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The transcriptional transactivator Tat selectively regulates viral splicing

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ABSTRACT

HIV-1 gene expression requires both viral and cellular factors to control and coordinate transcription. While the viral factor Tat is known for its transcriptional transactivator properties, we present evidence for an unexpected function of Tat in viral splicing regulation. We used a series of HIV-1 reporter minigenes to demonstrate that Tat's role in splicing is dependent on the cellular co-transcriptional splicing activators Tat-SF1 and CA150. Surprisingly, we show that this Tat-mediated splicing function is independent from transcriptional activation. In the context of the full-length viral genome, this mechanism promotes an auto-regulatory feedback that decreases expression of *tat* and favors expression of the *env*-specific mRNA. Our data demonstrate that Tat-mediated regulation of transcription and splicing can be uncoupled and suggest a mechanism for the involvement of specific transcriptional activators in splicing.

INTRODUCTION

HIV-1 gene expression is a highly regulated process composed of transcriptional and post-transcriptional processes that are mediated by both viral and cellular factors. The viral regulatory protein, Tat, stimulates transcription elongation of HIV-1 by binding a 59-nt stem loop structured RNA element (*trans*-activation-responsive region, TAR), located at the 5′-ends of all nascent HIV-1 mRNAs (1). Tat-TAR interactions promote the recruitment of the human positive transcription elongation factor b (P-TEFb) (2). Tat interacts directly with the cyclin T1 (CycT1) component of P-TEFb (3). The CDK9 kinase activity of P-TEFb results in hyperphosphorylation at Ser2 and Ser5 positions of the RNAPII CTD (4), which leads to efficient elongation (5). In addition, the cellular factor Tat-SF1 has been shown to be required for efficient transcriptional transactivation (6) and has also been found to interact with spliceosomal components (7). The association with both elongation and splicing factors has led to the suggestion that Tat-SF1 can couple these two processes. Tat-SF1 (Tat specific factor 1) has been shown to be associated with other transcription regulators such as Tat-CT1 (Tat cotransactivator 1) (8) and the transcription-splicing coupling factor, CA150 (TCERG1, transcription elongation regulator 1) (9). Overexpression of CA150 has been shown to reduce the ability of Tat to mediate viral transcription (10). This function is dependent on the association of CA150 with pre-mRNA splicing factors and the phospho-CTD of RNAPII (11) and may bridge splicing complexes to actively transcribing RNAPII (12). The cellular protein c-Ski-interacting protein, SKIP, has also been shown to regulate Tat-dependent viral transcription and interact with the U5 snRNPs and the tri-snRNP110K protein, but does not associate with a number of other splicing factors, including Tat-SF1 or CA150 (13).

The single HIV-1 primary transcript undergoes a complex series of splicing events to generate more than 40 mRNAs (14). These messages fall into three classes: (i) a ~2 kb class of multiply spliced mRNAs coding for the viral proteins Tat, Rev and Nef, (ii) a ~4 kb class of partially unspliced mRNAs coding for the viral proteins Env, Vpu, Vif and Vpr and (iii) the 9.2 kb unspliced transcript coding for the viral proteins Gag and Pol which are also packaged into new virions along with the viral genome (Figure 1A). Altering the balance of viral mRNA splicing can have dramatic effects on viral replication and infectivity (14,15). HIV-1 splicing regulation relies on the presence of intronic and exonic sequences as well as cellular splicing factors that interact with these elements. To date, five exonic splicing silencers (ESS), one intronic splicing silencer (ISS) and six exonic splicing enhancers (ESE) have been identified (16,17). Most cellular factors regulating viral mRNA processing belong to either the arginine–serine rich (SR) or the

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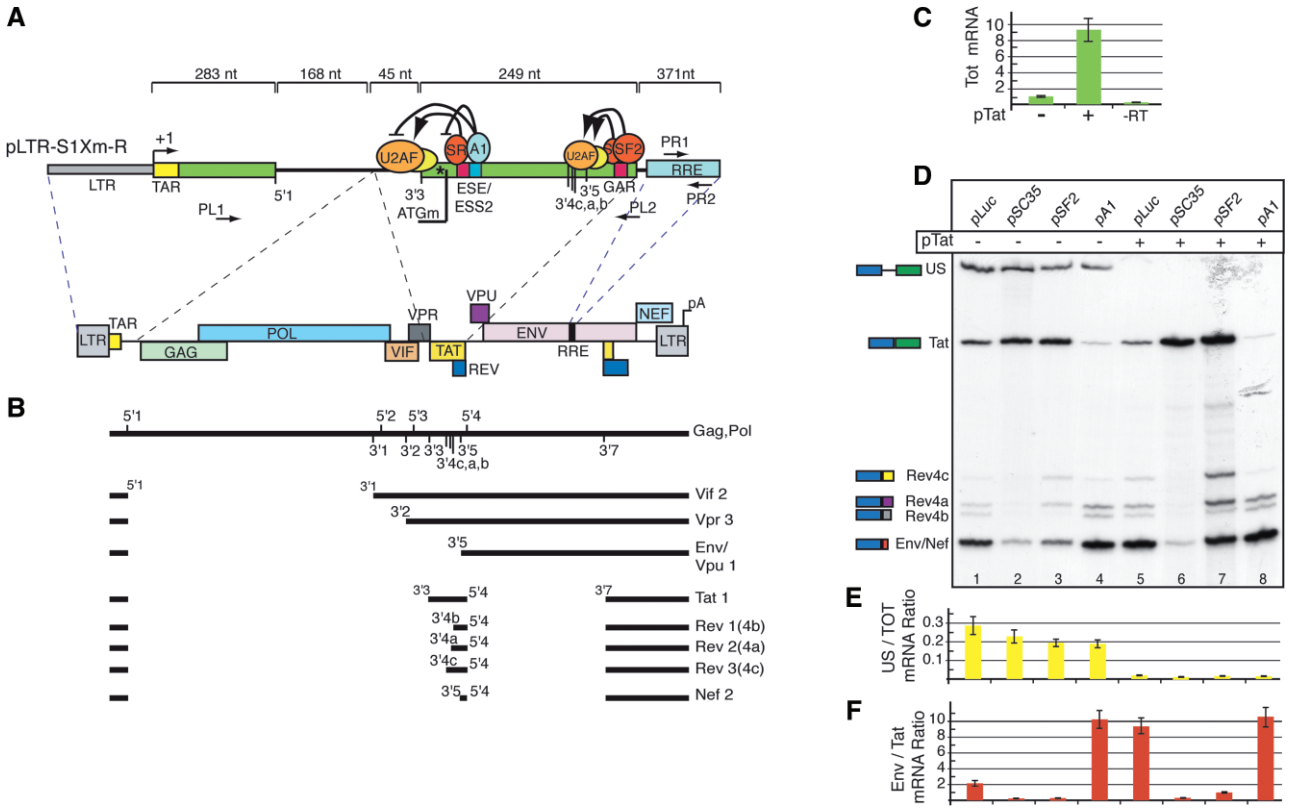

Figure 1. The reporter minigene mimics HIV-1 transcription and splicing regulation. (**A**) Schematic map of the pLTR-S1Xm-R reporter minigene. Dashed lines indicate the location within the viral genome of the sequences utilized to build the minigene. The size of the different fragments is indicated on top. The Tat start codon (asterisk) is mutated (ATGm). Location of the ESE/ESS2 and GAR splicing regulatory elements are indicated with a schematic representation of their functions. Location of the PCR primers utilized in the RT-PCR (PL1, PL2) and RT-qPCR (PR1, PR2) are indicated. (**B**) Schematic representation of the main HIV-1 RNA species. Exonic sequences present in each of the main viral mRNAs are drawn in correspondence of their location on schematic map on top. (**C**) Quantification of reporter transcripts. HEK-293T cells were transfected with pLTR-S1Xm-R, pEGFP-N1 (normalizing control) and the Tat coding plasmid pTAT. mRNAs generated by pLTR-S1Xm-R were quantified by RT-qPCR. The arbitrary value of 1 was assigned to the amount of reporter mRNA generated in the absence of Tat. An RNA sample, which was not reverse transcribed, was utilized as negative control (*−RT*). (**D**) Alternative spliced mRNAs generated by pLTR-S1Xm-R. HEK-293T cells were co-transfected with pLTR-S1Xm-R, and the vector indicated on top of the figure in the presence (+) or absence (−) of pTat. Samples were analyzed by RT-PCR, the cDNA input for each sample was normalized on the base of the total amount of transcripts generated by the reporter construct as determined by RT-qPCR. (**E**) Unspliced (US) mRNAs and the sum of all mRNA transcripts (TOT) are quantified and their ratio is indicated. (**F**) mRNAs representing splicing to the *tat* and *env* specific 3′ss #3 and 5 were quantified and their ratio is indicated. Data are represented as means ± SEM.

heterogeneous nuclear ribonucleoproteins (hnRNP) protein families (16,18). SR proteins are structurally and functionally related; they regulate splicing by binding enhancer elements and recruiting and stabilizing components of the core splicing machinery to nearby splice sites (19). Recent work revealed that SR proteins are co-transcriptionally recruited to RNAPII transcripts, coupling transcription to splicing *in vitro* (20) and may promote RNAP II elongation (21).

Work carried out in recent years indicates that transcription promotes splicing (20,22,23) and reciprocally splicing promotes transcription (7,21,24,25). The rate of elongation, the promoter type, the transcriptional activators present, and the chromatin remodeling factors nearby can all affect alternative splicing of a pre-mRNA (26,27). Studies have identified several splicing factors and spliceosomal components that interact either directly or indirectly with the transcription machinery (24). The carboxy-terminal domain (CTD) of the largest subunit (Rpb1) of RNA polymerase II (RNAPII) operates as a binding platform for components of the RNA processing machineries (28). Nevertheless, the functional significance of these associations has not been fully established. We demonstrate here that the viral factor Tat is a selective mediator of HIV-1 transcription and splicing via interactions with cellular cofactors that bridge to the RNAPII CTD. As proper splicing is critical for transition through stages of the viral lifecycle, this study highlights a critical role for Tat in the regulation of viral splicing.

MATERIALS AND METHODS

Plasmids and proviral vectors

Reporter plasmid pLTR-S1Xm-R was obtained by inserting the viral LTR promoter and the sequences upstream of the 5′ss #1 derived from the proviral clone pNL4-3 in the construct previously described as

pHS1-X (29). The 5′ splice site #4 was deleted and the RRE sequence was cloned downstream. The Tat start codon ATG was mutated into CTC. Plasmids pEVX1-S1Xm-R, pβglo-S1Xm-R, pCMV-S1Xm-R were obtained by substituting the LTR promoter for the *EVX1*, *β-globin* and CMV promoters, respectively. The *EVX1* and *β-globin* promoters were obtained by PCR amplification of genomic DNA utilizing primers EVX1-P5, EVX1-P3 and bglo-P5, bglo-P3, respectively (primer sequences are shown in Supplementary Table S1). The CMV promoter was obtained by digesting the pCDN3 vector (Invitrogen) with the restriction enzymes MluI, BamHI. Constructs pLTR-ΔTAR-S1Xm-R and pβGLO-ΔTAR-S1Xm-R were obtained by deleting the TAR region from the parent constructs. Construct pLTR-dsx-ΔE was obtained by substituting the viral sequences downstream the transcription start site in pLTR-S1Xm-R for the enhancer-dependent splicing reporter substrate derived from the *Drosophila melanogaster dsx* gene. Constructs pLTR-dsx-GAR and pLTR-dsx-GARm were obtained inserting the GAR and control mutated GAR sequences downstream the second exon of the reporter substrate as previously described (30). pLTR-Tm-dsx-GAR was obtained by inserting a synthetic DNA fragment carrying the deletion of the TAR stem. The pLuc plasmid was obtained by cloning the CMV promoter upstream the luciferase gene in the pGL4.72 vector (Promega). Plasmids pTat, pTat86, pTat86D(1–21) pTat86R(49–57)A have been previously described. Molecular clones pNL4-3 and pMtat(−), which is derived from pHXB2gpt, an infectious proviral clone of HIV-$1_{IIIB,}$ were obtained from the NIH AIDS Research and Reference Reagent Program.

Cell transfections and RT-PCR analysis

Cells were transfected with Lipofectamine 2000 (Invitrogen). pEGFP-N1 (Clontech) was added to each transfection mixture as a normalizer for transfection, RNA extraction and RT efficiency. Cells were harvested 18 h after transfection. Total RNA preparations were treated extensively with DNAse to eliminate traces of contaminating plasmids. Quantitative SYBR green PCR analysis was performed to measure transcription by the reporter construct with the primers PR1 and PR2 (Supplementary Table S1). qPCR analysis of the pMtat(−) viral transcripts was performed as previously described (15). Each sample was normalized by the relative content in *EGFP* mRNA. Each assay was carried out with a minimum of three independent transfections while qPCR assays were carried out in duplicates. Alternatively spliced mRNAs were detected using the primers PL1, PL2 for the pLTR-S1Xm-R derived constructs and primers PD1, PD2 for the pLTR-dsx-R derived constructs. Labeled ATPγ^{32}P PCR products were fractionated on polyacrylamide gels and visualized by autoradiography and quantified by Kodak 1D software. PCR analysis was performed with 26 cycles. The amount of input cDNA was adjusted for each sample based on the total amount of viral RNA as determined by the RT-qPCR reaction. RT-qPCR analysis of SC35, SF2 and hnRNP A1 mRNAs were carried out using the primer pairs 5SC35qPCR, 3SC35qPCR; 5SF2qPCR, 3SF2qPCR; 5A1qPCR, 3A1qPCR (see Supplementary Table S1).

siRNA assay

HEK-293T cells were transfected with a mixture containing 3 μl of Lipofectamine 2000 (Invitrogen) and a mixture of siRNAs 25 pM each. Qiagen HP validated siRNAs were utilized for each gene as follows: CA150 (cat. #SI04138554), (cat. #SI04328618), (cat. #SI0420 9037); Tat-SF1 (cat. #SI03154165), (cat. #SI00444479), (cat. #SI04312896); SKIP (cat. #SI00301812), (cat. #SI02655478), (cat. # SI00098609), Tat-CT1 (cat. #SI04142362), (cat. #SI04144308), (cat. #SI04155816). Forty-eight hours later cells were transfected with the reporter construct, the indicated expression vector and a mixture of siRNAs 10 pM each. Cells were harvested and RNA analyzed 18 h later.

Immunoprecipitations and western blots

Anti-CA150 antibodies were obtained from Bethyl biolabs (cat. #A300-360A), anti Tat-CT1 Bethyl biolabs (cat. #A300-868A), anti-Cyclin-T1 antibodies were obtained from Santa Cruz Biotechnology (cat. #SC1075), Tat antibodies were obtained from Immune Technologies (cat. #IT-001-041M), anti-αTubulin antibodies were obtained from Sigma Aldrich (cat. #SF2 T5168), antibody AK96 was a generous gift from Dr Adrian Krainer (Cold Spring Harbor Laboratories), anti-SC-35 antibodies were a generous gift from Dr J. Stevenin (INSERM, Strasbourg, France). A 50 μl volume of HeLa cell nuclear extract was diluted to 200 μl (final volume) with IP buffer [20 mM HEPES (pH 7.9), 150 mM KCl, 20% glycerol, 1 mM dithiothreitol, 1% Triton X-100, 0.5% NP-40, 0.2 mM EDTA]. Eight-hundred picomolar of RNA substrate was added to the reaction mixture in the samples indicated. RNA transcripts were synthesized *in vitro* utilizing T7 RNA polymerase and the following DNA oligoes substrates TAR-WT and TAR-mut. Recombinant Tat protein was obtained from the NIH AIDS Research and Reference Reagent Program (cat. #2222), 150 pM were added to the reaction mixture were indicated. Then 15 μg of anti-CA150 or anti-αTubulin antibodies were added and the mixture was incubated with end-over-end rotation at 4°C for 4 h. Immune complexes were collected by centrifugation. The pellets were washed four times with 1 ml of IP buffer by rotating for 5 min at 4°C, then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels (12.5% polyacrylamide) and analyzed by western blotting with the indicated antibody.

Chromatin immunoprecipitation (ChIP) assays

HEK 293T cells seeded in six-well plates at 60–70% confluence were transfected with 2 μg splicing reporter minigene in the precence or absence of 1 μg of Tat expression plasmid. Cells were fixed with formaldehyde (1% [vol/vol]) to crosslink the chromatin, and incubated at room temperature for 10 min. Cross-linking was

arrested by adding glycine (0.125 M), and incubated for an additional 5 min at room temperature. The cells were then pelleted, washed three times with phosphate-buffered saline, resuspended in SDS lysis buffer and incubated 10 min on ice. All solutions used prior to the collection of chromatin-antibody complexes contained protease inhibitor cocktail. The cell lysates were sonicated 6–8 times for 20 s bursts on ice. The sheared chromatin was diluted by the addition of 10 volumes of ChIP dilution buffer and pre-cleared with salmon sperm DNA–protein A/G agarose slurry for 2 h. Beads were removed by centrifugation, 10% of the pre-cleared chromatin supernatant was removed to serve as the pre-IP ('input') control, and the remaining pre-cleared chromatin incubated with either 10 μg/ml of anti-RNAPII (Millipore #05–623), anti-phospho ser2 RNAPII (Abcam #ab24758), or non-specific rabbit IgG (Bethyl #P120–301) overnight. Chromatin–antibody complexes were collected by incubation with salmon sperm DNA–protein A/G agarose (50% slurry) and subsequent collection of beads by centrifugation. Bead pellets were washed in low-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl), high-salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), 500 mM NaCl), and LiCl (0.25 M LiCl, 1% NP-40, 1% SDC, 1 mM EDTA, 10 mM Tris–HCl pH 8.0) immune complex wash buffers followed by two washes with TE buffer. Antibody–chromatin complexes were eluted from beads by incubation with elution buffer (0.1% SDS, 0.1 M $NaHCO_3$). NaCl was added to eluates (final concentration of 0.2 M) and incubated at 65°C for 4–6 h. The samples were then treated with RNase A and proteinase K, and the DNA was purified using a Qiaquick PCR purification kit (Qiagen). The pre-IP input sample was purified in a manner similar to the bound ChIP fraction described above.

Samples were amplified by PCR, with primers: LTR-Ch-5a and LTR-Ch-3a (LTR promoter), bglo-Ch-5a and bglo-Ch-3a (β-globin promoter), RRE-Ch-5a and RRE-Ch-3a (ORF). Immunoprecipitate (IP) (2 μl) or input (diluted 1:3) was used in 25-μl qPCR mixtures with primers LTR_P5ch, LTR_P3ch (LTR promoter), bglo-Chq-5, bglo-Chq-3 (*β-globin* promoter) and PR1, PR2 (ORF). Primer sequence are shown in Table 1. A fraction of input was used to standardize the values obtained. The relative proportions of coimmunoprecipitated DNA fragments were determined on the basis of the threshold cycle (C_T) for each PCR product. The data sets were normalized to input values (percent input; $2^{CT(\text{input}) - CT(\text{IP})} \times 100$).

RESULTS

Tat expression activates splicing to the *rev* and *env*/*nef* specific 3′ splice sites

To study the interactions between the transcription and splicing machineries in HIV-1, we developed the reporter system pLTR-S1Xm-R (Figure 1A). This construct contains the LTR promoter and a major deletion of the first tat intron followed by the first Tat coding exon. Since this reporter can code for a functional truncated Tat protein, we mutated the Tat start ATG codon. The mutation abrogated the Tat transactivation activity expressed by the reporter construct (Supplementary Figure S1). The Rev Response Element (RRE) was added to the 3′-end of the construct to provide for a region, included in all the spliced mRNAs, that can be detected by quantitative real time PCR (qPCR) (Figure 1A). To determine if the reporter minigene was responsive to Tat transactivation HEK-293T cells were cotransfected with the pLTR-S1Xm-R construct and either the Luciferase expression control vector pLuc or the Tat coding vector pTat. Tat expression resulted in a >9-fold increase in the level of the reporter minigene mRNA (Figure 1C).

Next, we confirmed that HIV-1 splicing regulation is reliably reproduced by the reporter minigenes. We tested the activity of cellular factors previously shown to regulate the usage of the five alternative 3′ splice sites (ss) present within the reporter construct. Splicing to the 3′ss #3 generates mRNAs coding for the Tat protein and its usage is regulated by hnRNP A1 through binding to the splicing silencer ESS2. The ESS2 silencer is juxtaposed to a splicing enhancer (ESE2), which is activated by SR proteins (Figure 1A) (31,32). Over-expression of SR proteins SC35 and SF2 caused a shift of the splicing pattern toward 3′ss #3 while over-expression of hnRNP A1 reduced it (Figure 1D, lanes 1–4). Although 3′ss #4a–c and 5 are regulated by an SR protein-dependent ESE termed GAR (Figure 1A) (30) addition of SF2 or SC35 did not markedly increase their usage (Figure 1D, lane 3). This is likely due to the relative strength of the competing splice sites and splicing enhancers. Finally, the relative ratio of the splicing isoforms observed was remarkably similar to that obtained by the whole virus (15). Thus, results indicate that the reporter construct can reliably mimic the complex regulation of HIV-1 splicing.

Surprisingly, expression of Tat in combination with the reporter transgene induced a switch in the splicing pattern of the reporter. Tat expression led to a decrease in the level of unspliced and mRNAs splicing to the 3′ss #3 and an increase in splicing to the 3′ss #4a–c and 5 (Figure 1D, lane 5; E and F). The splicing switch to the 3′ss #4a–c and 5 was not observed when SC35 was co-expressed with Tat (Figure 1D, lane 6). This is in agreement with the data showing that SC35 binds and activates the Tat specific 3′ss #3 (32).

Analysis of the endogenous mRNA for SC25, SF2 and hnRNP A1 showed that Tat did not alter their expression (Supplementary Figure S2). Furthermore, since a change in the mRNA steady-state and processing can be due to an alteration of its stability, we analyzed the relative stability of the viral mRNAs by adding actinomycin D, an inhibitor of RNAPII, to the cell culture media 18 h after transfection. The viral mRNAs did not show substantial differences in stability upon expression of any of the proteins known to alter its splicing (Supplementary Figure S3). These results suggested that Tat effect on splicing was not due a secondary effect on mRNA stability or expression of cellular splicing factors.

To test the specificity of the effect on splicing caused by expression of Tat, a series of Tat mutants carrying deletions in domains required for its RNA binding, nuclear localization and transactivation activities (Supplementary Figure S4A) was co-expressed with the reporter construct. Deletion of the Tat N-terminal domain and the arginine-rich domain abolished both Tat transactivation and splicing regulation activities (Supplementary Figure S4B–E). Tat splicing activity was also disrupted by the deletion of the TAR element, which is required for efficient recruitment of Tat (Supplementary Figure S5A–D). Thus, Tat activity in viral splicing modulation appears to be specific and dependent on a functional Tat protein and its interaction with the viral RNA.

Tat expression regulates env gene expression

We have previously shown that a splicing enhancer sequence, named GAR (Figure 1B), is required to stabilize the interaction between the U1 snRNP and the downstream 5′ss #4 and activates splicing to the upstream *rev*, *env* specific 3′ss #4a–c and 5 (30). This interaction is required for the efficient expression of *env* specific mRNAs, possibly by promoting their stability and export (33). Since our data indicate that Tat expression promotes activation of the 3′ss regulated by GAR, it is conceivable that it might also regulate expression of *env* mRNAs within the context of the complete virus. To confirm this hypothesis we utilized the proviral clone pMtat(−), which does not express Tat (Figure 2A) (34). HEK-293T cells were co-transfected with the proviral vector pMtat(−) and either the control expression plasmid pLuc or pTat. To determine the steady-state level of the total viral mRNA, we performed RT-qPCR with primers designed to anneal to a region common to all viral mRNAs (Figure 2A, see P3, P4). Tat expression increased the total viral mRNA level by 20-fold (Figure 2B).

To quantify the single viral mRNA isoforms we utilized primer sets spanning the splice junctions (Figure 2A). A fifth set of primers, specific for the *gag*/*pol* mRNA, was designed to anneal immediately downstream of the major HIV 5′ splice site. Since the total amount of viral mRNA increases upon expression of Tat, the relative amount of the single mRNA species was normalized for the total amount of viral mRNA present in each reaction. Tat expression induced a 3-fold increase in the relative amount of the *env* mRNA (Figure 2C) while decreasing the tat mRNA (Figure 2E). Tat expression did not alter the normalized amounts of *gag*/*pol*, *rev* and *nef* mRNAs (Figure 2D, F and G).

The level of Tat expression obtained by transfection of the pTat plasmid was comparable to the one observed in parent proviral vector pHIV-HXB2, which carries a functional Tat gene (Figure 2H). Thus, it is unlikely that the change in the *env* mRNA level is due to the expression of non-physiological amounts of Tat. Furthermore, since the export and stability of both *env* and *gag*/*pol* messages are regulated by Rev but only the relative amount of the *env* mRNA is altered by Tat expression, it is likely that the effect observed is not due to the increase of Rev following transcription transactivation. Thus, in the context of the full virus Tat affects transcription as well as expression of specific mRNA species, consistently with the data obtained in our reporter system.

The GAR ESE is required for Tat-mediated splicing

Splicing to the *tat*-specific 3′ss #3 is controlled by the juxtaposed ESE/ESS2 (31,32) while splicing to the 3′ss #4a–c and 5 is activated by the binding of SF2 to the splicing enhancer GAR (30) (Figure 3A). Tat-mediated splicing to the 3′ss #4a–c and 5 may be due to either activation of the GAR enhancer, repression of the upstream *tat* specific 3′ss #3, or down-regulation of ESE2, which activates the 3′ss #3. To test these hypotheses we mutated the different elements that control the splicing of this region (Figure 3A).

Mutation of the juxtaposed ESE/ESS2 decreases the overall amount of unspliced mRNA (Figure 3B and C) and increases usage of 3′ss #3, indicating that the silencing element is dominant toward the enhancer (Figure 3B–D, lanes 7–12). Addition of exogenous Tat increased splicing to the 3′ss #4a–c, suggesting that Tat activates splicing independently of the presence of the ESE/ESS2. Furthermore, upon mutation of the *tat*-specific 3′ss #3 addition of exogenous Tat strongly increased splicing to the Rev/Env splice sites (Figure 3B–D, lanes 19–24). This indicates that splicing activation by Tat is independent from the presence of the upstream 3′ss #3. On the contrary, mutation of the GAR enhancer abolished Tat-mediated splicing to the 3′ss #4a–c and 5 (Figure 3B–D, lanes 13–18). These data suggest that Tat is likely to regulate splicing by activating the SF2-dependent GAR enhancer rather than repressing usage of the upstream 3′ss #3 or the ESE/ESS2 element.

Next, we sought to determine whether the GAR enhancer might be sufficient to confer Tat-mediated splicing responsiveness to a heterologous gene. To this end, we substituted the viral sequences present between the TAR region and the GAR element for a splicing reporter substrate derived from the *D. melanogaster doublesex* (*dsx*) gene (construct pLTR-dsx-GAR-R in Figure 4A). Splicing of the parental *dsx* substrate (dsx-Δ*E*) is weak (Figure 4C, lanes 7 and 8) because of a non-consensus 3′ss. Insertion of an SR-dependent splicing enhancer activates splicing by aiding in the recognition of the weak splice site (35). Tat activated transcription of the pLTR-dsx-GAR-R reporter construct similarly to the viral reporter pLTR-S1Xm-R (Figure 4B) and induced an increase in splicing to the weak 3′ss in the construct carrying the wild-type GAR sequence (Figure 4C and D, lanes 1 and 2). This effect was lost upon mutation of the GAR sequence (Figure 4C and D, lanes 3–8) or mutation of the TAR region.

Tat-mediated splicing is independent from transactivation and Pol II CTD phosphorylation

Since Tat transactivation is dependent on enhancer motifs present within the LTR promoter and the viral TATA box (36), we asked if the structure of the LTR promoter would influence Tat-mediated splicing as well. To this end,

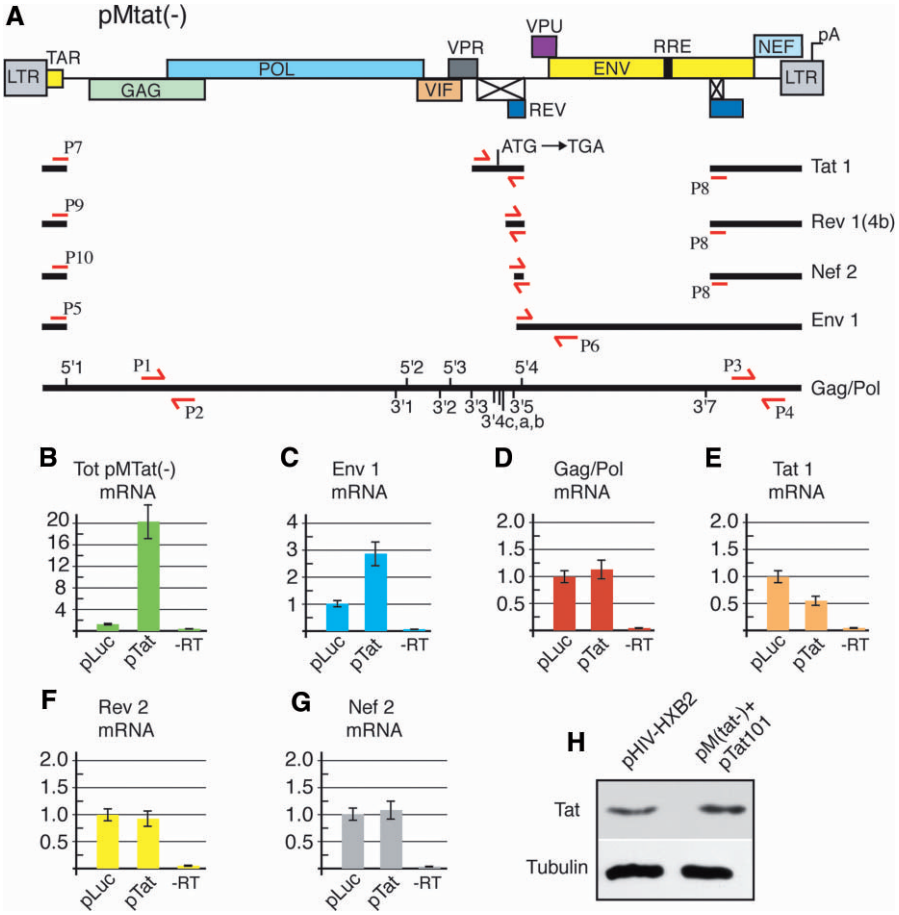

Figure 2. Tat upregulates *env* mRNA splicing. (**A**) Schematic representation of the pMtat(−) proviral construct. The relative position of the viral genes is indicated on the map on top. The Tat start codon (ATG) is mutated. The main mRNAs analyzed by RT-qPCR are indicated with the solid line representing the exons. Location of the primers utilized in the qPCR assays is also indicated (arrows P1 through P10). 5′ and 3′ splice sites are indicated on the unspliced (gag/pol) mRNA. (**B–G**) HEK-293T cells were transfected with the proviral clone pMTat(−), the control pLuc or pTat. Each graph summarizes the quantification by RT-qPCR of the indicated mRNA species. The amount of each mRNA species generated in the transfection containing the control pLuc was assigned the value 1. (B) The total viral mRNAs (primers P3–P4) data were normalized for the EGFP mRNA content of each sample. (C–G) gag/pol mRNA (primers P1–P2), env1 mRNA (primers P5–P6), tat1 mRNA (primers P7–P8), rev2 mRNA (primers P9–P8) and nef2 mRNA (primers P10–P8) were normalized on the base of the total viral mRNA content of each sample. A RNA sample, which was not reverse transcribed, was utilized as negative control (*−RT*). (**H**) Tat expression in 293T cells transfected with the proviral clone pHIV-HXB2 or cotransfected with pMTat(−) and pTat. Data are represented as means ± SEM.

we substituted the LTR promoter sequences upstream of TAR with either the strong viral CMV promoter or weaker cellular promoters derived from the *β-globin* and *EVX1* (even-skipped homeo box homolog 1 gene) genes (Figure 5A). The transcription level from the CMV promoter was roughly 2-fold higher than that generated by the LTR, but the CMV promoter driven minigene was only marginally stimulated by addition of exogenous Tat (Figure 5B). The relative amount of transcripts generated from the *β-globin* and *EVX1* promoters were roughly 3–4-fold lower than that generated by the LTR; addition of exogenous Tat did not increase the reporter mRNA level of these minigenes (Figure 5B). Surprisingly, analysis of the alternatively spliced mRNAs shows that Tat can strongly mediate splicing to the 3′ss #4a–c and 5 in the construct under the control of the *β-globin* promoter (Figure 5D–F, lanes 13 and 14) but has a modest effect on splicing of the transcripts generated by the CMV and the *EVX1* promoters (Figure 5D–F, lanes 7, 8, 19 and 20).

Deletion of TAR in the reporter gene driven by the *β-globin* promoter strongly decreased Tat mediated splicing activity (Supplementary Figure S6) but did not abolish it completely. A similar effect was also observed with the deletion of TAR within the reporter driven by the LTR promoter (Supplementary Figure S5). It is possible that a suboptimal Tat binding site distinct from TAR may be present within the reporter transcript, thus supporting Tat splicing activity but not its role in transcription, which also requires the binding of Cyclin T1.

Previous work indicated that phosphorylation at Ser2 position of the RNAPII CTD stimulates transcription elongation and pre-mRNA splicing (37,38). Since Tat promotes RNAPII CTD phosphorylation at position Ser2 and Ser5 (4), we investigated the phosphorylation states of the CTD on the viral LTR versus *β-globin*

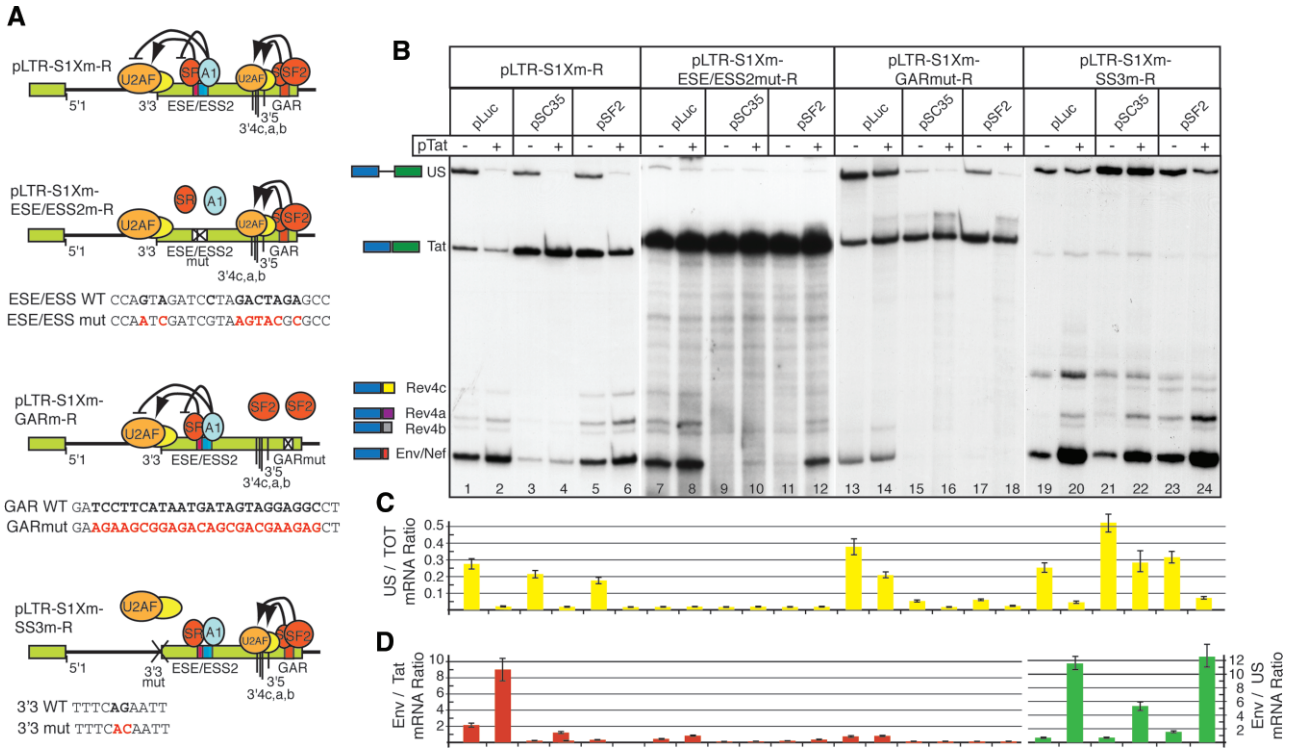

Figure 3. Tat-mediated splicing is dependent on the GAR ESE. (**A**) Schematic representation of the reporter constructs. Mechanism of ESE/ESS2 and GAR splicing splicing regulation are schematically represented. Wild-type (black bold font) and mutated (red bold font) sequences are indicated below each construct. (**B**) RT-PCR analysis of alternative spliced mRNAs generated by the indicated reporter construct (top of the panel) transfected in combination with the control (pLuc), splicing factors (pSC35, pSF2) and pTat as indicated, (**C**) Unspliced (US) mRNAs and the sum of all mRNA transcripts (TOT) are quantified and their ratio is indicated. (**D**) The splicing products representing splicing to the *tat* and *env* specific 3′ss #3 and 5 were quantified and their ratio is indicated on the left. For the pLTR-S1Xm-SS3m-R construct, products representing splicing to the *env* specific 3′ss #5 and unspliced mRNAs were quantified and their ratio is indicated on the right. Data are represented as means ± SEM.

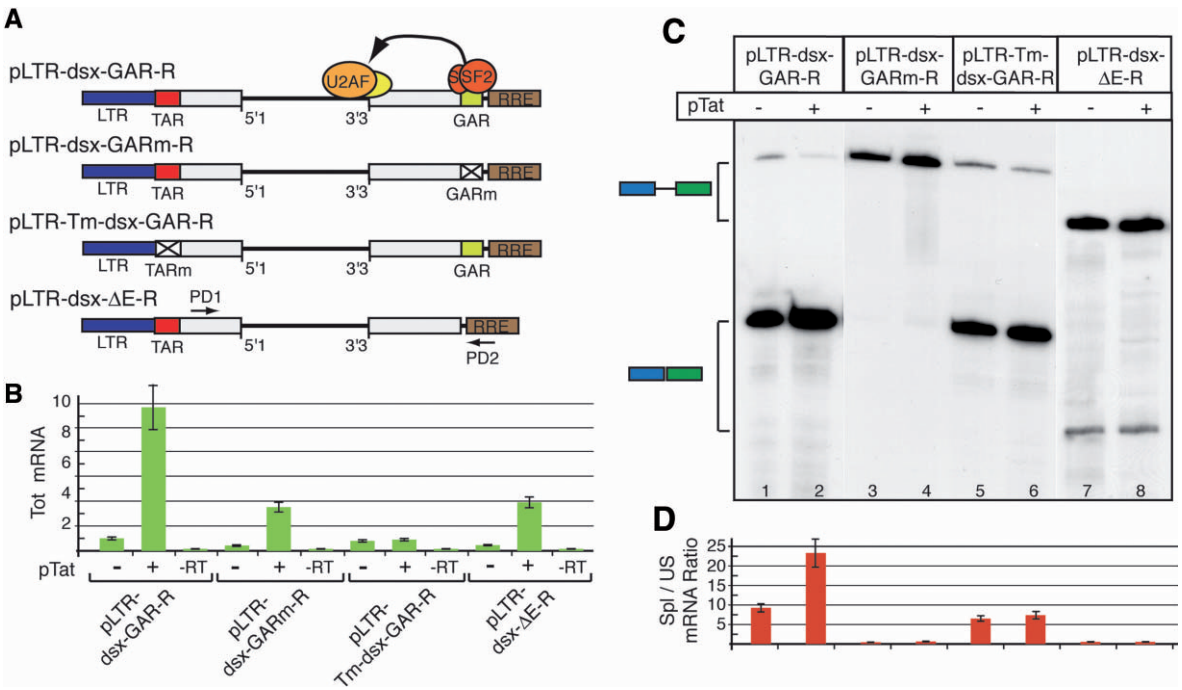

Figure 4. Tat promotes splicing of a heterologous substrate. (**A**) Schematic representation of the pLTR-dsx-R minigenes. Dsx exonic sequences (light boxes), LTR promoter and RRE sequences (dark boxes) are shown. GAR splicing enhancer, TAR region and respective mutations are indicated. The GAR wild type and mutated sequences are shown in Figure 3A. (**B**) Quantification of reporter transcripts. HEK-293T cells were co-transfected with the indicated reporter construct and pTat. Transcripts were quantified by RT-qPCR. A RNA sample, which was not reverse transcribed, was utilized as negative control (−RT). (**C**) RT-PCR analysis of alternative spliced mRNAs generated by the indicated reporter construct (top of the panel). (**D**) Spliced and unspliced mRNAs were quantified and their ratio is indicated. Data are represented as means ± SEM.

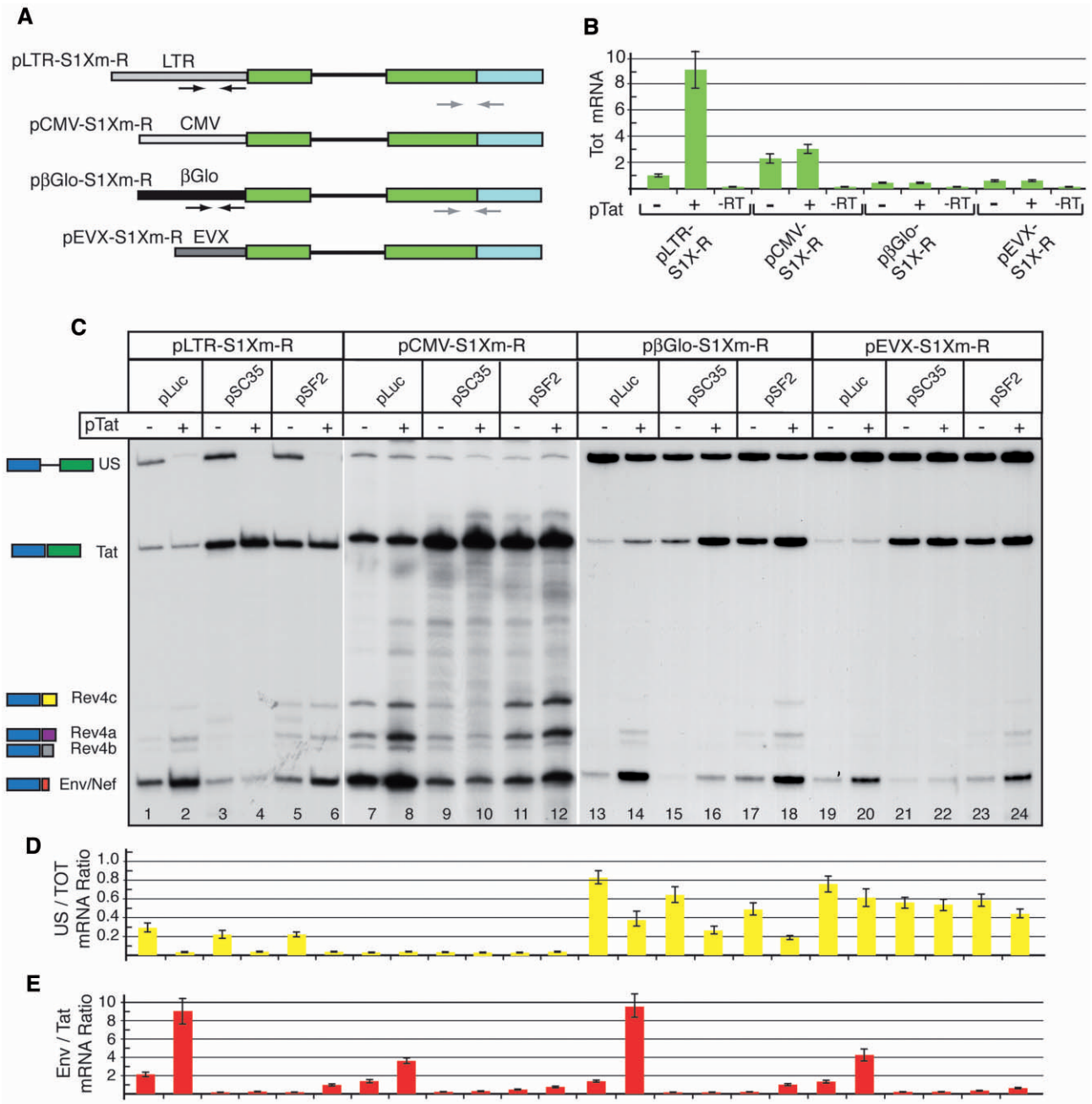

Figure 5. The structure of the promoter affects Tat-mediated splicing. (**A**) Schematic representation of the reporter constructs utilized. (**B**) Quantification of reporter transcripts by RT-qPCR. HEK-293T cells were co-transfected with the indicated reporter construct, and pTat. (**C**) RT-PCR analysis of alternative spliced mRNAs generated by the indicated reporter construct (top of the panel) transfected in combination with the control (pLuc), splicing factors (pSC35, pSF2) and pTat as indicated. A RNA sample, which was not reverse transcribed, was utilized as negative control (*−RT*). (**D**) Unspliced (US) mRNAs and the sum of all mRNA transcripts (TOT) are quantified and their ratio is indicated. (**E**) The splicing products representing splicing to the *tat* and *env* specific 3′ss #3 and 5 were quantified and their ratio is indicated. Data are represented as means ± SEM.

cellular promoter in the presence or absence of Tat. ChIP assays were performed utilizing antibodies against the phosphorylated form of the CTD Ser2 and against Pol II (Figure 6). When the assay was performed on the constructs carrying the LTR promoter in the absence of Tat, there was association of RNAPII but not phosphorylated Ser2 at the promoter while both, RNA pol II and the phosphorylated Ser2, were not associated with the downstream ORF [Figure 6A (right), B and C]. However, following addition of Tat, there was a 10-fold increase in phosphorylated Ser2 at the promoter and an increase in both RNAPII and its phosphorylated form at the ORF. This indicates that Pol II stalls at the promoter in an unphosphorylated state in the absence of Tat. Addition of Tat promotes promoter clearance and elongation through Ser2 phosphorylation. In the presence of

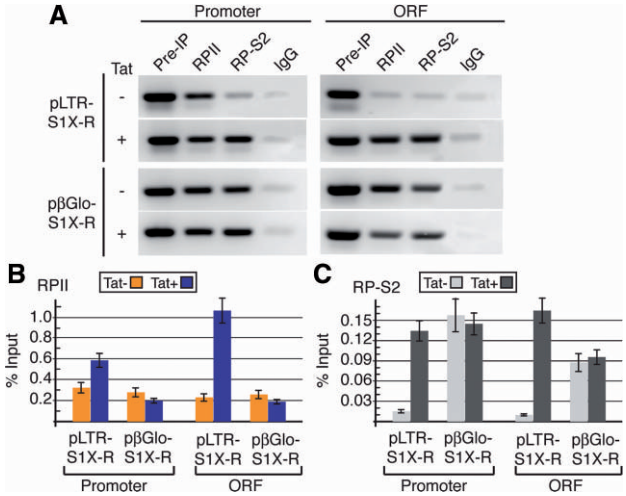

Figure 6. (**A**) RNAPII assembly and phosphorylation was analyzed by a ChIP assay using the indicated antibodies. The location of the primers used in the ChIP assay to analyze the promoter (black) or ORF (gray) is schematically indicated in Figure 5A. The reporter plasmids indicated at the left of the panel were transfected in combination or absence of Tat as indicated. Antibodies recognizing RNAPII (RPII) and the phosphorylated form of RNAPII at Ser2 (RP-S2) together with a control anti-IgG are indicated on top. qPCR analysis of the RPII (**B**) and RP-S2 (**C**) assays at the promoter and ORF in the presence or absence of Tat. The ChIP values are expressed as percentages of input. Data are represented as means ± SEM.

the non-Tat responsive *β-globin* promoter, the association of both RNAPII and the Ser2 phophorylated form with the promoter and the ORF did not vary in the absence or presence of Tat. Thus, indicating that Tat is necessary to promote RNAPII CTD phosphorylation only in the presence of the LTR promoter. Moreover, these differences in promoter architecture reveal that Tat-mediated splicing activation is independent from transcriptional transactivation and phosphorylation of the RNAPII CTD. Analysis of the relative ratios between spliced and unspliced mRNA species (Figure 5E) also indicates that the structure of the promoter and possibly its strength can influence the overall splicing efficiency of the reporter minigene. Indeed, the relative amount of unspliced mRNA increased in the transcripts generated by the *β-globin* and *EVX1* promoters and decreased in the transcripts generated by the CMV and LTR promoters (Figure 5D and E).

Tat-mediated splicing is dependent on splicing associated factors Tat-SF1 and CA150

Several studies have indicated a role for splicing-associated proteins in the Tat transcription activation complex (7,8,10,13). We investigated if proteins associated with both the Tat transcription activation complex and spliceosomal components are required for Tat-mediated splicing. SiRNAs directed against components of the Tat transactivation complex CA150, Tat-SF1, Tat-CT1 and SKIP were co-transfected with the reporter construct in the absence or presence of Tat. Expression of the targeted genes was reduced by at least 70% at both RNA and protein level (Supplementary Figure S7). Down-regulation of Tat-SF1, induced a decrease in transcription of the reporter minigenes (Figure 7A and B) and reduced Tat-mediated splicing (Figure 7C–E, lanes 7 and 8). Reduction of CA150 expression increased basal transcription of the reporter construct (Figure 7A) and partially reduced Tat-mediated splicing (Figure 7C–E, lanes 9 and 10). These data suggest that Tat-mediated splicing is dependent on both CA150 and Tat-SF1, which appear to differentially affect viral transcription confirming that the two Tat activities, transcription and splicing, might be functionally uncoupled. This observation is confirmed by the SKIP and Tat-CT1 knockdowns, which results in a loss of basal transcription activity (Tat-CT1 and SKIP) and transcriptional transactivation (SKIP) (Figure 7A and B) but not of Tat-mediated splicing (Figure 7C–E, lanes 3–6).

Previous studies have shown that CA150 interacts *in vivo* with Tat-SF1 (9). Since splicing to the 3′ss #4a–c and 5 is regulated by the SR protein-dependent splicing enhancer GAR it is plausible that the CA150:Tat-SF1 complex might recruit SR proteins. To explore this possibility we performed immunoprecipitation assays in nuclear extracts utilizing CA150 antibodies. The assay showed that both SR proteins, SF2 and SC35, were co-precipitated with CA150 but not with the control antibody (Figure 7F).

Addition to the assay mixture of an exogenous RNA containing the TAR sequence and purified exogenous Tat protein further increased the precipitation of SF2 and marginally SC35 (Figure 7F–H, lane 4). The increase in the recruitment of SR proteins was lost in the presence of a control RNA carrying a mutated TAR sequence suggesting that a complex containing CA150/Tat-SF1 and SR proteins, in particular SF2, might be promoted by the Tat:TAR interaction.

DISCUSSION

In this study, we show that Tat is a mediator of viral splicing and its functions in transcriptional activation are independent from its role in splicing regulation (summarized in Figure 8). These observations provide support for a general mechanism whereby transcriptional activators, viral or cellular, can selectively regulate splicing processes.

Prevailing models, for the coupling between the transcription and the splicing machineries, propose that splicing factors are recruited to the promoter (recruitment model) or, alternatively, that RNAP II elongation rates regulate the availability of competing splice sites to the splicing machinery (kinetic model) (28,39). Both models assign a central role to the RNAPII CTD. Differential phosphorylation of the CTD is thought to determine the association/dissociation of transcription and RNA processing factors throughout the transcription cycle and to regulate RNAPII processivity. Although, Tat promotes the recruitment of P-TEFb to the viral promoter and the CDK9 kinase activity of P-TEFb results in hyperphosphorylation of the RNAPII CTD at Ser5 and Ser2,

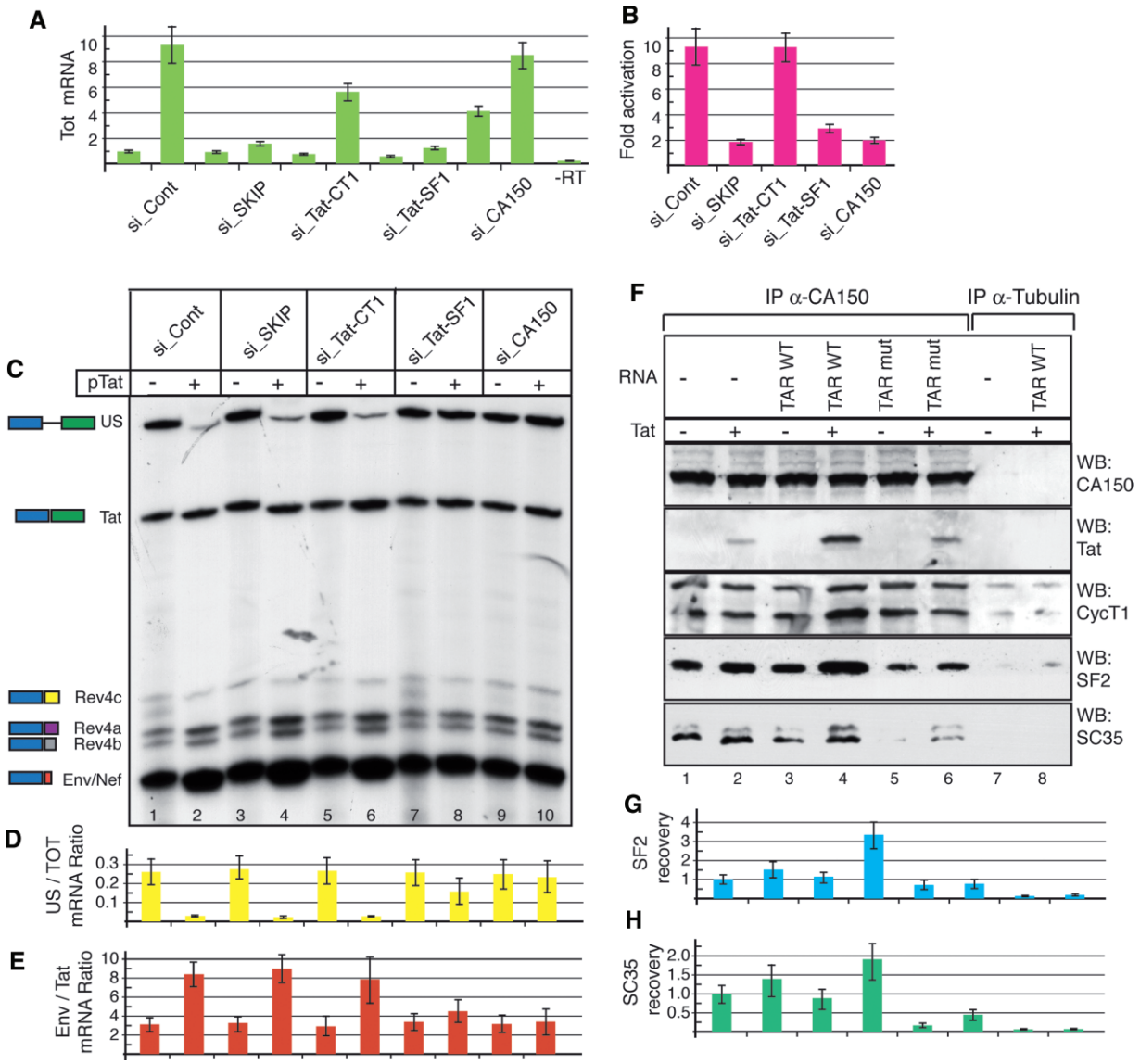

Figure 7. Splicing is dependent on Tat-associated cellular factors. (**A**) Down-regulation by siRNA of cellular transcription-splicing factors. HEK-293T were transfected with the reporter construct pLTR-S1Xm-R, pTat and the indicated a control siRNA (si_Cont) or siRNAs to Tat-SF1 (si_Tat-SF1), CA150 (si_CA150), SKIP (si_SKIP), Tat-CT1 (si_Tat-CT1). Transcripts were quantified by RT-qPCR. A RNA sample, which was not reverse transcribed, was utilized as negative control (*−RT*). (**B**) Tat transcription transactivation. Fold activation of transcription in the presence of Tat and the indicated siRNAs. (**C**) RT-PCR analysis of the alternative spliced mRNAs generated by pLTR-S1Xm-R cotransfected with either the control pLuc or pTat and the indicated siRNA (top of the panel). (**D**) Unspliced (US) mRNAs and the sum of all mRNA transcripts (TOT) are quantified and their ratio is indicated. (**E**) The splicing products representing splicing to the *tat* and *env* specific 3′ss #3 and 5 were quantified and their ratio is indicated. (**F**) SF2 and SC35 co-immunoprecipitate with CA150 in HeLa nuclear extracts. CA150 was immunoprecipitated with anti-CA150 antibodies and anti-tubulin antibodies as control (indicated on top). Short RNA transcripts containing TAR or a control sequence were added to the assay mixture as indicated. Recombinant GST-Tat protein was also added to the incubation mixture as indicated. The immunoprecipitates were analyzed by western blotting with the indicated antibodies (panels from top to bottom). Quantification of (**G**) SF2 and (**H**) SC35 recovered in each immunoprecipitation assays. The arbitrary value of 1 was assigned to the amount recovered by precipitation with anti-CA150 antibodies in the absence of exogenous RNA and Tat. Data were quantified by optical density analysis. Data are represented as means ± SEM.

our data indicate that Tat-mediated splicing may not be dependent on this activity. Here we have shown that Tat transactivation can be uncoupled from its splicing activity. Substitution of the LTR promoter for heterologous cellular (*β-globin*, *EVX1*) or viral (CMV) promoters or, down-regulation of Tat transcription co-factors cofactors (SKIP, Tat-CT1) blocks Tat transactivation but not splicing. Since Tat transactivation is characterized by the hyperphosphorylation of the RNAPII CTD, which in turn leads to efficient elongation (5), ChIP assays confirmed that Tat does not promote RNAPII elongation and CTD phosphorylation when the *β-globin* promoter is substituted for the LTR. These data indicate that Tat may modulate splicing independently from its ability to functionally engage the transcription machinery and alter the phosphorylation state of the RNAP II CTD.

Tat function in splicing depends on Tat-SF1 and CA150, two cellular factors that can engage both the

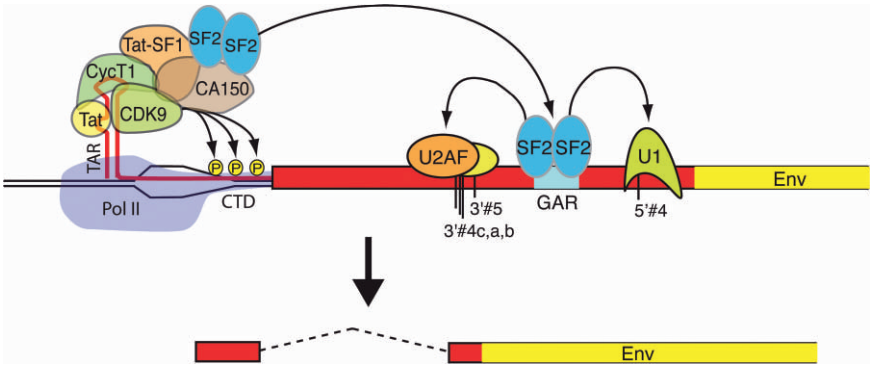

Figure 8. Proposed model for Tat-mediated splicing in the viral context. Tat binding to TAR aids in the recruitment of the pTEF-b complex that in turn helps the recruitment of Tat-SF1 and CA150, which stimulates the assembly of SF2 onto the GAR enhancer. SF2 interaction with GAR promotes the upstream *rev*, *env* specific 3′ss and recruitment of U1 snRNP to the downstream 5′ss, which allows for *env* mRNA expression.

splicing and transcription machineries (6,9–12). Co-immunoprecipitation data (Figure 7F) indicate that CA150 assembles into a complex containing SR proteins SC35, SF2 and cyclin T1, the latter likely recruited through the bridging activity of Tat-SF1 (40). Furthermore, addition of Tat in the presence of the TAR RNA appears to increase the recruitment of SF2. Tat specifically activates the SF2 dependent GAR enhancer but not the upstream ESE2, which is also responsive to SF2 (Figure 3B). It is conceivable that the relative strength and affinity of the different *cis*-acting elements (ESE, ESS and splice sites) could influence this process. Alternatively, structural constraints might also influence the positioning of splicing factors onto the nascent pre-mRNA. Activation of the GAR enhancer leads to usage of the nearby 3′ss #4a–c and 5 and recruitment of U1 snRNP to the downstream 5′ss #4, which has been shown to lead to an increase expression of the *env* mRNA (30). The mechanism by which U1snRNP binding to 5′ss #4 increases expression of the *env* mRNA is still unclear but it is thought to increase the stability of the viral mRNA and possibly have a synergistic effect with the Rev-dependent mRNA nuclear export (33,41). In agreement with this model, our data show that Tat expression specifically increases the expression of *env* mRNA in the context of the complete replicating virus. This suggests a feedback model in which higher Tat expression levels induce a splicing-dependent increase in the relative level of envelope protein mRNAs, which is key in infectious virion production and viral pathogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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