat under the control of the CMV promoter (lanes 2–4) alongside two different m.o.i.s of lentiviral vectors expressing either shRNA against DNA-PK (lanes 3 and 4) or control scrambled shRNA (lane 2). After 24 h, cells were superinfected with lentiviruses expressing a luciferase gene under the control of the HIV LTR promoter (lanes 1–4). (c) Latently infected Jurkat J1.1 T cells were superinfected with different m.o.i.s of lentiviral vectors expressing either shRNA against DNA-PK (lanes 4 and 5) or control scrambled shRNA (lane 3). After 36 h, cells were treated with TNF-α (lanes 2–5). Gag (p24) production in the supernatants of cells was measured by ELISA after 51 h. The data represent the results of three separate experiments; bars indicate SD.
peptides, each 20 aa long, spanning the entire Tat sequence and overlapping by 10 aa. Phosphorylation assays were carried out in the presence or absence of DNA, and the results are shown in Fig. 5. The peptides encompassing the serine residues at positions 16 or 62 were phosphorylated by DNA-PK in the presence of DNA (lanes 2, 4, 12 and 13), whereas the peptide containing the DNA-PK target site at position 75 was not (lane 16). Thus, our results demonstrate that, of three putative sites, only two are actually functional, which is not surprising, as a similar pattern of DNA-PK phosphorylation has also been observed for other proteins (Anderson & Lees-Miller, 1992).

**Physiological significance of Tat phosphorylation by DNA-PK**

Serine at position 62 lies immediately adjacent to the basic region of Tat, suggesting that phosphorylation of Tat at this site could modulate its HIV-1 LTR promoter transactivation activity in different ways. These include by affecting its interaction with TAR sequences, inducing aberration in its nuclear localization (Karn, 1999; Tyagi et al., 2001), or both. The serine residue at position 16 lies in the N-terminal domain, which is also an essential domain for efficient Tat transactivation activity (Karn, 1999), further suggesting the impact of Tat phosphorylation on HIV gene expression. The wild-type and mutated GST–Tat proteins were used in a standard LTR transactivation assay using HL3T1 cells, a HeLa fibroblast-based cell line harbouring an integrated LTR–CAT construct. We generated Tat constructs mutated from serine to alanine at either position 16 or 62, or at both positions. As shown in Fig. 6(a), all three mutant proteins were modestly impaired in LTR transactivation compared with wild-type Tat.

To confirm the functional significance, further similar experiments were performed in Jurkat-pHRluc cells, a T-cell line (a natural HIV target cell line) carrying HIV provirus with luciferase as reporter (Kim et al., 2006; Tyagi & Karn, 2007). Cells were transfected with 100 ng of plasmids expressing different Tat constructs (mutant

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**Serine residues at positions 16 and 62 are the functional target sites for DNA-PK phosphorylation**

To map the position(s) of the phosphorylation site(s) in the HIV-1 Tat protein precisely, we utilized a set of eight peptides, each 20 aa long, spanning the entire Tat sequence and overlapping by 10 aa. Phosphorylation assays were carried out in the presence or absence of DNA, and the results are shown in Fig. 5. The peptides encompassing the serine residues at positions 16 or 62 were phosphorylated by DNA-PK in the presence of DNA (lanes 2, 4, 12 and 13), whereas the peptide containing the DNA-PK target site at position 75 was not (lane 16). Thus, our results demonstrate that, of three putative sites, only two are actually functional, which is not surprising, as a similar pattern of DNA-PK phosphorylation has also been observed for other proteins (Anderson & Lees-Miller, 1992).

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To confirm the functional significance, further similar experiments were performed in Jurkat-pHRluc cells, a T-cell line (a natural HIV target cell line) carrying HIV provirus with luciferase as reporter (Kim et al., 2006; Tyagi & Karn, 2007). Cells were transfected with 100 ng of plasmids expressing different Tat constructs (mutant
or wild-type) under control of the CMV promoter (Fig. 6b). The graph shows the luciferase activity obtained from duplicate experiments, repeated three times. As expected, similar to the CAT assays (Fig. 6a), the Tat mutants clearly demonstrated modestly impaired transactivation capability. Nevertheless, this effect was reproducible in different experiments, as evidenced by short error bars. Hence, comparable results were obtained when Tat was expressed endogenously (Fig. 6b) or provided exogenously (Fig. 6a).

Altogether, our results show clearly that DNA-PK facilitates HIV-1 transcription essentially by phosphorylating the RNAP II CTD, and that Tat phosphorylation also contributes significantly to HIV replication.

**DISCUSSION**

In this study, we have demonstrated for the first time the recruitment of DNA-PK at the HIV LTR. The parallel recruitment of both DNA-PK and RNAP II at the HIV LTR demonstrates clearly that DNA-PK is an integral part of a larger RNAP II complex assembled at the HIV LTR. Our observation is supported by another study that also reported similar conclusions for another gene (An et al., 2011). Although the precise role of DNA-PK in the RNAP II complex recruited at the LTR remains the subject of our future, more detailed investigation, the results obtained in this study represent the elucidation of a potentially novel mechanism underlying HIV-1 gene expression.

The observations that DNA-PKcs and Ku (p70/p80) are components of an RNAP II holoenzyme (Maldonado et al., 1996) encouraged us to determine whether, along with RNAP II, DNA-PK is also present in its functional holoenzyme form at the HIV LTR. As expected, levels of RNAP II at the latent provirus were highly restricted but, upon proviral reactivation by TNF-α treatments, the levels of RNAP II were increased severalfold, suggesting the induction of HIV gene expression (Fig. 1a). Interestingly, alongside this increase, the levels of DNA-PK also rose in parallel upon TNF-α activation. Furthermore, our analysis of DNA-PK levels at different LTR regions, such as the promoter region, where recruitment identifies factors involved in transcriptional initiation, and the nucleosome-2 region, which indicates factors involved in transcriptional elongation, revealed similar increases in the levels of DNA-PK at both regions following TNF-α stimulation. Thus, these data argue strongly for the involvement of DNA-PK during both the initiation and the elongation phases of transcription. In addition, the observed enrichment of DNA-PK at the LTR during HIV transcriptional activation clearly suggests its important role in HIV transcription.

By running high-resolution ChIP assays, we found a parallel distribution of both RNAP II and DNA-PK along the HIV provirus both before and after reactivation of latent provirus (Fig. 1b). In addition, we also found their association during both the initiation and the elongation phases of transcription. Thus, our results confirm that DNA-PK is a component of a larger RNAP II complex that
is involved in both the initiation and the elongation phases of HIV transcription.

Furthermore, our analysis of the phosphorylation pattern of the CTD by DNA-PK unravelled the facilitatory roles of this kinase complex in HIV transcription. Phosphorylation of Ser-5 at the RNAP II CTD is linked to transcriptional initiation (Kim et al., 2002; Yamamoto et al., 2001), whilst Ser-2 hyperphosphorylation is found to be correlated with the elongation phase of transcription (Kim et al., 2006; Price, 2000; Saunders et al., 2006). The CTD of RNAP II is a known phosphorylation target of several kinases, including DNA-PK, and it is also known that knockdown of any individual kinase does not confer complete aberration of RNAP II function (Trigon et al., 1998; Zhang & Corden, 1991). Phosphorylation of the CTD by DNA-PK in the absence of the kinase consensus target sequences appears to be in line with previous studies reporting that certain proteins can be phosphorylated at other sites by this DNA-dependent protein kinase (Anderson & Lees-Miller, 1992). Although phosphorylation of the serine residues at positions 2 and 5 of the CTD by DNA-PK emphasizes its role during both the initiation and the elongation phases of transcription, the preferential phosphorylation of the residue in position 2 over that in position 5 clearly suggests a more prominent role for the kinase during elongation. These results are also supported by similar observations in the context of other cellular promoters (Arias et al., 1991), where the presence of DNA-PK in pre-initiation complexes and resultant CTD phosphorylation have been reported.

Our results from employing shRNA to deplete endogenous DNA-PK clearly define the physiological relevance of DNA-PK in both HIV gene expression and replication. In fact, upon DNA-PK knockdown, remarkably we found not only a significant dose-dependent specific reduction in HIV gene expression, but also a parallel reduction in HIV replication. Furthermore, the shRNA knockdown experiments demonstrated clearly that cellular levels of DNA-PK are sufficiently high to contribute significantly to facilitating HIV replication.

Overall, these results demonstrate that DNA-PK is an integral part of RNAP II complexes recruited at the HIV LTR and that DNA-PK plays significant roles in HIV replication.

Prompted by an earlier observation that Tat forms direct contacts with DNA-PK (Chun et al., 1998), we explored the possibility of its phosphorylation by DNA-PK. Interestingly, upon sequence analysis of the Tat protein from the HXB2 strain of HIV-1, we discovered three preferred DNA-PK target sites. The results of our subsequent phosphorylation assays (shown in Figs 4 and 5) demonstrate clearly that HIV Tat is a bona fide substrate of DNA-PK. As other authors have also observed, DNA-PK does not always recognize all sequences conforming to the target consensus sequences (reviewed by Smith & Jackson, 1999). We believe that the arginine residues of the neighbouring basic domain of Tat might adversely affect the phosphorylation process at position 75 by DNA-PK. Previously, positively charged amino acids have been suggested to negatively affect DNA-PK recognition on neighbouring serines or threonines (Bannister et al., 1993), probably due to conformational constraints. By analogy with the observation made by the Nekhai group (Ammosova et al., 2006), protein sequence analyses revealed that the serine residues at positions 16 and 62 are fairly conserved among Tat proteins from different HIV-1 isolates, but the same is not true in the case of HIV-2 and simian immunodeficiency virus isolates.

We determined the physiological relevance of Tat phosphorylation by DNA-PK in HIV transcription by comparing the transactivation capabilities of wild-type Tat and of Tat mutants carrying alanine instead of serine residues. The modestly, but reproducibly lower HIV transactivation activity of mutant Tat proteins, provided either exogenously (Fig. 6a) or expressed endogenously (Fig. 6b), clearly demonstrates the wider role of DNA-PK in regulating HIV gene expression and further reinforces the role of Tat phosphorylation in HIV transactivation. However, it should be noted that the only phenotype so far studied for these mutants has been LTR transactivation. Understanding the possible extent to which these mutations could interfere with other, non-transcriptional functions of the Tat protein, including its neurotoxicity, would require further investigation.

Interestingly, DNA-PK is not the only protein kinase able to phosphorylate the Tat protein; at least three other kinases [CDK2, protein kinase C (PKC) and the interferon-induced dsRNA-activated kinase (PKR)] have also been shown to phosphorylate HIV Tat (Ammosova et al., 2006; Brand et al., 1997; Endo-Munoz et al., 2005; Holmes, 1996). In addition, the Nekhai group has also confirmed the presence of the phosphorylated form of Tat inside cells (Ammosova et al., 2006).

Altogether, our results demonstrate that DNA-PK is an integral part of RNAP II complexes recruited at the HIV LTR and is able to facilitate both the initiation phase and (primarily) the elongation phase of HIV transcription by phosphorylating both Ser-2 and Ser-5, but mainly Ser-2 residues of the CTD of RNAP II. The influence of this protein kinase complex on initiation and elongation is also facilitated significantly by phosphorylation of the Tat protein by DNA-PK.

These results are supported by a recent study (An et al., 2011) that reported the interaction of DNA-PK with cyclin T2, a subunit of P-TEFb, during transcription-coupled repair in the dihydrofolate reductase gene and suggested that this repair pathway could be a general feature of normal transcription. Furthermore, the Rice laboratory has also reported the interaction of Ku with CDK9, another subunit of P-TEFb (Liu et al., 2010). CTD phosphorylation induced by P-TEFb is well recognized to play an important role during HIV transcriptional elongation. Clearly, these
observations suggest an additional role of DNA-PK in HIV transcription, probably by regulating P-TEFb recruitment. Thus these observations substantiate our results and suggest that, besides modulating CTD phosphorylation, Tat phosphorylation and P-TEFb recruitment, DNA-PK also facilitates HIV replication by supporting transcription-coupled repair during HIV gene expression.

Finally, the successful uncovering of the different pathways that significantly affect HIV replication may provide additional important and valuable targets for potential drug molecules in the search and development of novel anti-HIV-1 therapeutics.

METHODS

Cells and transfection. HL3T1 cells, kindly donated by B. Felber (Felber & Pavlakis, 1988), are a HeLa-derived cell line containing an integrated LTR–CAT construct. HL3T1 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen); Jurkat cells were maintained in RPMI medium (Invitrogen). Both media were supplemented with 10% FCS, 2 mM glutamine and 50 μg gentamicin ml⁻¹.

Cells were transfected by standard procedures. All transfections were adjusted to the same amount of DNA by compensating with empty plasmid. CAT and luciferase assays were performed 48 h after transductions or transfections. The corresponding results represent the mean values obtained in several (at least three) independent experiments.

HIV replication assays. Latently infected J1.1 cells (2 x 10⁶) were superinfected with vesicular stomatitis virus G protein-pseudotyped lentiviruses carrying either shRNA sequences against DNA-PK or scrambled control shRNA sequences. After 36 h superinfection, cells were treated in triplicate with 10 ng TNF-α ml⁻¹ and incubated for an additional 51 h. Subsequently, virus levels in the supernatant were determined by measuring the HIV-1 p24 Gag protein by ELISA, following the protocol suggested by the manufacturer (ZeptoMetrix).

Preparation of Tat mutants. The Tat mutants employed in the experiments were generated by PCR using mutated primers, and the presence of the desired mutation at the appropriate site was confirmed by sequencing. These mutants were then cloned either in the pcDNA3 vector (Invitrogen) to express Tat proteins or in the pGEX-2T vector (GE Healthcare) to generate GST–Tat fusion proteins or in the pGEX-2T vector (GE Healthcare) to generate GST–Tat fusion products. Association with transcriptional initiation.

ChiP assays. Quantitative ChiP assays were carried out using a standard protocol (Kim et al., 2006; Pearson et al., 2008; Tyagi & Karn, 2007; Tyagi et al., 2010). The ChiP assays were performed before and after activating a latently infected E4 Jurkat cell line with 10 ng TNF-α ml⁻¹ for either 30 min or 3 h. Most of the antibodies used were purchased from Santa Cruz Biotechnology unless otherwise mentioned. On every run, 5% of each sample was analysed by quantitative real-time PCR to determine the amount of sample immunoprecipitated by each individual antibody, using different primer sets to examine >4000 nt of proviral genome. The reading obtained with pre-immune serum was subtracted from each sample value as background counts.

DNA-PK phosphorylation assay. DNA-PK was either purified using our published protocol (Ochem et al., 2008) or purchased from Promega. Reaction mixtures were prepared by mixing the protein substrates with 1 ng DNA and 1 μl [γ-32P]ATP (Amersham Biosciences; 3000 Ci (111 TBq) mM⁻¹; 10 mCi (370 MBq) ml⁻¹] in a DNA-PK reaction buffer (Gottlieb & Jackson, 1993). Phosphorylation was initiated by adding either 1 μg (in-house-purified) or 200–400 U (commercial; Promega) DNA-PK to the reaction mixtures. Control reactions were carried out in the absence of DNA. Reaction mixtures were incubated for 1 h at 37 °C and the reaction was stopped by boiling in denaturing SDS-PAGE gel loading buffer. The phosphorylated products were analysed by electrophoresis either on a 4–20% gradient Tris/glycine SDS-PAGE gel or on phosphate buffer–polyacrylamide gel at pH 6.0 (Trigon et al., 1998). Protein phosphorylation was quantified by densitometry.

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REFERENCES


