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Transcriptional Activators Differ in Their Abilities to Control Alternative Splicing*

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Promoter and enhancer elements can influence alternative splicing, but the basis for this phenomenon is not well understood. Here we investigated how different transcriptional activators affect the decision between inclusion and exclusion (skipping) of the fibronectin EDI exon. A mutant of the acidic VP16 activation domain called SW6 that preferentially inhibits polymerase II (pol II) elongation caused a reduction in EDI exon skipping. Exon skipping was fully restored in the presence of the SW6 mutant by either the SV40 enhancer in cis or the human immunodeficiency virus (HIV) Tat in trans, both of which specifically stimulate pol II elongation. HIV Tat also cooperated with the Sp1 and CTF activation domains to enhance transcript elongation and EDI skipping. The extent of exon skipping correlated with the efficiency with which pol II transcripts reach the 3′ end of the gene but not with the overall fold increase in transcript levels caused by different activators. The ability of activators to enhance elongation by RNA polymerase II therefore correlates with their ability to enhance exon skipping. Consistent with this observation, the elongation inhibitor dichlororibofuranosylbenzimidazole (DRB) enhanced EDI inclusion. Conversely, the histone deacetylase inhibitor trichostatin A that is thought to stimulate elongation caused a modest inhibition of EDI inclusion. Together our results support a kinetic coupling model in which the rate of transcript elongation determines the outcome of two competing splicing reactions that occur co-transcriptionally. Rapid, highly processive transcription favors EDI exon skipping, whereas slower, less processive transcription favors inclusion.

The production of mature messenger RNA in most eukaryotic cells requires transcription and three pre-mRNA processing reactions: capping, splicing, and cleavage/polyadenylation. For years transcription and processing have been thought to be independent events until a series of biochemical, cytological, and functional experiments demonstrated that all three processing reactions are tightly coupled to RNA polymerase II (pol II)[1] transcription. Coupling is in part due to the ability of pol II to bind and "piggyback" some of the processing factors in a complex known as the "mRNA factory" (for reviews see Refs. 1–5). The carboxyl-terminal domain (CTD) of pol II plays a central role in the coupling process: truncation of the CTD causes defects in capping (6), cleavage/polyadenylation, and splicing (7). CTD phosphorylation/dephosphorylation at specific serines influences the recruitment of capping enzymes and cleavage/polyadenylation factors to the mRNA factory during transcript elongation (8–10).

We have previously shown that differences in promoter structure cause conspicuous differences in alternative splicing of the transcript (11). We analyzed mammalian cells transfected with minigenes carrying the EDI exon of fibronectin (FN), which can be either included or excluded from the mRNA by exon skipping. EDI contains an exonic splicing enhancer (12), which is a target for the splicing factors SF2/ASF and 9G8 that belong to the SR ((Ser/Arg)-rich) family of proteins. Overexpression of SF2/ASF and 9G8 stimulates EDI inclusion, but the effect of these proteins critically depends on the promoter (13). Modulation of alternative splicing by the promoter is not the consequence of differential promoter strengths but depends on some qualitative properties conferred by promoters to the transcription/RNA processing machinery. One model suggests that promoters might control alternative splicing via the regulation of pol II elongation or processivity. Low pol II processivity or internal pauses for elongation would favor the inclusion of alternative exons such as EDI, whereas a highly processive form of pol II, or the absence of internal pauses, would favor exclusion of this type of exon (5). The model is supported by the following evidence. (*a*) Promoters eliciting higher EDI inclusion levels (such as the FN or CMV promoters) determine lower pol II processivities than promoters eliciting lower EDI inclusion (such as the α-globin promoter), as revealed by the measurements of pol II densities along the minigene templates by chromatin immunoprecipitation (14). (*b*) A few rounds of minigene replication, which causes a decrease in pol II processivity presumably by chromatin compaction, provokes higher inclusion of the alternative EDI exon (15). (*c*) The

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[1] The abbreviations used are: pol II, polymerase II; DRB, dichlororibofuranosylbenzimidazole; TSA, trichostatin A; HIV, human immunodeficiency virus; e/o, enhancer + origin; CTD, carboxyl-terminal domain; FN, fibronectin; CMV, cytomegalovirus; RT, reverse transcription; RPA, RNase protection assay; EDI, extra domain I.

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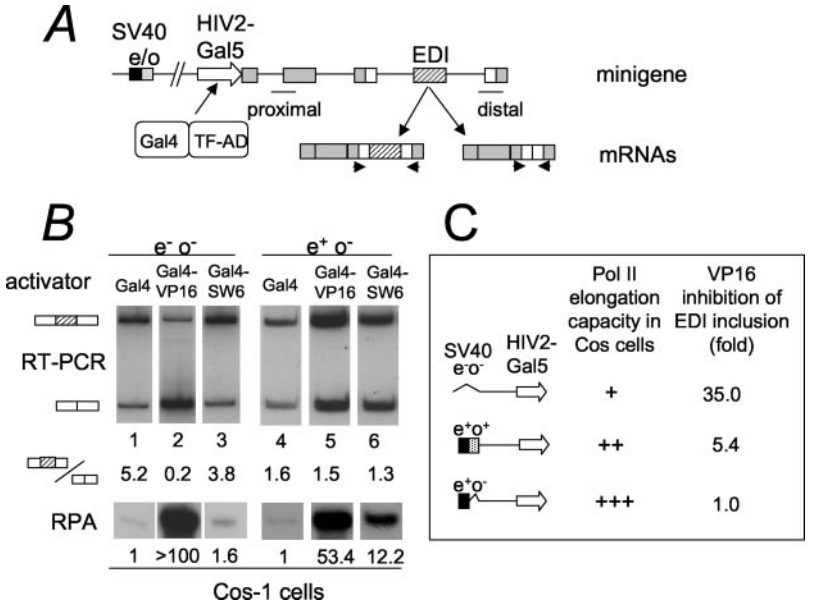

FIG. 1. **The effect of Gal4-VP16 on alternative splicing depends on the template.** *A*, scheme of the minigene transfected to assess alternative splicing. *Gray exons*: human α-globin; *empty* and *dashed exons*, human FN; *black* and *dotted boxes*, SV40 enhancer and origin of replication, respectively. *TF-AD*, transcription factor activation domain. *Arrows*, primers used to amplify the mRNA splicing variants by RT-PCR. *Lines*, proximal and distal probes used for RPA. *B*, COS-1 cells were transfected with 600 ng of pSVEDA/Gal_5-HIV-2 plus 400 ng of pCMVβgal as a control for transfection efficiency. RNA splicing variants were detected by radioactive RT-PCR and analyzed in 6% native polyacrylamide gels (*top*). Ratios between radioactivity in EDI^+ bands and radioactivity in EDI^- bands are shown under each lane. *Bottom*, RPA with the distal probe shown in *A* to measure total levels of transcripts. RT-PCR and RPA ratios correspond to a representative experiment of at least three independent transfections. *C*, correlation between inhibition levels of EDI inclusion by Gal4-VP16 and different combinations of the SV40 enhancer and origin of replication in the template minigene.

SV40 transcriptional enhancer, which stimulates pol II elongation (16), stimulates EDI exon skipping (14). (*d*) VP16, a herpesvirus transcriptional activator that stimulates pol II elongation (16), also provokes exon skipping (15).

The use of a promoter swapping strategy proved to be very useful to define the transcriptional control of alternative splicing. However, promoters are not swapped in nature. Pursuing a more "physiological" approach, we study here the effects of different transcriptional activators acting on a single promoter. Three functional classes of transcriptional activation domains have been defined according to their abilities to stimulate the initiation and elongation steps of pol II transcription *in vivo* (17). Class I activators, such as Sp1 and CTF/NF1, stimulate initiation; class IIA activators, of which HIV-1 Tat is the best example, stimulate predominantly elongation (18, 19); and class IIB activators, which include VP16, p53, and E2F1, stimulate both initiation and elongation. In this report we show that whereas Gal4-VP16 provokes lower inclusion of the EDI exon relative to Gal4 DNA binding domain, class I and class IIA activators alone have little effect on alternative splicing of the EDI exon. However, class I and IIA activators synergize with one another in provoking exon skipping (*i.e.* lower exon inclusion), which correlates with their reported synergism in stimulating pol II elongation.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pSVEDA/Gal_5-HIV-2 has been described previously (13). This plasmid contains the α-globin/FN minigene reporter for alternative splicing of the EDI exon under the control of the HIV-2 promoter fused to five copies of the target site for the DNA binding domain of *Saccharomyces cerevisiae* Gal4. This plasmid generates transcripts with the Tar sequence at their 5′ ends, which is a binding site for HIV-1 or HIV-2 Tat. The construct contains a SV40 enhancer/origin (e/o) located approximately at −600 bp with respect to the transcriptional start site. Strategies for disruption/deletion of either the enhancer or the origin elements individually or together have been described (14, 15). Expression vectors for Gal4 fusion proteins Gal4-VP16-(410–490), Gal4-SW6, Gal4-Sp1B-(263–499), and Gal4-CTF-(399–499) and the control vector Gal4-(1–147) were described previously (17).

Transfections and Alternative Splicing Assay—Culture of Hep3B and COS-1 cells, transfections with LipofectAMINE (Invitrogen), RNA preparation (20), and radioactive RT-PCR amplification of splicing isoforms using specific primers were described previously (11, 13).

RNase Protection Assay (RPA)—The design of distal and proximal riboprobes and RPA conditions were described previously (15).

RESULTS

VP16, SV40 Enhancer, T-antigen-dependent Replication, and Alternative Splicing—When α-globin/FN minigenes containing the SV40 enhancer and origin of replication are transfected into COS-1 cells, which constitutively express SV40 T-antigen, RNA pol II elongation is controlled by both viral elements. On one hand, the origin of replication allows 2–3 rounds of minigene replication which in turn provokes a decrease in pol II processivity with a concomitant increase in EDI exon inclusion (15). On the other hand, the transcriptional enhancer provokes more efficient pol II elongation with a concomitant decrease in EDI exon inclusion (14). In a previous report (15) we showed that transcriptional activation by Gal4-VP16 targeted to pSVEDA/Gal_5-HIV-2 minigene (Fig. 1*A*) inhibits EDI inclusion in COS-1 cells. This inhibition was observed both in the presence (by 5-fold) and in the absence (by 35-fold) of the intact e/o element. The VP16 effect on splicing was attributed to its ability to promote elongation because a VP16 mutant with four Phe-Ala substitutions (21), named SW6, which predominantly promotes initiation (17), has no important effect on EDI splicing.

VP16 and the SV40 enhancer stimulate elongation whereas T-antigen-dependent replication inhibits it. If elongation efficiency is inversely proportional to EDI inclusion, then abolition of replication but preservation of the enhancer effect should lead to a situation of efficient elongation where additional effects of VP16 would be small or negligible. To test this prediction we assessed the effects of VP16 and SW6 on COS-1 cells transfected with Gal_5-HIV-2 promoter minigene lacking the SV40 origin of replication but carrying the SV40 transcriptional enhancer (e^+/o^-). Fig. 1*B*, *lanes 4–6*, shows that al-

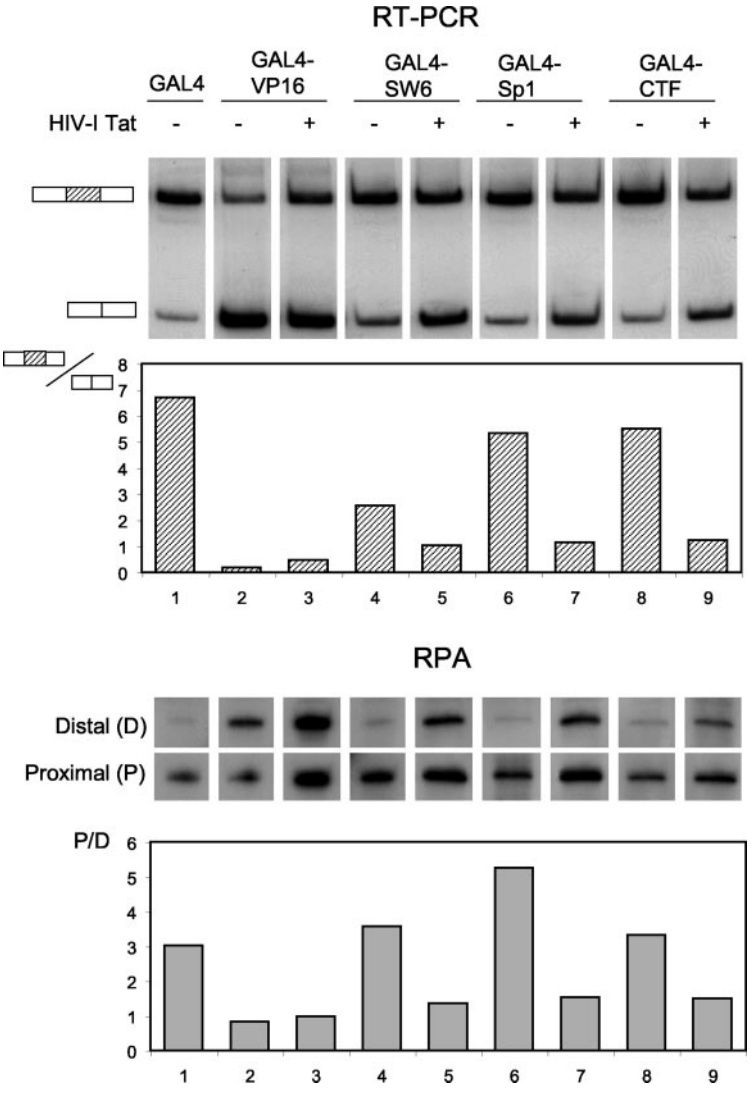

FIG. 2. **Tat synergism in the transcriptional control of EDI splicing.** COS-1 cells were transfected with 600 ng of pSVEDA/Gal_5-HIV-2 plus 10 ng of the corresponding expression plasmid for Gal4 fusion proteins and 50 ng of a plasmid expressing HIV-1 Tat (*lanes 3*, *5*, *7*, and *9*) or its empty vector (*lanes 1*, *2*, *4*, *6*, and *8*). Transfections also contained 340 ng of pCMVβgal as a control for efficiency. RT-PCRs and RPAs were as in Fig. 1. Ratios shown as *bars* correspond to a representative experiment of at least three independent transfections.

though Gal4-VP16 activates transcription by 53-fold as determined by RNase protection assay, there is no effect on EDI inclusion as determined by RT-PCR (*lane 5*). Gal4-SW6 has practically no effect on splicing and, although less potently than Gal4-VP16, still activates transcription by 12-fold. Positive controls for VP16 action on splicing are shown in *lanes 1–3*; as reported previously (15), in the absence of both the enhancer and origin of replication Gal4-VP16 activates transcription by more than 100-fold but inhibits EDI inclusion by 26-fold.

HIV-1 Tat, Sp1, and CTF Activation Domains Alone Have No Effect on EDI Splicing—Fig. 1*C* shows that stimulation of exon skipping by Gal4-VP16 correlates directly with pol II elongation capacity. We extended these experiments to assess the effects of other transcriptional activators on the e^-o^- construct. Gal4-VP16 stimulates initiation and elongation (Type IIB) whereas Gal4-SW6 only stimulates initiation (Type I). In this context it remained important to analyze the effects on splicing of activators that stimulate only elongation (Type IIA) and of other Type IIB activators. COS-1 cells were transfected with pSVEDA/Gal_5-HIV-2 and expression vectors for Gal4 fusion proteins or a Gal4 empty vector. Overexpression of HIV-1 Tat either in a soluble form or targeted to the promoter via a Gal4 DNA binding domain had no effect on EDI splicing (results not shown). Neither Gal4-Sp1 (type I, glutamine-rich) nor Gal4-CTF (type I, proline-rich) caused a significant inhibition of EDI inclusion, compared with the effects of Gal4-VP16 (Fig. 2, compare RT-PCRs of *lanes 1*, *2*, *6*, and *8*). This indicates that, independently of the nature of the transactivation domain (acidic, glutamine-rich, or proline-rich), type I activators, which preferentially stimulate initiation, have no effect on EDI splicing.

Tat Synergizes with Type I Activators to Enhance EDI Exon Skipping—Blau *et al.* (17) showed that type I activators such as Sp1, SW6, and CTF specifically synergize with Tat in transcriptional activation by increasing the efficiency of elongation whereas type IIB activators such as VP16 do not, when present in multiple copies at the promoter. To assess Tat synergism on alternative splicing, COS-1 cells were transfected with the alternative splicing reporter pSVEDA/Gal_5-HIV-2, expression vectors for Gal4 fusion proteins, and a Tat expression plasmid. Results in Fig. 2 show that Tat does not further enhance the stimulation of EDI skipping caused by Gal4-VP16 (*lane 3*) consistent with the fact that Tat and VP16 do not synergize at the transcriptional level. In contrast, type I activators Gal4-SW6, Gal4-Sp1, and Gal4-CTF synergize strongly with Tat in enhancing EDI skipping and reducing EDI inclusion (*lanes 5*, *7*, and *9*).

Tat Synergism Correlates with Shorter/Longer Transcript Ratios—Transcriptional processivity is defined as the ability to elongate through sites where polymerase is prone to pause or terminate prematurely. Nonprocessive polymerases arrest or are released at some positions within the transcription unit, generating an excess of short promoter proximal transcripts relative to long promoter distal transcripts in the steady state.

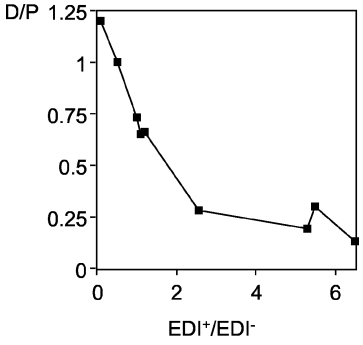

FIG. 3. **pol II processivity correlates inversely with EDI inclusion.** D/P (distal/proximal) and EDI$^+$/EDI$^-$ ratios correspond to data in Fig. 2.

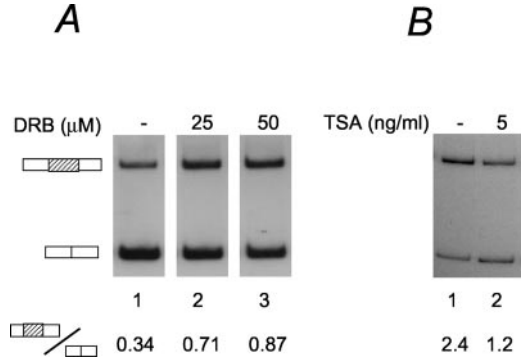

FIG. 4. *A*, DRB stimulates EDI inclusion. Hep3B cells were transfected for a total of 36 h with 600 ng of pSVEDA/FN (FN promoter) (11) plus 400 ng of pCMVβgal as a control for transfection efficiency. DRB was added to the culture medium at the indicated concentrations for the last 9 h of transfection. *B*, TSA inhibits EDI inclusion. COS-1 cells were transfected for a total of 48 h with 600 ng of pSVEDATot (α-globin promoter, containing the SV40 origin of replication) (9) plus 400 ng of pCMVβgal as a control for transfection efficiency. TSA was added to the culture medium at the indicated concentration for the last 24 h of transfection. RT-PCRs were as in Fig. 1. A representative experiment of at least 3 independent transfections is shown.

Relative proportions of these transcripts can be estimated by quantitative RPA performed with 5′ and 3′ probes. This assay is based on various observations that prematurely terminated RNAs are stable (17, 22). We have recently shown that in our system RPA proximal/distal ratios correlate with ratios of RNA pol II densities upstream and downstream of the EDI exon, determined by chromatin immunoprecipitation with an antibody directed to pol II (14). RPAs in Fig. 2 show that proximal/distal ratios decrease when Tat synergizes with an activator (note the similarity between the bar patterns of RT-PCR and RPA quantifications), consistent with the fact that Tat synergism promotes mainly pol II elongation (17). In Fig. 3 we plotted pol II processivity (distal/proximal ratios, *D/P*) against EDI$^+$/EDI$^-$ in nine independent conditions with different activator combinations for a single promoter (Fig. 3). D/P ratios decrease nearly linearly with the increase of EDI inclusion up to EDI$^+$/EDI$^-$ ratios around 2. For higher EDI$^+$/EDI$^-$ ratios negative correlation is less pronounced.

DRB Increases EDI Inclusion—Phosphorylation of the pol II CTD by the kinase activity of P-TEFb, CDK9-CycT (23), is involved in the stimulation of elongation by activators. P-TEFb converts the polymerase from a nonprocessive to a processive form, which is consistent with the fact that inhibitors of this kinase, such as dichlororibofuranosylbenzimidazole (DRB) inhibit pol II elongation (24). Cells transfected with an EDI-containing minigene under the control of the FN promoter were treated with DRB. Concentrations of DRB as low as 25 μM, known to be more specific to inhibit CTD phosphorylation (25, 26), caused a 2-fold increase in EDI inclusion (Fig. 4*A*).

Histone Acetylation Inhibits EDI Inclusion—The mechanism by which VP16 activates elongation involves the promotion of histone acetylation far downstream of promoters (27). We reasoned that inhibition of histone deacetylation might mimic the VP16 effect on EDI splicing. Fig. 4*B* shows that trichostatin A (TSA), a potent inhibitor of histone deacetylation, inhibits EDI inclusion by 2-fold. This effect is observed only when the minigene template is allowed to replicate (COS-1 cells, plasmid containing the SV40 origin of replication) a condition that favors a more compact chromatin organization. EDI splicing elicited by templates lacking the origin of replication transfected in COS-1 cells, or possessing the origin but transfected in Hep3B cells, is not affected by TSA (results not shown).

DISCUSSION

Until recently it was assumed that proteome complexity in higher eukaryotes was mainly achieved through the differential transcriptional regulation of a large number of genes. The observation that the human genome contains a smaller number of genes than foreseen enhances the contribution of alternative splicing to the observed complexity. In this context, the mechanisms that control tissue-specific and developmentally regulated alternative splicing are poorly understood and deserve more investigation. The finding that promoter structure is important for alternative splicing confirmed the importance of the coupling between transcription and pre-mRNA processing in the generation of protein diversity. The promoter effect was put in evidence through a strategy of promoter swapping (11, 13). However, since most genes have a single promoter, the only conceivable way by which promoter architecture could control alternative splicing *in vivo* should be based on the differential occupation of promoters by transcription factors of different natures and mechanisms of transcriptional activation. Such a differential occupation, either tissue-specific or signal transduction-dependent, could also involve proteins that act as couplers between the transcription and splicing machineries or with dual functions both as splicing and transcription factors (28, 29).

In this report we analyzed the individual and combined effects on alternative splicing of five transcription factors (VP16, SW6, Sp1, CTF, and Tat) acting on a single promoter. To guarantee that the observed effects were direct, we used Gal4 fusion proteins prepared with the transactivation domains of these factors to target them specifically to the promoter driving transcription of the alternative splicing reporter. Results strikingly indicate that the effects of these factors on splicing correlate strictly with their previously reported abilities to control RNA pol II elongation (17).

The original finding that promoter strength was irrelevant for the control of EDI alternative splicing (11) is illustrated here (Fig. 1*B*) with much higher levels of transcription. In two different conditions (one in the absence and the other in the presence of the SV40 e), VP16 dramatically activates overall transcription by 50–100-fold. Nevertheless, whereas VP16 greatly inhibits EDI inclusion in the absence of the enhancer, it has no effect in its presence.

Transcriptional activation by VP16, under conditions where it enhances pol II processivity (e$^-$/o$^-$), leads to a significant inhibition of EDI inclusion (26-fold), whereas activation on constructs that have high basal pol II processivity (e$^+$/o$^-$) has no effect on EDI splicing (Fig. 1*C*). In the same direction, the VP16 mutant SW6 activates transcription by 12-fold in the presence of the enhancer, having practically no effect on splicing.

The experiments in Figs. 2–4 using different effectors of transcriptional initiation and elongation lead to similar conclusions. Class I activators like SW6 (acidic), Sp1 (glutamine-

rich), and CTF/NF1 (proline-rich) that predominantly promote initiation had little effect on EDI splicing. On the other hand, VP16 (acidic), which promotes both initiation and elongation, stimulated EDI exon skipping. HIV-1 Tat, which has little effect on transcription in the absence of other activators, had no effect on EDI splicing either as a "soluble" factor nor as a Gal4 fusion protein targeted to the promoter (not shown). Stimulation of both initiation and elongation can be reconstituted by the synergistic activities of Tat with class I activators (17). In agreement with these observations, Tat synergizes with SW6, Sp1, and CTF but not with VP16 in promoting transcriptional processivity in our system and therefore in inhibiting EDI inclusion (Fig. 2). HIV-1 Tat controls transcriptional elongation in part through a complex named Tat-SF (30). *In vitro* Tat-SF supports basal, Sp1-activated, and Tat-activated transcription (31). Our results are consistent with the suggested role of Tat-SF in transcription/splicing coupling via its association with spliceosomal U small nuclear ribonucleoproteins (32).

Elongation factor P-TEFb converts the polymerase from a nonprocessive to a processive form, which is consistent with the fact that inhibitors of this kinase such as DRB inhibit pol II elongation (24). Cells transfected with EDI splicing reporters and treated with DRB displayed a 2-fold increase in EDI inclusion compared with untreated cells (Fig. 4*A*).

Our finding that trichostatin A, an inhibitor of histone deacetylases, inhibits EDI inclusion (Fig. 4*B*) favors the hypothesis that acetylation of the core histones would facilitate the passage of the transcribing polymerase, which is in turn consistent with the proposal of chromatin opening mediated by DNA tracking (2) by a transcribing pol II complex piggybacking a histone acetyltransferase activity (33).

It is important to bear in mind that the effects of DRB and TSA on EDI splicing are consistent with those of the Gal4 fusion proteins but, while the latter are clearly direct effects, we cannot rule out an indirect mechanism for the former. The mechanism by which elongation affects EDI splicing is conditioned by pre-mRNA sequence constraints as suggested before for other genes (34, 35). EDI exon skipping occurs because the 3′ splice site of the upstream intron is suboptimal compared with the 3′ splice site of the downstream intron. If the polymerase pauses anywhere between these two sites, only elimination of the upstream intron can take place. Once the pause is passed or the polymerase proceeds, there is no option for the splicing machinery but to eliminate the downstream intron, which leads to exon inclusion. A highly processive elongating pol II, or the absence of internal pauses, would favor the simultaneous presentation of both introns to the splicing machinery, a situation in which the stronger 3′ splice site of the downstream intron outcompetes the weaker 3′ splice site of the upstream intron, resulting in exon skipping (5).

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