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Mutation of the major 5′ splice site renders a CMV-driven HIV-1 proviral clone Tat-dependent: connections between transcription and splicing

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Abstract Efficient transcription from the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR) promoter is dependent on the viral transactivator Tat. To generate a Tat-independent proviral plasmid, we replaced the promoter in the HIV-1 LTR with the immediate early promoter of cytomegalovirus. Transfection of this plasmid yielded Tat-independent production of infectious HIV-1. Tat-independent expression was lost, however, when the major 5′ splice site in the HIV-1 genome was mutated and no HIV-1-specific RNA or protein was detected. This defect was restored when a Tat expression plasmid was cotransfected. Our results support recent reports indicating an influence of the recognition of splice sites on efficient transcriptional elongation.

Key words: Human immunodeficiency virus; Transcription; Splicing; Cytomegalovirus promoter; Tat

1. Introduction

Cellular homeostasis requires balanced gene expression which is partly regulated at the level of mRNA transcription. Transcriptional control occurs at the level of initiation, but also at the level of elongation as shown by the isolation of positive and negative elongation factors [1]. An early example for transcriptional control by regulating elongation has been human immunodeficiency virus (HIV) type 1. After infection of a susceptible cell, the RNA genome of HIV-1 is reverse-transcribed into DNA and subsequently integrated into the cellular genome. The integrated provirus is transcribed by cellular RNA polymerase II (pol II), yielding a single primary transcript of approximately 9 kb [2]. At early times of infection, however, very few full-length transcripts are synthesized due to non-processive pol II complexes initiating from the viral promoter [3]. These transcripts are completely spliced leading to translation of the viral transactivator Tat [4]. The Tat protein binds to a unique RNA structure termed TAR (transactivation response element) at the 5′ end of all HIV-1 transcripts [5]. This complex then recruits cyclin T1 and the associated kinase CDK9 which are part of the cellular elongation factor pTEFb [6]. Subsequently, CDK9 phosphorylates the C-terminal domain (CTD) of RNA pol II, thus generating highly processive elongation complexes and stimulating HIV-1 transcription by a factor of up to 100 [7].

The molecular mechanism of Tat function has been elucidated in recent years, but it is still largely unknown why initiation at the HIV-1 long terminal repeat (LTR) promoter gives rise to non-processive RNA pol II complexes. Clearly, HIV-1 gene products can be produced independent of Tat when placed under control of a heterologous, Tat-independent promoter, e.g. the immediate early promoter of cytomegalovirus (CMV). To generate a Tat-independent proviral plasmid, we placed the HIV-1 transcription unit under control of the CMV promoter. Here, we show that the CMV-driven proviral plasmid leads to efficient Tat-independent production of infectious HIV-1. However, Tat-independent gene expression was lost when a mutation was introduced into the major 5′ splice site (5′ss) of HIV-1. This result supports other reports indicating that recognition of 5′ss may regulate transcriptional elongation.

2. Materials and methods

2.1. Plasmids

To replace the U3 region in the 5′ LTR of the proviral HIV-1 plasmid pNL4-3 [8] by the CMV promoter, an overlap polymerase chain reaction (PCR) was performed. The CMV promoter was amplified from pC1-green fluorescent protein (GFP) (Clontech) using primers 5′CMV+ (GTG AAC CGG GTC TCT CTG GTT AGA CCA GAT CTG AGC C), introducing a unique *Bst*1107I site and 3′CMV+ overlap (GGT CTA ACC AGA GAG ACC CGG TTC ACT AAA CCA GCT CTG), containing 10 nucleotides at the 5′ end which are complementary to the transcription start site of NL4-3 (nt 451). The HIV-1-specific sequence was amplified from pNL4-3 using primers 5′CMV overlap (CGG AGT ATA CTA GTT ATT AAT AGT AAT CAA TTA CG) and RW MAR2 (CCC TTT CTT TGT TAT A). Both products were re-amplified with primers 5′CMV+ and RW MAR2. The product was cloned into a derivative of pNL4-3 containing a silent *Ngo*MI site immediately adjacent to the major 5′ss [9]. The PCR-derived region in the resulting plasmid, termed pNLC4-3, was confirmed by sequence analysis. To construct a *tat*/*rev*-negative derivative, the *Eco*RI/*Nhe*I fragment from a *tat*/*rev*-negative subgenomic HIV-1 plasmid (kindly provided by H. Schaal, Düsseldorf, Germany) was transferred into pNLC4-3 to yield pNLC4-3^{tr-}. To introduce the M3 mutation into the major 5′ss, the *Bss*HII/*Eco*RI fragment of pNL4-3^{tr-}M3 [9] was transferred into pNLC4-3^{tr-}.

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Abbreviations: HIV, human immunodeficiency virus; CMV, cytomegalovirus; LTR, long terminal repeat; Tat, transactivator of transcription; TAR, transactivation response element; pol II, RNA polymerase II; ss, splice site; CTD, C-terminal domain

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2.2. Cells and transfection

HeLa P4 and 293T cells were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Sup-T1 cells were cultivated in RPMI 1640 medium with the same additives. For transient transfection, 5×10^5 HeLa P4 or 293T cells were seeded and transfected with 10 μg of the respective plasmid and 1 μg of a reporter construct encoding GFP using the modified calcium phosphate coprecipitation technique [10]. To assess transfection efficiency, coverslips were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline and fluorescence-positive cells were counted. For transfection of Sup-T1 cells, 5×10^6 cells were electroporated with a Bio-Rad gene pulser in the presence of 15 μg DNA using 250 V and 960 μF. Cells electroporated with a GFP expression plasmid served as transfection controls and were analyzed by fluorescence-associated cell sorting to determine transfection efficiency.

2.3. Western blot analysis

Cells were harvested at 60 h post transfection. Cell lysates were normalized for transfection efficiency and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting using antisera against HIV-1 capsid (CA; 1:10 000), Env (1:5000, kindly provided by V. Bosch, Heidelberg, Germany) or Nef proteins (1:5000) and an antibody directed against Erk2 as a loading control (Santa Cruz, Santa Cruz, CA, USA) and peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany). Enhanced chemiluminescence (Amersham) was used for detection according to the manufacturer's protocol. Blots were exposed to Kodak Biomax films.

2.4. RNA preparation and analysis

Preparation of total RNA, gel electrophoresis, blotting and detection with a radiolabeled probe were performed as described [11]. Blots were washed, sealed and exposed to X-ray films (Kodak X-omat-AR) or quantified by phosphoimage analysis (Bio-Rad).

2.5. Virus titration

Filtered supernatants from transfected 293T cells were analyzed for HIV-1 CA antigen by enzyme-linked immunosorbent assay and virus inocula corresponding to 0.1–100 ng CA antigen were used to infect HeLa P4 cells. After 60 h, cells were fixed and HIV-1-infected cells were detected by immunostaining with a monoclonal antibody against CA and a β-galactosidase-conjugated goat anti-mouse secondary antibody.

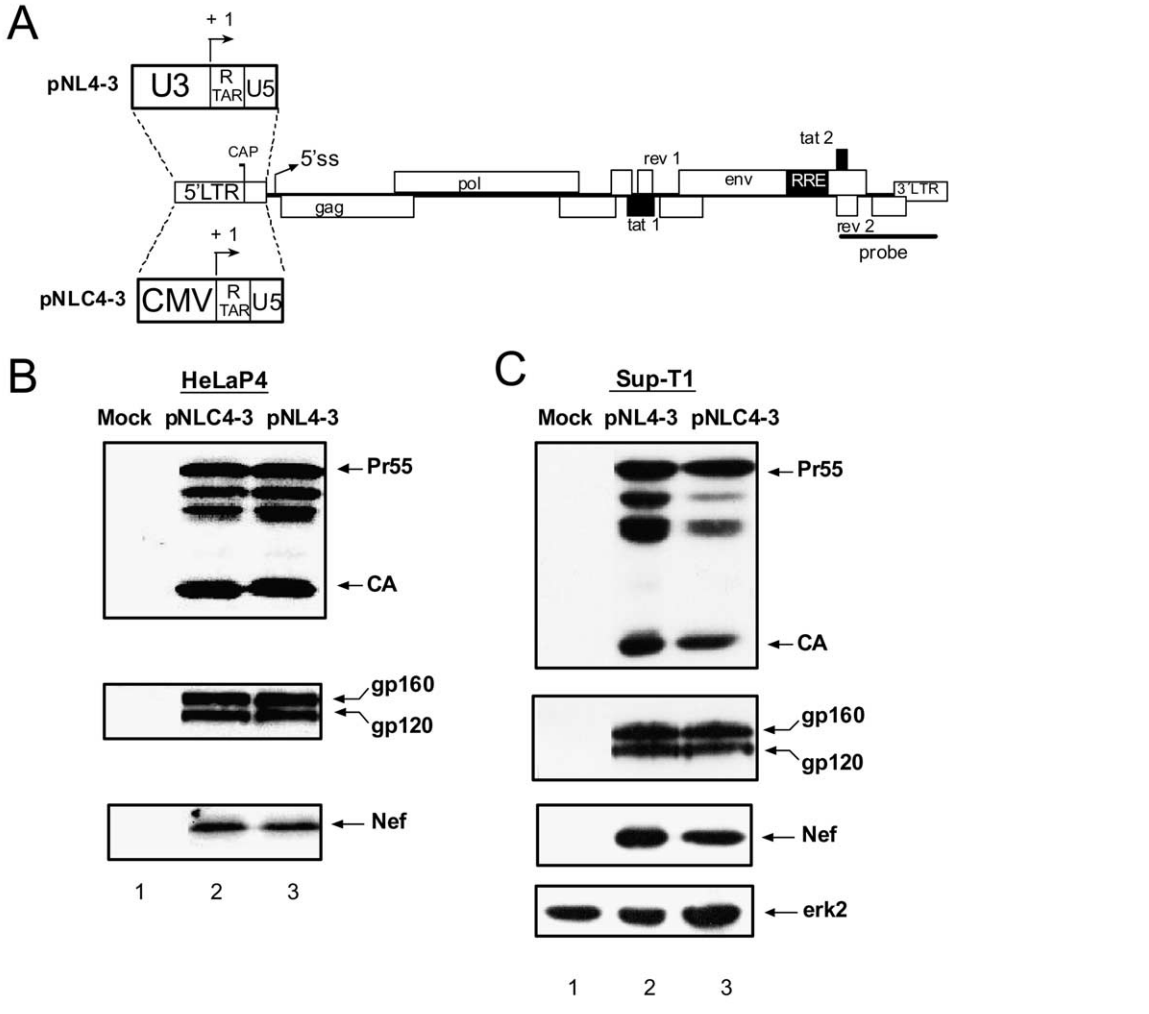

Fig. 1. CMV-driven HIV-1 expression. A: Schematic depiction of the HIV-1 proviral plasmid pNL4-3 (middle); the wild-type LTR region (pNL4-3, top) and the CMV-containing LTR region (pNLC4-3, bottom) are expanded. The positions of the *gag*, *env*, *rev*, and *tat* genes are indicated. The transcriptional start site (+1), the major 5′ss and the region detected by the probe used for Northern blotting are shown. B: Immunoblot analysis of HeLa P4 cells mock-transfected (lane 1) or transfected with 10 μg pNLC4-3 (lane 2) or pNL4-3 (lane 3). Equal loading was checked by staining the blot with Ponceau S. HIV-1-specific proteins were detected with antiserum against CA (top), gp120 (middle) or Nef (bottom). Relevant HIV-specific proteins are identified on the right. C: Immunoblot analysis of Sup-T1 cells mock-transfected (lane 1) or transfected with 15 μg of pNL4-3 (lane 2) or pNLC4-3 (lane 3). Analysis was as in Fig. 2B, detection with antibodies against Erk2 is included as a loading control.

3. Results

3.1. A CMV-driven HIV-1 proviral plasmid yields production of infectious HIV-1

Efficient transcription from the HIV-1 5′ LTR promoter is completely dependent on the viral transactivator protein Tat. In order to generate a Tat-independent proviral plasmid, we replaced the U3 region in the HIV-1 5′ LTR, which contains the viral promoter and enhancer, with the immediate early promoter of CMV. In the resulting plasmid pNLC4-3, the transcription start site of the HIV-1 genome is placed directly adjacent to the CMV promoter such that the resulting transcript is identical to the HIV-1 genomic RNA (Fig. 1A). Transfection of pNLC4-3 is expected to yield infectious virus, since the U3 region will be regenerated upon reverse transcription in the newly infected cell [2] and the integrated proviral DNA should be identical with that obtained after wild-type HIV-1 infection.

Immunoblot analysis of HeLa P4 cells transfected with pNL4-3 or pNLC4-3 showed a very similar pattern of protein expression in both cases (Fig. 1B). Western blots stained with antisera detecting the HIV-1 CA protein revealed equal expression levels for CA, the Gag precursor protein Pr55 and intermediate cleavage products in pNLC4-3- and pNL4-3-transfected cells, respectively (Fig. 1B, upper panel, lanes 2 and 3). Analysis with antisera against the viral envelope proteins (middle panel) and the HIV-1 accessory protein Nef (lower panel) showed equal expression for the glycoproteins gp160 and gp120 and for the Nef protein as well. To test the relative infectivity of particles produced from pNLC4-3-trans-

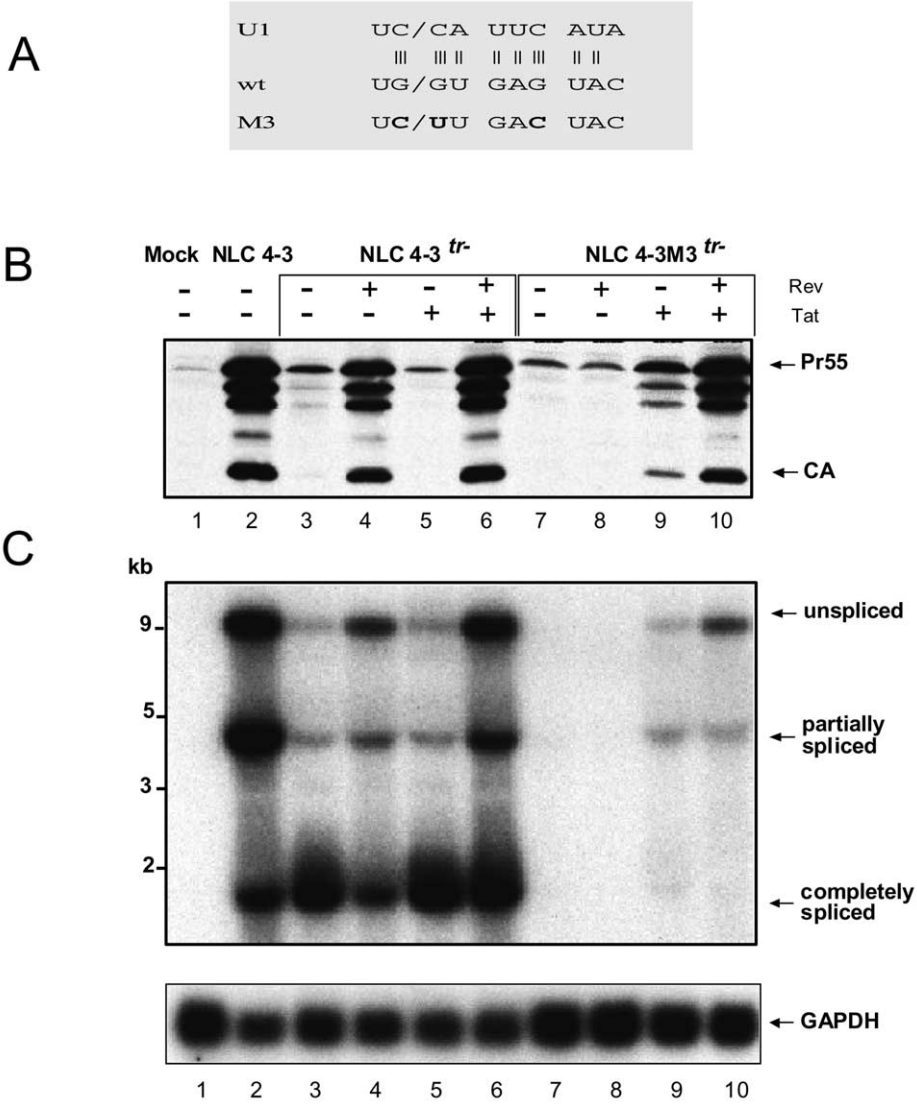

Fig. 2. Analysis of the Tat and Rev dependence of HIV-1 gene expression from pNLC4-3 and a derivative with a mutation in the major 5′ss. A: The sequence of the HIV-1 major 5′ss is shown in the middle and the sequence of the M3 mutant at the bottom. Altered nucleotides are in bold. The complementary region of U1 snRNA is shown in the top line and hydrogen bonds with the 5′ss are depicted as vertical lines. The slash indicates the exon/intron junction. B: Immunoblot analysis of HeLa P4 cells mock-transfected (lane 1) or transfected with plasmids pNLC4-3 (lane 2), pNLC4-3^{tr-} (lanes 3–6) or pNLC4-3^{tr-}M3 (lanes 7–10) carrying the M3 mutation in the 5′ss. Expression vectors for HIV-1 Tat and Rev were cotransfected as indicated above each lane. Equal loading was checked by staining the blot with Ponceau S. HIV-1 Gag proteins were detected with antiserum against CA. C: Northern blot analysis of cells derived from the same transfection experiment as in B. HIV-1-specific RNA was detected with a labeled probe against the 3′ LTR (indicated in Fig. 1A). The blot was reprobed for GAPDH as loading control (lower panel). Lanes are as in B.

fected cells, 293T cells were transfected with pNL4-3 or pNLC4-3 in triplicate and filtered media were tested for HIV-1 antigen release and for infectious titer on HeLa P4 cells. Media from pNLC4-3-transfected cells contained slightly higher antigen levels than media from pNL4-3-transfected cells (821 ng versus 610 ng CA/ml), while their infectious titer was approximately twofold lower (55 infectious units versus 132 infectious units per ng CA). To assess the expression levels in a HIV-infectable T cell line, Sup-T1 cells were transfected with pNL4-3 and pNLC4-3, respectively, and viral protein expression was analyzed by immunoblot (Fig. 2C). Similar to the result observed for HeLa P4 cells, expression levels of the Gag, Env and Nef proteins were approximately equal in both cases. Thus, transfection of the CMV-driven HIV-1 proviral plasmid yielded efficient release of infectious HIV-1 particles and produced an expression pattern virtually indistinguishable from wild-type virus.

3.2. *HIV-1 expression from pNLC4-3 is Tat-independent*

Following transfection of pNLC4-3 endogenous Tat is produced. To determine whether transcription from pNLC4-3 is Tat-independent, we constructed plasmids pNL4-3^{tr-} and pNLC4-3^{tr-}, which carry mutations in the start codons of the *rev* and *tat* genes. Accordingly, expression from the LTR-driven proviral clone pNL4-3^{tr-} was completely dependent on cotransfection of expression vectors for Tat and Rev (data not shown). In the case of pNLC4-3^{tr-}, on the other hand, only cotransfection of a Rev but not of a Tat expression plasmid is required. In the absence of Rev, only trace amounts of Pr55 were detected (Fig. 2B, lane 3), while cotransfection of a Rev expression plasmid largely restored production of HIV-1-specific proteins (Fig. 2B, lane 4). Cotransfection of a Tat expression vector in the absence of Rev had no effect (Fig. 2B, lane 5), while cotransfection of Rev and Tat expression plasmids stimulated Gag expression twofold compared to cotransfecting only a Rev expression vector (Fig. 2B, lane 6). A similar picture was seen when total RNA from the same transfection experiment was analyzed by Northern blot using a probe specific for the HIV-1 3′ LTR (Fig. 2C). This probe recognizes all three classes of HIV-1-specific RNA. Following transfection of pNLC4-3, the unspliced genomic RNA (ca. 9 kb), partially spliced RNAs (ca. 4 kb), and completely spliced RNAs (ca. 2 kb) were observed (Fig. 2C, lane 2). This result was very similar to that observed for wild-type pNL4-3-transfected cells (data not shown). In the absence of Rev, the amount of multiply spliced RNA was strongly increased at the expense of unspliced and singly spliced RNA (Fig. 2B, lanes 3 and 5). Importantly, however, efficient RNA production was seen in all cases, independent of the presence or absence of Tat (Fig. 2B, lanes 3–6). Again, cotransfection of a Tat expression plasmid stimulated RNA production approximately twofold as determined by phosphoimage analysis of the respective products normalized for GAPDH levels. Thus, transcription of HIV-1 genomic RNA from pNLC4-3 does not require Tat but is slightly stimulated by Tat.

3.3. *Mutation of the major 5′ss of HIV-1 renders pNLC4-3 Tat-dependent*

In another study we had shown that mutating the major 5′ss in the proviral plasmid pNL4-3 led to partially Rev-independent expression of the HIV-1 structural proteins [9]. The major 5′ss located upstream of the *gag* open reading frame (Fig. 1A) was inactivated by three point mutations, reducing the number of hydrogen bonds between the 5′ss and U1 snRNA (Fig. 2A). In addition, the GU dinucleotide as well as a cryptic 5′ss four nucleotides downstream were eliminated. Transfection of the proviral plasmid pNL4-3^{tr-}M3 led to production of genomic RNA without further splicing and to partially Rev-independent Gag expression [9]. A very different picture was observed when the same M3 mutation in the major 5′ss was analyzed in the context of the CMV-driven plasmid pNLC4-3^{tr-}. Virtually no Gag expression and no HIV-1-specific RNA was observed following transfection of pNLC4-3^{tr-}M3, both in the absence (Fig. 2B,C, lanes 7) and in the presence (Fig. 2B,C, lanes 8) of Rev. Cotransfection of a Tat expression plasmid together with pNLC4-3^{tr-}M3 and a Rev expression plasmid, on the other hand, restored production of HIV-1 genomic RNA (Fig. 2C, lane 10) and of viral Gag proteins (Fig. 2B, lane 10). Furthermore, cotransfection of a Tat expression vector partially restored genomic RNA and Gag protein expression even in the absence of Rev (Fig. 2B,C, lanes 9).

4. Discussion

In this report, we have shown that infectious HIV-1 can be produced from a proviral plasmid containing a heterologous promoter independent of Tat only when the major 5′ss in the 5′ untranslated region of the genome is retained. Mutation of this 5′ss renders transcription Tat-dependent with no specific RNA detected in the absence of Tat. Thus, in this context, transcription from the CMV promoter requires either a functional 5′ss or the stimulatory effects of Tat for elongation. This result raises the question how a signal for RNA processing may influence transcriptional elongation. It is generally accepted that splicing occurs cotranscriptionally [12,13], and splicing factors associate with the nascent transcript immediately after the RNA emerges from the polymerase [14]. In the presence of Tat, cyclin T1 and CDK9 co-assemble with Tat on the TAR region of the nascent RNA, thus facilitating hyperphosphorylation of the pol II CTD. The CTD of cyclin T1 also binds the protein Tat-SF1 [15], which is believed to be a general elongation factor. Interestingly, Tat-SF1 shows homologies to the yeast splicing factor CUS2, and Fong and Zhou showed that Tat-SF1 links the splicing machinery to transcription via cyclin T1 [16]. One may speculate, therefore, that Tat-SF1 may be recruited to the major 5′ss of HIV-1 leading to the further recruitment of cyclin T1 and CDK9 to TAR and to Tat-independent expression of HIV-1 from the CMV promoter (Fig. 3A). If a Tat expression vector is cotransfected, elongation is further stimulated by a factor of 2 due to the direct effects of Tat (Fig. 3B). If the major 5′ss is mutated, however, Tat-SF1 or other factors cannot be recruited and elongation-competent complexes are not formed (Fig. 3C). In the presence of Tat, on the other hand, co-assembly of Tat, cyclin T1 and CDK9 on the TAR region and thus elongation is restored (Fig. 3D).

Tat and the major 5′ss appear to perform redundant functions in the CMV-driven HIV-1 proviral plasmid, leading to the question whether expression from the CMV promoter needs associated 5′ss also in other cases and why the HIV-1 promoter is Tat-dependent even when a functional 5′ss is present. The group of Proudfoot observed that a mutation of a 5′ss close to the CMV promoter in a synthetic minigene

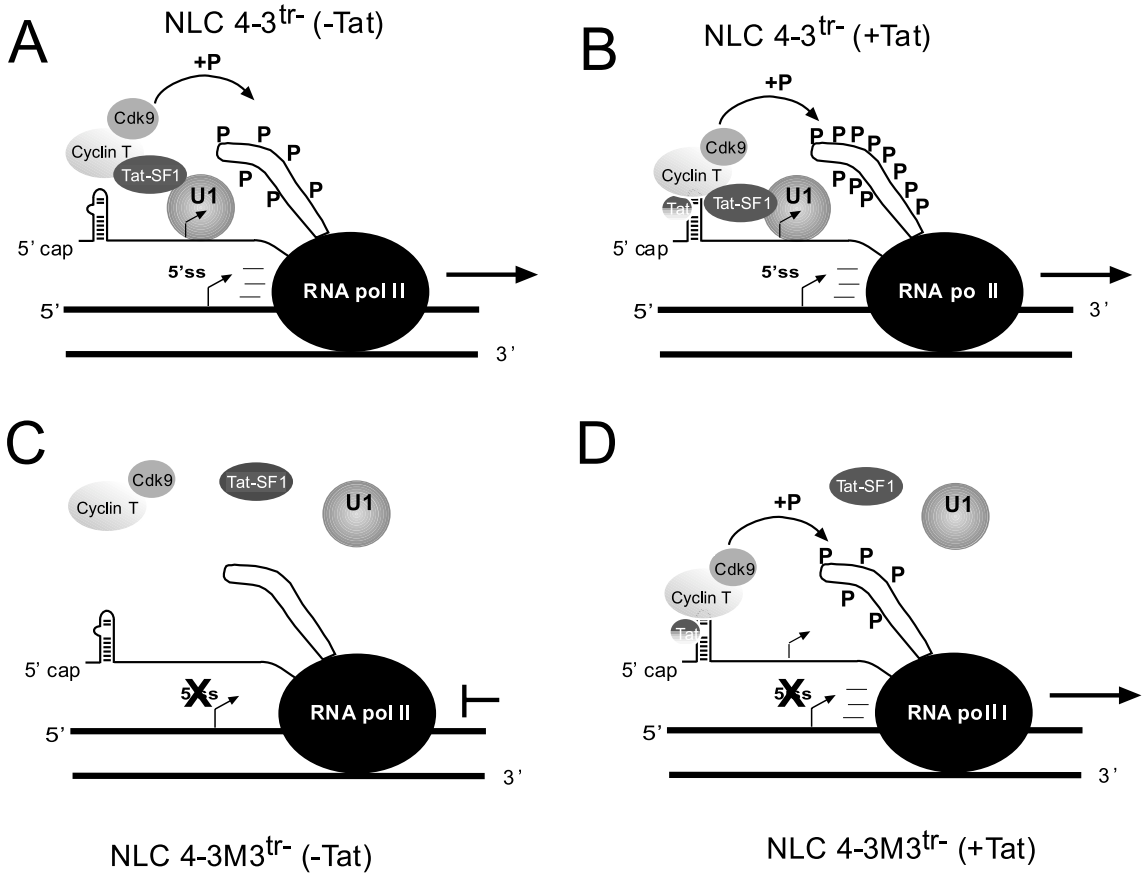

Fig. 3. Model for the molecular basis of Tat dependence of pNLC4-3^{tr-}M3. The figure shows RNA pol II complexes with their differentially phosphorylated CTDs which have initiated at the promoter region of different constructs. The DNA template is shown as thick lines, the nascent RNA as a thin line. The cap site, TAR region and major 5′ss are indicated. Differential phosphorylation of the CTD is noted by the number of P residues. Recruitment of Tat as well as of cellular cofactors cyclin T1/CDK9, Tat-SF1 and of the U1 snRNP is also shown. Panels A and B correspond to pNLC4-3^{tr-} in the absence (A) or presence (B) of Tat. Panels C and D correspond to pNLC4-3^{tr-}M3 in the absence (C) or presence (D) of Tat.

construct inhibited transcription [17], indicating that our observation may not be restricted to the specific case of HIV-1. It is clear, however, that many CMV-driven genes can be expressed without an adjacent 5′ss and the molecular reason for this difference is currently not clear. An interesting parallel to our system exists in the genome of CMV itself. The immediate early protein 1, which is synthesized from the CMV immediate early promoter, is encoded by three exons with a functional intron in the 5′ untranslated region [18]. Conceivably, splice sites may also be important for transcriptional elongation in this case and it would be interesting to test the effects of splice site mutations in this context. By analogy, all retroviruses contain a functional 5′ss in their 5′ untranslated region and the presence of this 5′ss has been shown to be important for efficient gene expression from retroviral vectors [19]. The situation is different in the case of HIV-1, however, where the viral LTR promoter requires Tat even when a functional splice donor is present. This requirement must be dependent on the promoter region since CMV-driven expression is Tat-independent provided the 5′ss is present. Accordingly, differences in the promoter structure are likely to determine the different requirements for efficient elongation.

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