Transcriptional activators control splicing and 3´-end cleavage levels

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SUMMARY

We have investigated whether transcriptional activators influence the efficiency of constitutive splicing and 3'-end formation, in addition to transcription levels.

Remarkably, strong activators result in higher levels of splicing and 3'-cleavage than weak activators and can control the efficiency of these steps in pre-mRNA processing separately. The pre-mRNA processing-stimulatory property of activators is dependent on their binding to promoters, but is not an indirect consequence of the levels of transcripts produced. Moreover, stimulation of splicing and cleavage by a strong activator operates by a mechanism that requires the carboxyl-terminal-domain (CTD) of RNA pol II. The splicing-stimulatory property of activators was observed for unrelated transcripts and for separate introns within a transcript, indicating a possible general role for strong activators in facilitating pre-mRNA processing levels. The results suggest that the efficiency of constitutive splicing and 3'-end cleavage is closely coordinated with transcription levels by promoter-bound activators.
INTRODUCTION

The generation of mature mRNA involves the addition of an m7G cap at the 5′-end, splicing, and cleavage and polyadenylation at the 3′-end of precursor (pre-)mRNAs. Although these steps can occur in isolation of each other, the majority of pre-mRNA processing occurs as RNA polymerase II (pol II) is transcribing. This is evident from numerous microscopy studies, in which the removal of most introns is observed to coincide with nascent transcript synthesis, as well as from more recent studies which demonstrate that transcription and pre-mRNA processing steps are physically coupled and can influence one another (1-3).

The largest subunit of pol II contains a carboxyl terminal domain (CTD), which in mammals consists of 52 repeats of the heptapeptide consensus sequence YSPTSPS (4,5). Pol II at the stage of transcription initiation is associated with a large holoenzyme complex, which is recruited to promoters by sequence-specific transcriptional activators (6-10). In some cases, stimulation of transcription by activators requires the CTD (11-13). Recent work has demonstrated that the CTD is also important for pre-mRNA processing. Truncation of the CTD in vivo reduces the efficiency of capping, splicing and 3′-end formation (14-16). Moreover, purified pol II, or the CTD itself, can stimulate splicing as well as the 3′-end processing of transcripts in vitro (17-19). Consistent with
these results, factors involved in capping, splicing and 3´-end formation have been found to interact with the CTD (1,3,20).

It is not known at which stage of transcription different pre-mRNA processing components are recruited to transcription complexes, nor how CTD-associated components influence pre-mRNA processing. The 3´-end cleavage-polyadenylation stimulatory factor (CPSF) interacts with the general transcription factor TFIID during transcription initiation (21), as well as with the CTD (15), indicating that it is recruited to transcription initiation complexes prior to its role in cleavage. A number of splicing or splicing-related factors have recently been detected in association with holoenzyme complexes involved in transcription initiation, further suggesting that specific pre-mRNA processing components may be recruited to active genes at the stage of transcription initiation (22,23). Recent work has also shown that the type of promoter or activator used to drive transcription can influence the level of inclusion of alternative exons in transfected pre-mRNA reporters (24-27), suggesting that events occurring as early as promoter recognition may play an important role in the regulation of splicing. However, whether transcriptional activators can also influence the efficiency of constitutive pre-mRNA processing reactions, including splicing and 3´-end formation, has not been investigated.

In the present study we provide evidence that tethering transcriptional activators of different strengths to the promoter of pre-mRNA reporters can modulate the efficiency
of splicing and 3′-end cleavage. Stronger activators resulted in higher levels of splicing of constitutive introns, as well as higher levels of 3′-end cleavage, than weak activators. Stronger transcriptional activators were also found to stimulate 3′-end cleavage independently of splicing. The differential stimulation of splicing and cleavage by these activators was not a consequence of the different levels of transcripts produced but, instead, appeared to be due to an inherent functional property of the transcriptional activator. Our results also demonstrate that the increased level of splicing and cleavage promoted by a strong activator is dependent on the CTD of pol II, indicating an important role for this domain in mediating the effects of promoters on pre-mRNA processing. In summary, our results suggest that an important role of transcriptional activators is to modulate pre-mRNA processing efficiency in accordance with transcript levels.

**EXPERIMENTAL PROCEDURES**

**Transfections and RNA Analysis.**

Human 293 cells grown on 15 cm plates were transfected using FuGENE-6 (Roche) as per the manufacturer’s instructions, with 5 µg reporter, 0.5 µg of Gal4-(fusion) expression plasmid (or as indicated in Fig. 2), 0.5 µg pSP-VA1, and the appropriate empty vector to maintain equal amounts of DNA between samples. Cells were harvested 48 hours post-transfection and RNA was isolated as previously described.
In Fig. 4, 5 µg of α-amanitin-resistant pol II expression plasmid or the corresponding empty vector was transfected in addition to the above plasmids. 2.5 µg/mL α-amanitin was added to the medium after 16 hours and RNA was isolated after another 48 hours. RNase protection assays were performed as previously described. RNase protection analysis products were detected by autoradiography and quantified using a BioRad phosphorimager, after electrophoresis on a 6% polyacrylamide-urea gel.

Spliced:unspliced and cleaved:uncleaved RNA ratios were calculated as follows. Band intensities were first normalized to the number of U residues; the corrected unspliced or uncleaved band intensities were divided by the corresponding corrected spliced or cleaved band intensities, respectively. Primer extension analysis was performed using 10 µg of total RNA and the oligonucleotide primer sequence 5´-GAGGTTTTTAAAAGCAAGCAAGCGTGGGGCCG—3´ which is complementary to a sequence located 148 bases downstream of the expected transcriptional start site, within the HIV-TAR region of the Gal5HIV2dsx pre-mRNA reporters.

**Plasmids.**

Previously described plasmids include pSVEDA Gal5HIV2 (24) and expression plasmids for Gal4-FBP (29), Gal4-VP16 (pSGVPΔ490), Gal4-SW6 (pSGSW6), Gal4-TAT (pHKG Tat1-48), and Gal4-p53 (pGalM p53(1-92)) (30).
Pre-mRNA reporters and RNase protection probes:

Gal5HIV2dsx(+/-ESE) were constructed by replacing the Bgl II - EcoR I segments of pAD2MLdsxΔ and pAD2MLdsx(GAA)₆ plasmids (31) with a Bgl II - EcoR I PCR fragment corresponding to the Gal5HIV2Tar portion of plasmid pGal5HIV2CAT (28), using PCR primers 5'-GAAGATCTCTCGAGCAGCTGAAGCTTGCA-3' and 5'-GCTGATGAATTCGAGCTCGG-3'. Gal5HIV-dsx-Δint(+/-ESE) reporters were constructed by ligation of PCR products generated in Pfu polymerase reactions containing the Gal5HIV2dsx(+/-ESE) plasmids, a reverse primer that hybridizes specifically to exon 3 sequences immediately upstream of the intron, and a forward primer to exon 4 sequences immediately downstream of the intron. The following primer sequences were used: 5'-CCTCTTCGATTCGCCGGG-3' and 5'-GCCAATACGTTGTGAATG-3'.

pSVEDaGal5HIV2 was a generous gift from A. Kornblihtt, and has been previously described (24).

RNase protection probes for 5’-end, splicing, and VA-RNA analysis (Figs. 1–3) have been previously described (28,31). The RNase protection probe for 3’-end cleavage analysis (Figs. 1 and 3) was constructed by PCR amplification of sequences including 40 bp downstream of the cleavage position of the SV40 late polyadenylation signal, and 196 bp upstream including sequences in dsx exon 4 and the ESE using PCR primer sequences 5’-GGGGGGGAGTACTCCCCTCAACATAATTGGA-3’ as a primer forward, and 5’-
GGGGGGGTAATACGACTCAGCTATAGGCTCGAGCCCCCCTGAACCTGAAACAT
TAAAATG-3’ as a reverse primer, which contains the T7 RNA polymerase promoter
sequence for in vitro antisense transcription from the PCR product. The PCR product
was digested with Sca I and Xho I, and subcloned into the Sma I and Xho I sites of the
polylinker in pBluescript SK+ (Stratagene). Digesting the resulting plasmid with Not I,
and transcribing with T7 RNA polymerase generates an RNase protection probe that will
result in protected fragments from Gal5HIV2dsx(+ESE) reporter transcripts with sizes of
236 bases for uncleaved and 196 bases for cleaved RNA.

The RNase protection probe for detecting splicing of α-1 globin introns 1 and 2
from pSVEDaGal5HIV2 (Fig. 3) was generated by PCR amplification of the 205 bp of α-
1 globin exon 2 and the flanking intronic sequences including 113 bp upstream and 35 bp
downstream. The PCR primer sequences used were 5’-
CGGGGATCCCGCTCCCTCCCTGCTCCGACCC-3’ as a forward primer, and 5’-
CGGTAATACGACTCAGCTATAGGGCTTGATATCGAATTCCTGCACCCTCGACC
CAGATCGCTCC -3’ as a reverse primer, which contains the T7 RNA polymerase
promoter sequence for in vitro antisense transcription from the PCR product. However,
the PCR product was digested with EcoR I and BamH I, and was subcloned into the
corresponding sites in the polylinker of pBluescript SK+ (Stratagene). Upon digestion
with Xba I and transcription with T7 RNA polymerase, the resulting plasmid yields an
RNase protection probe of length 424 bases, and will protect RNAs generated from
pSVEDaGal5HIV2 with the following sizes: fully unspliced, 353 bases; unspliced at intron 1 only, 318 bases; unspliced at intron 2 only, 240 bases; and fully spliced, 205 bases.

Pol II expression plasmids:

Pol IIΔ was constructed by PCR amplification and sub-cloning of nucleotides 1 to 4767 of an α-amanitin resistant form of the largest subunit of pol II from pAT7GRPBIαAm (32) into mammalian expression plasmid pcDNA3.1(-) (Invitrogen). The coding sequence is flanked by an upstream FLAG epitope sequence and downstream Xba I and Cla I restriction sites. Coding sequences for CTD heptapeptide 1 to 52 (for pol II 1-52) were generated by PCR amplification and sub-cloned into the Xba I and Cla I sites of pol IIΔ. Primer sequences and more detailed descriptions of the construction of pol II expression plasmids are available from the authors upon request.

RESULTS

Transcriptional activators influence splicing and 3'-end cleavage levels

To investigate whether transcriptional activators influence pre-mRNA processing levels, we tethered different transactivation domains to the promoter of a model pre-mRNA reporter (Gal5HIV2dsx) derived from exons 3 and 4 of the Drosophila doublesex
(dsx) gene (Fig. 1A). The dsx pre-mRNA requires an exonic splicing enhancer (ESE) in exon 4 for efficient processing in vitro and in vivo (31,33-36). To determine whether activators might affect ESE-dependent or ESE-independent processing differently, we compared splicing and 3′-end cleavage levels of Gal5HIV2dsx(+intron) transcripts, with or without a typical mammalian ESE consisting of six GAA repeats inserted into exon 4. The Gal5HIV2dsx pre-mRNA reporter contains an HIV2 promoter and five upstream binding sites for the yeast Gal4 DNA binding domain (DBD), and can be activated by Gal4 DBD fusion proteins containing different transcriptional activation domains. The Gal5HIV2dsx(+/- ESE) reporters were transiently transfected into human 293 cells, initially with expression plasmids for Gal4 DBD fusion proteins containing either a strong activation domain, derived from the Herpes virus VP16 protein (Gal4-VP16), or a relatively weak activation domain corresponding to a mutant derivative of Gal4-VP16, “Gal4-SW6” (37) (refer to Fig. 1B legend). RNA isolated from the transfected cells was analyzed by RNase protection using probes for detection of splicing or 3′-end cleavage (Fig. 1B; refer to Fig. 1A), as well as a probe for detection a pol III VA-RNA (refer to Experimental Procedures), which served as an internal control for transfection efficiency and RNA recovery. Primer extension analysis, and RNase protection with an antisense probe spanning the transcription start site of the dsx reporters, were also performed to assess whether initiation occurred at the correct position (Fig. 1E and data not shown).
Ratios of spliced to unspliced and cleaved to uncleaved RNA, as well as transcript levels, were routinely determined from three independent transfection experiments.

In addition to stimulating transcription greater than ten-fold (as determined by summation of unspliced and spliced bands) compared to Gal4-SW6, Gal-VP16 also resulted in approximately a four-fold higher ratio of spliced:unspliced RNA and a similar higher ratio of cleaved:uncleaved RNA of the Gal5HIV2dsx(+ESE) pre-mRNA (Fig. 1B, compare lanes 4 and 6; refer also to the bar graph in Fig. 1C, which represents quantification of the data in Fig.1B as well as three separate experiments analyzing Gal4-VP16, Gal4-SW6 and additional activators described below). Gal4-VP16-dependent stimulation of splicing and 3'-end cleavage, by approximately five-fold and six-fold, respectively, was also observed in the absence of the ESE (compare Fig. 1B lanes 3 and 5; refer also to data shown in Fig. 1C). The differences in levels of splicing and cleavage resulting from activation of transcription by Gal4-VP16 vs. Gal4-SW6 was not due to differential stability of transcripts. Actinomycin-D-chase experiments revealed no difference in the half-life of unspliced or spliced transcripts generated by these activators (data not shown).

To investigate whether other activators can influence pre-mRNA processing, we next tested Gal4-fusions containing activation domains from the HIV-1 Tat transactivator (TAT), c-myc Far upstream element binding protein (FBP) and p53 tumor suppressor.
protein (Fig. 1C). Compared to each other as well as to Gal4-VP16 and Gal4-SW6, each activation domain resulted in a different level of splicing and 3′-end cleavage of the Gal5HIV2dsx(+/-ESE) substrates, as well as different transcript levels. The highest levels of splicing and cleavage were observed consistently with strong activation domains, derived from p53 and VP16, whereas lower levels were observed with relatively weak activation domains derived from SW6, TAT and FBP (refer to the bar graph in Fig. 1C). Splicing and cleavage levels paralleled each other and varied by as much as 18-fold between the different transactivation domains. Independent of the specific activator used to drive transcription, the presence of an ESE consistently stimulated splicing (~four-fold) and 3′-end cleavage (~five to ten-fold) (compare bar graphs in Fig. 1C). Thus, irrespective of which activator is used to drive transcription, splicing and cleavage levels are closely coordinated. However, the particular activator used to drive transcription, independently of the presence of an ESE, markedly affects the level of splicing and cleavage of a transcript.

**Transcriptional activators influence 3′-end cleavage in the absence of splicing**

We next determined whether the Gal4-activators can modulate 3′-end formation in the absence of splicing (note: since splicing is prevented in the absence of cleavage on the dsx pre-mRNA (31), it was not possible to determine whether activators modulate splicing independently of cleavage for this substrate). Each Gal4-activator was expressed
with an intronless version of the Gal5HIV2dsxΔInt(+ESE) pre-mRNA reporter and RNase protection assays were performed to measure cleavage levels (Fig. 1D). Analysis with the 3´-end protection probe revealed that different activators resulted in cleavage levels of the intronless substrate that were similar to those observed with the intron-containing reporter (compare lanes 1-5); Gal4-VP16 was the exception, resulting in even greater stimulation of cleavage of the intronless reporter (compare data in Figs. 1D and 1C). Thus, transcriptional activators can modulate 3´-end cleavage levels in the absence of splicing.

Transcripts generated by different activators are accurately initiated

The activator-dependent changes in splicing and 3´-end cleavage efficiency were not a consequence of altered initiation of transcription. The 5´ ends of RNAs generated by Gal4-SW6 or VP16-activated transcription of the Gal5HIV2dsx(+ESE) reporter were mapped by primer extension (Fig. 1E; refer to IA). Initiation was only detected at the expected position (Fig. 1E, compare lanes 2,3). Analysis of the 5´ ends of transcripts generated by each Gal4-activator by RNase protection, using an antisense probe spanning the transcription start site, also showed that initiation occurred at the correct position and not at spurious upstream sites (data not shown). Thus, the different levels of splicing and 3´-end cleavage observed with each activator are not a consequence of altered transcription initiation.
Promoter-bound activators influence splicing and cleavage levels independently of transcript levels

None of the activators described above resulted in a significant change in the level of splicing or cleavage of the dsx reporter transcripts when driven by an adenovirus major late (Ad2ML) promoter which lacks Gal4 binding sites (data not shown; see also Fig. 4). Moreover, the increased levels of splicing and cleavage were not an indirect consequence of the increased levels of transcripts. For example, splicing and cleavage were more efficient when the dsx(+/-ESE) pre-mRNAs were transcribed from the Ad2ML promoter which, compared to the Gal4-VP16-activated Gal5HIV2 promoter, produced ~10-fold lower levels of transcripts (Fig. 1C; fold activation of transcription and splicing/cleavage levels are indicated). Furthermore, VP16 and p53 activation domains resulted in similar levels of splicing and cleavage-stimulation of the dsx pre-mRNAs, but VP16 consistently yielded at least two-fold higher levels of transcripts than p53.

In order to further establish whether the level of splicing and cleavage of reporter transcripts reflects the type of activator used to drive transcription, rather than the specific level of transcripts produced, we determined whether the efficiency of splicing and cleavage is altered when Gal4-VP16 is expressed at reduced levels (Fig. 2). Reducing Gal4-VP16 expression to an extent where transcript levels are less than Gal4-SW6-
activated levels did not significantly alter the ratios of spliced to unspliced RNA, or cleaved to uncleaved RNA (Fig. 2 and data not shown). These results demonstrate that the higher levels of splicing and cleavage resulting from Gal4-VP16 compared to Gal4-SW6-activated transcription reflect an intrinsic functional property of the VP16 activation domain rather than an indirect consequence of its activity in promoting increased transcript levels.

A role for the pol II CTD in mediating activator-dependent stimulation of splicing and cleavage

The experiments described so far suggest that strong activators could result in increased recruitment of pre-mRNA processing components to promoters, thereby facilitating more efficient processing of the transcripts that are subsequently produced. If this is the case then it is possible that recruitment operates in a manner that is dependent on the pol II CTD, which has been shown previously to facilitate splicing and 3´-end cleavage (refer to Introduction). To investigate whether increased pre-mRNA processing levels dependent on a strong activator is mediated by the CTD, we compared splicing and cleavage levels of the Gal5HIV2dsx(+ESE) pre-mRNA transcribed by α-amanitin-resistant and Flag epitope-tagged derivatives of pol II, with or without a CTD, and when activated with either Gal4-VP16 or Gal4-SW6 (Fig. 3). Immunoblotting confirmed...
similar levels of expression of these pol II derivatives (data not shown). Consistent with a previous report (38), full levels of Gal4-VP16-activated transcription required the CTD (Fig. 3, lanes 7-9). Whereas deletion of the CTD did not have a pronounced effect on the level of splicing or cleavage of transcripts generated by the Gal4-SW6 activator, Gal4-VP16 resulted in approximately a four-fold increase in splicing and a six-fold increase in cleavage that was dependent specifically on the CTD (Fig. 3, compare lanes 6,9 with 5,8; refer to adjacent bar graphs). This indicates an important role for the CTD in mediating the increased splicing and cleavage levels that are specifically dependent on a strong activator.

**Activator-dependent stimulation of splicing occurs on unrelated transcripts**

To establish whether the increased level of dsx pre-mRNA processing observed with strong versus weak activators reflects a more general effect of activators on pre-mRNA processing, we next determined the influence of activator-stimulated transcription on the splicing of an unrelated pre-mRNA reporter, containing sequences from the constitutively spliced human α-1 globin gene, which contains two introns (Fig. 4). This reporter, which also has an HIV2 promoter and five upstream Gal4 DNA binding sites, contains an alternative EDI exon and flanking constitutive exonic sequences from the human fibronectin gene downstream of the α1-globin minigene (pSVEDaGal5HIV2, see
Fig. 4A) (24,25) (see below). Consistent with the results observed with the dsx pre-mRNA reporters, when transcription was driven by the Gal4-VP16 and Gal-p53 activators, splicing of α-1 globin introns 1 and 2 was stimulated by approximately eight- and four-fold, respectively, compared to when transcription was driven by the Gal4-SW6, -TAT, and -FBP activators (Fig. 4B, refer to adjacent bar graph). Since there was a Gal4-VP16 and Gal4-p53-dependent increase in splicing of both α-1 globin introns 1 and 2, it is possible that the effects of activators on splicing is not an indirect consequence of any effects they may have on 5’-end cap formation, as well as effects they have on 3’-end cleavage, since intron 2 is spatially (and most likely temporally) separated from these 5’- and 3’-end processing steps (see Discussion below). Moreover, the increased splicing levels were not an indirect consequence of expression of the activators. As observed for the dsx pre-mRNA reporter, expression of the different activators did not alter splicing efficiency of the α-1 globin introns when the reporter was transcribed from a promoter lacking Gal4 DNA binding sites, in this case the native α-1 globin promoter (Fig. 4C, compare lanes 1-6). The results with the α-1 globin pre-mRNA reporter therefore indicate a more direct and general role for activators in modulating pre-mRNA processing levels.

DISCUSSION
A new role for transcriptional activators in gene expression

Our results establish for the first time a role for transcriptional activators in controlling constitutive splicing and 3′-end cleavage levels. In particular, we provide evidence that there is a parallel relationship between the strength of a promoter-bound activator and the efficiency of constitutive splicing and 3′-end cleavage. These findings extend recent studies in which the effect of different promoters and activators on pre-mRNA processing was studied specifically in the context of alternative splicing regulation (24-27). Several of these studies investigated effects on the inclusion level of the fibronectin EDI exon in the same reporter as we have examined in the present study (pSVEDaGal5HIV2) (24, 25, 27, 35). Stronger promoters or activators resulted in the decreased inclusion of the EDI alternative exon (25,27) (our unpublished observation). This effect was attributed to an altered elongation rate of pol II (27,39). In contrast, we show that strong activators promote increased levels of splicing of the constitutive α1-globin introns in this reporter, as well as the ESE-independent levels of splicing of the dsx pre-mRNA reporter. Thus, stronger activators can result in an increased level of splicing of introns for unrelated transcripts and for different constitutive introns within a transcript. Moreover, we also show for the first time that activator strength can determine the efficiency of 3′-end processing and that this function of activators can occur independently of splicing. Thus, our results provide evidence that an additional and
possible general role for transcriptional activators is the stimulation of constitutive
splicing as well as 3´-end cleavage.

The splicing-stimulatory property of a strong activator occurs independently of
the total levels of transcripts produced, yet requires the CTD of pol II. An attractive
possibility is that activators result in increased pre-mRNA processing levels by recruiting
pre-mRNA processing factors to promoters. These pre-mRNA processing factors could
be recruited by activators directly or indirectly, for example, via association with a pol II
holoenzyme. Association of these factors with the pol II CTD during transcription
elongation would then facilitate the assembly of splicing and 3´-end processing
complexes on nascent transcripts. Consistent with this model, several reports have
described interactions between pre-mRNA processing factors and transcriptional
activators (40), coactivators (41-43), and the holoenzyme (22,23). Thus, it is possible that
increased splicing and cleavage levels promoted by transcriptional activators is the
consequence of increased recruitment of pre-mRNA processing components to
promoters. It is also interesting to speculate that the increased level of splicing and 3´-
end cleavage may be due, at least in part, to increased levels of 5´-end cap formation
resulting from activator-dependent transcription. For example, formation of a cap-
binding complex is known to stimulate the splicing of cap-proximal introns, as well as 3´-
end processing of intronless transcripts (44,45). Future work will be directed at

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determining the mechanism(s) by which activators modulate pre-mRNA processing levels.

We conclude that the efficiency of multiple steps in gene expression, including transcription, splicing and 3′-end cleavage, is determined in part by the strength of promoter-bound activator(s). Such a mechanism may be important to ensure the efficient processing of increased levels of transcripts generated by strong transcriptional activators.
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FIGURE LEGENDS

Fig. 1. Transcriptional activators modulate splicing and 3’-end cleavage levels.

A, Diagram of the Gal5HIV2dsx(+/-ESE) (exonic splicing enhancer) pre-mRNA reporter plasmid. RNase protection probes used to monitor transcription initiation, splicing and 3’-end cleavage, and the position of the oligonucleotide primer used to map the 5´ end of transcripts (left-pointing arrow) are indicated (refer to Experimental Procedures for details).

B, RNase protection analysis of RNA isolated from 293 cells transfected with the Gal5HIV2dsx(+/-ESE) reporters, together with an expression vector for the yeast Gal4 DNA binding domain (Gal4) without an activation domain (N.A.) (lanes 1,2), or Gal4 fused to amino acids 410-490 of VP16 (Gal4-VP16) (lanes 5,6), or the same region of SW6 (Gal4-SW6) (lanes 3,4), which is identical to the VP16 activation domain except for four Phe substitutions (residues 422, 473, 475, and 479) which reduce transactivation levels (30). Each transfection contained a pol III-VA reporter (pSP-VA) as an internal control for transfection efficiency and recovery of RNA. Ten-fold less RNA was analyzed from cells expressing Gal4-VP16 in order to facilitate direct comparison of splicing and cleavage levels. Ratios of spliced/unspliced and cleaved/uncleaved RNAs were quantified and are shown in C (refer to Experimental Procedures for details).

C, Additional Gal4 fusion proteins containing activation domains from TAT (amino acids 1-48), FBP (amino acids 44-490) and p53 (amino acids 1-92) transactivators were
analyzed for effects on splicing and cleavage, as described in B. Splicing and cleavage levels were also analyzed for the dsx(+/ESE) pre-mRNAs transcribed from reporters driven by an adenovirus promoter (Ad2MLdsx(+/ESE)). Spliced/unspliced and cleaved/uncleaved ratios, represented as average values from three independent experiments, are shown as bar graphs with the standard deviations indicated. Ten-fold less RNA was analyzed from cells expressing Gal4-VP16, and five-fold less RNA for cells expressing Gal4-p53, in order to facilitate comparison of splicing and cleavage levels. Relative levels of the transcripts generated by each activator, adjusted according to VA RNA levels, are indicated below the upper bar graph. These values were determined by the addition of unspliced and spliced transcript levels and represented as a percentage of the maximal level obtained by Gal4-VP16-activated transcription.

D. Transcriptional activators influence cleavage in the absence of splicing. 3'-end cleavage levels of Gal5HIV2dsx(-intron)(+ESE) reporter transcripts activated by the different Gal4-activator proteins as indicated were assayed by RNase protection using the cleavage probe shown in A. 100-fold less, and five-fold less RNA was analyzed from cells expressing Gal4-VP16, and Gal4-p53, respectively, to facilitate direct comparison. (*) Indicates irrelevant undigested probe fragment. Adjacent bar graph shows corresponding cleaved/uncleaved ratios, determined from the average values from three independent experiments.
Activated transcripts from the Gal5HIV2dsx (+ESE) reporters are correctly initiated. Transcripts activated by the Gal4-SW6 (lane 2), Gal4-VP16 (lane 3), or Gal4-DBD (N.A., lane 1), were analyzed by primer extension using a primer complementary to a sequence 148 bases downstream of the start site (indicated in A). The expected size of the extension product ("5´-end"), and positions of molecular size markers (in bases) are indicated. Ten-fold less total RNA was analyzed for Gal4-VP16 than Gal4-SW6 or Gal4-DBD samples.

**Fig. 2. Activators influence splicing and cleavage independently of transcript levels.**

Levels of Gal4-VP16 and Gal4-SW6 were titrated by transfection of decreasing amounts of the corresponding expression plasmids, with constant amounts of pre-mRNA reporter and pSP-VA plasmids. RNase protection analysis of splicing levels of Gal5HIV2dsx(+ESE) with no activator (N.A., lane 1), decreasing levels of Gal4-VP16 (0.5 µg, 0.1 µg, 0.05 µg, 0.03 µg, or 0.01 µg in lanes 2-6, respectively), or decreasing levels of Gal4-SW6 (1 µg, or 0.5 µg in lanes 7 and 8, respectively). The adjacent bar graph shows average transcript levels (black bars; determined by addition of spliced and unspliced band intensities) and the corresponding spliced/unspliced ratios (grey bars with standard deviations) (refer to Experimental Procedures).
Fig. 3. The CTD of pol II is required for VP16-stimulated splicing and 3´-end cleavage.

RNase protection analysis of splicing and cleavage, as described in Fig.1B, for Gal5HIV2dsx(+ESE) reporter transcripts generated by α-amanitin-resistant RNA pol II containing either a wild type CTD (pol II 1-52) (lanes 3,6,9) or no CTD (pol II Δ) (lanes 2,5,8), and when activated by either Gal4-SW6, Gal4-VP16, or Gal4 alone (N.A.), as indicated. Controls represent background levels of reporter transcripts in cells not expressing α-amanitin-resistant pol II (lanes 1,4,7). A shorter exposure of the gel is shown for lanes 7-9 to facilitate comparison with lanes containing weaker signals. Spliced/unspliced and cleaved/uncleaved ratios are shown in the adjacent bar graphs, calculated as in IC. Bands present in lane 7 likely represent residual stable transcripts generated by endogenous pol II, and activated by VP16, in the first few hours of the experiment before complete depletion of endogenous pol II as a consequence of α-amanitin treatment. Signals in lanes 8 and 9 are essentially entirely due to plasmid-derived α-amanitin resistant pol II Δ and pol II 1-52, respectively, since the levels of these polymerases are in vast excess of endogenous pol II levels.
Fig. 4. Splicing of different pre-mRNA introns are stimulated by strong transcriptional activators.

A, Diagram of the pSVEDaGal5HIV2 reporter (28). The reporter promoter contains five Gal4 DNA binding sites upstream of an HIV2 promoter, human α-1 globin gene exons (shaded grey), and fibronectin exonic sequences (shaded black). The RNase protection probe used to assay splicing of α-1 globin introns 1 and 2 is shown. B, RNase protection analysis of splicing levels of α-1 globin introns 1 and 2 (as indicated diagrammatically next to gel) of pSVEDaGal5HIV2, when activated by Gal4-SW6, Gal4-TAT, Gal4-FBP, Gal4-VP16, Gal4-p53, or Gal4 alone (N.A.). 100-fold and 50-fold less RNA was analyzed for Gal4-VP16 and Gal4-p53, respectively, than for the other activators in order to facilitate comparison of splicing levels on the same gel. Adjacent bar graphs show the average ratios of spliced/unspliced RNA for each α-1 globin intron, with standard deviations calculated from two independent experiments (ratios were determined as described in the Experimental Procedures. C, Gal4 activation domain fusion proteins were expressed with the same pre-mRNA reporter as in A, but driven instead with the α-1 globin promoter “pSVEDa-Tot”. Splicing levels of α-1 globin introns 1 and 2 were analyzed by RNase protection using the same probe as used in B (refer to A).
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