VP16 activates transcription by stimulating initiation, and for this function the aromatic residue at position 442 within its activation domain is critical. Recent studies have suggested that VP16 also stimulates transcriptional elongation. It has been shown that VP16 can activate transcription tethered downstream of the transcription start site to RNA. Here, we analyze the synergistic activation features of hybrid VP16 fusion proteins when tethered simultaneously to RNA downstream of the start site and to DNA upstream of a promoter in order to investigate its role in postinitiation control of transcription. Upon targeting the VP16 activation domain simultaneously to both DNA and RNA, high levels of transcriptional synergism is observed. Importantly, a transcription-defective VP16 minimal activation domain (amino acids 413–453) mutated at critical residue 442 (phenylalanine → proline) maintained synergism, when bound to RNA, with the DNA-bound wild-type VP16 minimal activation domain. Targeting of this “functionally defective” VP16 minimal activation domain via RNA and an intact activation domain via DNA allowed us to uncover a postinitiation activity for VP16 not previously detected in DNA targeting studies. We suggest that, in addition to stimulating initiation, VP16 also acts at a postinitiation step involving residues other than the critical residue at position 442 within the same 42-amino acid minimal activation domain (amino acids 413–453) required for initiation.

VP16 protein is a component of the herpes simplex virus virion and in addition to its structural role selectively activates transcription of the viral immediate early genes during lytic infection by interacting with host cellular proteins that bind to specific enhancer elements located upstream of each immediate early gene (reviewed in Thompson and McKnight (1992)). The C-terminal 78-amino acid region of VP16 (amino acids 413–490) is highly acidic, and when fused to the DNA binding domain of the heterologous yeast protein GAL4 can activate transcription potently from target promoters containing GAL4-binding sites (Sadowski et al., 1988; Cousens et al., 1989). Mutational analysis of the C-terminal activation domain of VP16 suggests that this domain can be subdivided into the proximal subdomain extending from amino acids 413–456 and the most C-terminal distal subdomain spanning amino acids 457–490. It is concluded, from studies using mammalian cells, that both subdomains are required for overall efficient transcriptional activation by VP16 (Regier et al., 1993; Walker et al., 1993). However, the proximal subdomain (amino acids 413–456) of VP16 constitutes the minimal activation domain and, when fused to GAL4 DNA binding domain, can activate transcription from a minimal promoter, retaining about 50% of the activity of the full-length domain (Triebeneger et al., 1988; Greaves and O'Hare, 1989). Studies have shown that the aromatic residue phenylalanine at position 442 (Phe442) is critical for the function of this truncated activation domain rather than its net negative charge (Cress and Triebeneger, 1991; Regier et al., 1993). Mutation of Phe442 to proline (F442P) within this minimal activation domain abolished transcriptional activity in both in vivo and in vitro transcription assays even when tethered to multiple DNA-binding sites highlighting the critical role of this residue and subdomain in the activation process (Cress and Triebeneger, 1991; Regier et al., 1993; Walker et al., 1993; Jang et al., 1994). This region of VP16 interacts in vitro with components of the basal transcription complex, TFIIID1 (Stringer et al., 1990) and TFIIIB (Lin and Green, 1991; Lin et al., 1991). Mutation of phenylalanine to proline (F442P) disrupted the interaction with both TFIIIB and TBP in vitro (Ingles et al., 1991; Lin and Green, 1991; Lin et al., 1991). It is, therefore, believed that VP16 stimulates transcription by interactions with TFIIID and/or TFIIIB to increase assembly of the preinitiation complex (Choy and Green, 1993; Chi et al., 1995). VP16 has also been shown in vitro to bind basal transcription factors, TFIIH (Xiao et al., 1994) and human positive coactivator 4 (PC4) (Ge and Roeder, 1994), and mutation at position 442 (F442P) substantially reduced these interactions.

The distal most C-terminal 39-amino acid region of VP16 has been shown to constitute an independent domain and to have the ability to activate transcription, but poorly compared to the proximal region (Regier et al., 1993; Walker et al., 1993). Interestingly, this region of VP16 was found in vitro to directly bind dTAF1,40 and nTAF1,32, subunits of Drosophila and human TFIIID, respectively, both of which in turn bind to TFIIB, suggesting a ternary interaction among the activator VP16, the coactivator TAF, and the basal transcription initiation factor TFIIIB (Goodrich et al., 1993; Kimm et al., 1995). Thus, this subdomain may also contribute to VP16’s transcriptional activity by stimulating initiation of transcription. Recent studies

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have suggested that, in addition to stimulating transcription initiation, VP16 also plays a role in transcription elongation (Yankulov et al., 1994; Krumm et al., 1995).

Here, in order to investigate VP16’s role in postinitiation control of transcription, we have taken an independent in vivo functional approach and have analyzed the synergistic activation features of VP16 when tethered simultaneously upstream of a promoter to DNA and downstream of the transcription start site to RNA. Cullen and colleagues reported earlier that the VP16 activation domain could activate transcription from the human immunodeficiency virus-1 long terminal repeat (HIV-1 LTR) when tethered to a promoter-proximal RNA element (Tiley et al., 1992). We have constructed a modified HIV-1 LTR reporter plasmid, pG6RRE (see Fig. 1A) which allowed us to target fusion protein derivatives of VP16 simultaneously to RNA and DNA in transfection experiments in mammalian cells. We find that RNA-bound VP16 can cooperate with DNA-bound VP16 to activate transcription synergistically. Furthermore, the minimal activation domain of VP16 (amino acids 413–453) was found to be sufficient for synergistic activation of transcription via RNA and DNA. Activation was abolished when the DNA-bound VP16 minimal activation domain was mutated at critical residue 442 (F442P). However, the RNA-bound VP16 minimal activation domain mutated at critical residue 442 maintained synergism with DNA-bound VP16. Our studies suggest that the 41-amino acid minimal activation domain of VP16 (amino acids 413–453), which plays an important role in transcription initiation, also acts at a postinitiation step to increase rates of transcription. Furthermore, this postinitiation activity of the minimal activation domain does not involve the critical residue at position 442 required for initiation. Thus, VP16 activates transcription by a sequence of at least two major events. The first event involves stimulation of initiation upon interaction of both the proximal and distal subdomains of VP16 activation domain with components of the basal transcription initiation machinery at the promoter. Next, distinct determinant(s) within the proximal 41-amino acid activation region of VP16 activation domain act at a postinitiation step to enhance rates of transcription leading to the overall potent activation characteristic of VP16.

MATERIALS AND METHODS

Cell Culture and Transient Transfection Assays—Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics in tissue culture flasks. HeLa and CV1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics on 100-mm Corning tissue culture plates. All were incubated at 37°C in a humidified atmosphere containing 5% CO2. Jurkat cells were transfected with plasmid DNA using the DEAE-dextran method (Sambrook et al., 1989). Briefly, 1 x 10^6 cells were washed with phosphate-buffered saline and then incubated with 10 μg of target reporter and 5 μg of effector plasmids in 2 ml of serum-free RPMI 1640 medium containing 300 μg of DEAE-dextran/ml for 2 h at 37°C. Carrier DNA (pGem7Zf+; Promega) was added where necessary to maintain the same total amount of DNA in every transfection. Subsequently cells were pelleted and resuspended in 100 μl dimethyl sulfoxide (made in phosphate-buffered saline) and incubated for 2 min. Cells were then washed twice with phosphate-buffered saline, and RPMI supplemented with 10% fetal calf serum was added. Cells were harvested 48 h after transfection, cell lysates prepared, and chloramphenicol acetyltransferase (CAT) assays performed on 25 μg of lysate.

Transfection of HeLa and CV1 cells were performed using the DEAE-dextran method as described earlier (Ghosh et al., 1993). CAT assays were performed by the variants partition method (Neumann et al., 1987) where CAT activity is expressed as the rate of formation of 3H-labeled acetylated chloramphenicol and is normalized to the amount of protein in the lysates. Protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad). Transfection efficiency was examined by cotransfection of RSV-luciferase reporter plasmid, and luciferase assays were performed using the luciferase assay systems kit (Promega).

RESULTS

Synergistic Activation of Transcription by VP16 Tethered Simultaneously to Downstream RNA and Upstream DNA—VP16 has been shown to activate transcription from the HIV-1 LTR when tethered downstream of the transcription start site to a promoter-proximal RNA element (Tiley et al., 1992). In order to determine if VP16 tethered downstream to RNA can synergize with VP16 tethered to DNA upstream of a promoter, we constructed a modified HIV-1 LTR reporter plasmid, pG6RRE, which allowed us to target the TAR/RRE (SLIIB) subdomain of the HIV-1 RRE. The TAR element is a target for the HIV-1 Rev RNA-binding protein. pG6RRE was created first by digesting G6(-119)deltatAR (HIV-1 LTR sequence with TAR element deleted and six GAL4-binding sites placed upstream of the promoter at 30 to +35) by sequence coding for the most C-terminal 39-amino acid activation domain of VP16 to Rev and GAL4 (1-147), respectively. All sequences were verified by double-stranded DNA sequencing (U. S. Biochemical Corp. Sequenase kit).

Plasmid Constructions—The reporter plasmid pG6RRE is a derivative of plasmids G6(-119)deltatAR (Southgate and Green, 1991) and pSLIIB/CAT (Tiley et al., 1992) and contains HIV-1LTR sequences from positions 119 to +80 (Sanchez-Pescador et al., 1985) linked in cis to the CAT gene. Upstream of the NF-kB sequences at –119 are introduced six synthetic GAL4 DNA-binding sites (Southgate and Green, 1991). Downstream, the apical essential region of the TAR element (+30 to +35) is replaced by sequences that introduced the 29-nucleotide SLIIB high affinity Rev-binding site and the last pyrimidine residue (+25) of the TAR bulge is deleted resulting in a nonfunctional TAR element while the Rev response element (RRE) (SLIIB) sequence forms an in vivo target for the HIV-1 Rev RNA-binding protein. pG6RRE was created first by digesting G6(-119)deltatAR (HIV-1 LTR sequence with TAR element deleted and six GAL4-binding sites placed upstream of the promoter at 119) with XhoI and HindIII, filling in to create blunt ends, and religating the plasmid, resulting in the removal of the PvuII site located in between the XhoI and HindIII restriction sites upstream of the GAL4 DNA-binding sites to give G6(-119)deltatAR (PvuII). This plasmid was next digested with PvuII to remove all sequences located between the PvuII site 3′ of the TATA element in the LTR and the PvuII site within the downstream CAT coding sequences and replaced with a PvuII fragment derived from pSLIIB/CAT which restored HIV-1 LTR and RRE coding sequences and now also introduced the TAR/RRE (SLIIB) sequence to give the reporter plasmid pG6RRE used in this study.

The effector plasmids GAL4-VP16N and GAL4-VP16N/m442 have been described previously as SW3 and SW5, respectively, and so has SW22 (Walker et al., 1993). GAL4 and GAL4-VP16 have also been reported earlier (Lin and Green, 1991). Rev, Rev-VP16, and Rev-VP16m442 have been described earlier as pRev, pRev/VP16, and pRev/ΔPlp16, respectively (Tiley et al., 1992). Rev-VP16N and Rev-VP16N/m442 were created by introducing translational downstream sequence coding for the most C-terminal 39-amino acid activation domain of VP16 to Rev and GAL4 (1-147), respectively. All sequences were verified by double-stranded DNA sequencing (U. S. Biochemical Corp. Sequenase kit).
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Fig. 1. VP16 tethered simultaneously to RNA and DNA act synergistically in trans-activating the HIV-1 LTR. A, schematic drawing of the reporter and effector plasmids used in cotransfections. In the pG6RRE reporter, HIV-1 LTR (−119 to +80 relative to transcription start site) is linked to the reporter gene CAT and has six synthetic GAL4-binding sites introduced upstream of NF-κB sequences and downstream the apical region of TAR element (+30 to +35) is replaced by the SL1IB subdomain of the HIV-1 RRE. The TAR element is rendered nonfunctional while the SL1IB RRE sequence forms an in vivo RNA target for the HIV-1 Rev RNA-binding protein (Tiley et al., 1992). GAL4-VP16 directs the synthesis of a hybrid protein containing the DNA-binding domain of GAL4 (amino acids 1–147) fused to the C-terminal acidic activation domain of VP16 (amino acids 413–490), while Rev-VP16 expresses the 113-amino acid Rev protein fused in-frame with the C-terminal acidic activation domain of VP16 (amino acids 413–490). B, Jurkat cells were transfected with pG6RRE and either GAL4-VP16 or Rev-VP16, or both. GAL4-VP16 fusion targets VP16 to GAL4 DNA and Rev-VP16 targets VP16 to RRE (SL1IB) RNA. At 48 h after transfection, cells were harvested, cell lysates were prepared, and the level of CAT enzyme activity was determined by the solvent partition method of Neumann et al. (1987) where CAT activity is expressed as the rate of formation of δH-labeled acetylated chloramphenicol (cpm/min) and is normalized to the amount of protein in the lysate. Transactivation of the HIV-1 LTR reporter was monitored by CAT enzyme activity, and the data are reported as fold-trans-activation, where levels of CAT activity in the presence of the effector are divided by the basal activity level of the reporter in the absence of the effector and in the presence of carrier DNA (pGEM-7Zf+ vector DNA, Promega). When Rev-VP16 and GAL4-VP16 were cotransfected alone, GAL4 and Rev, respectively, or vector DNA were used as carrier. The basal activity of the pG6RRE reporter plasmid as measured by CAT activity is 1.85 cpm/min. Synergistic activation was obtained when GAL4-VP16 and Rev-VP16 were cotransfected. GAL4 and Rev did not have any effects on activation indicating that the synergism obtained with the hybrid fusions is specifically due to the activation domains of VP16. The data presented are representative of three transfections, each done in duplicate. Transfection efficiency was examined by cotransfection of RSV-luciferase reporter plasmid. Less than 10% variation in luciferase activity from the mean was detected among the different transfections.
Rev-VP16N alone) when cotransfected into Jurkat cells. The C region tethered to RNA (Rev-VP16C) did not synergize with a GAL4-VP16N or a GAL4-VP16C fusion. Thus the determinants for synergistic activation by VP16 are located within the 41 amino acid proximal minimal activation domain (N region).

Transcription-defective VP16 Minimal Activation Domain Mutated at Critical Residue 442 Maintains Synergism when Tethered to RNA with Wild-type VP16 Activation Domain Tethered to DNA—To define the nature of the synergism observed with VP16, we used truncated VP16N fusion derivatives where the critical aromatic residue phenylalanine at position 442 is mutated to proline (Fig. 3, A and B). GAL4-VP16Nm442 and Rev-VP16Nm442 have the mutated N region of VP16 activation domain fused to GAL4 and Rev, respectively. In context of the truncated minimal activation domain of VP16, mutation of the aromatic residue at 442 to proline abolished activation when this region was targeted to promoter DNA in both in vivo and in vitro studies (Regier et al., 1993; Jiang et al., 1994). Consistent with previous results, no activation was obtained with the GAL4-VP16Nm442 fusion even when targeted via multiple GAL4-binding sites. Neither did Rev-VP16Nm442 activate transcription via RNA. However, when Rev-VP16Nm442 was cotransfected with GAL4-VP16N, once again synergistic levels of activation were obtained (Fig. 3C and Table IIB). The full-length activation domain of VP16 (amino acids 413–490) mutated at residue 442 (Phe→Pro) and targeted to RNA as a Rev-VP16Nm442 fusion and which did not significantly activate transcription by itself, also maintained synergism with the full-length intact GAL4-VP16N fusion. Fivefold synergistic levels of activation were obtained when Rev-VP16Nm442 was coexpressed with GAL4-VP16 (Fig. 3C and Table IIA). Thus the "functionally defective" VP16 activation domain when targeted via RNA can cooperate with a functionally intact DNA bound VP16 activation domain leading to higher levels of activation. No significant synergism was detected between a wild-type Rev-VP16N fusion targeted to RNA and a mutated VP16N region (GAL4-VP16Nm442) targeted to upstream DNA. Also mutated VP16N activation domains did not synergize with each other when simultaneously targeted to DNA and RNA via GAL4-VP16Nm442 and Rev-VP16Nm442 fusions, respectively. These results indicate that for synergism to occur the aromatic residue at position 442 in the DNA-bound VP16 activation domain is critical, while this same residue is dispensable for the RNA-bound VP16. The data taken together suggest that synergistic activation by VP16 targeted simultaneously to DNA and RNA occurs by a sequence of at least two events. First, VP16 acts to stimulate rates of initiation upon interaction with basal components of the transcription initiation machinery. Thus, the requirement for the critical aromatic residue at position 442 within the minimal activation domain which has been extensively documented to play a crucial role in initiation and has been shown in vitro to contact directly components of the basal transcription complex. Next, residues within the same 41-amino acid minimal activation domain required for initiation, but not including the critical aromatic residue at position 442, interact with the transcription complex to enhance overall rates of transcription. The mutated minimal activation domain is incapable of stimulating activation from the promoter by itself, and its activity is dependent and follows the stimulation of initiation by this domain mediated by the aromatic residue at 442. This second step activity of VP16 is most likely a postinitiation event, since targeting the mutated VP16 activation domain via DNA upstream of the promoter did not synergize with a functional VP16 activation domain targeted downstream via RNA. Cotransfection of GAL4-VP16Nm442 and Rev-VP16N did not lead to any further increase in activation levels over that of Rev-VP16N alone (Fig. 3C and Table IIB). If this second pathway for VP16 functioned to activate the initiation step rather than act on a postinitiation stage, then one would have expected GAL4-VP16Nm442 to have synergized with Rev-VP16, similar to that observed upon cotransflecting GAL4-VP16N and Rev-VP16Nm442. Our studies, therefore, suggest that the 41-amino acid minimal activation domain of VP16, which plays an important role in transcription initiation, also acts at a postinitiation step to increase overall rates of transcription.

**Table I**

| Target | Basal CAT activity | Effectors | Trans-activated CAT activity | Fold-trans-activation |
|--------|-------------------|-----------|-----------------------------|----------------------|
| A. pG6RE | 1.85 ± 0.62 | GAL4 | 1.75 ± 0.21 | 0.94 |
|        |               | Rev     | 1.90 ± 0.07 | 1.02 |
|        |               | GAL4 + Rev | 1.95 ± 0.49 | 1.05 |
|        |               | GAL4-VP16 | 14.69 ± 0.70 | 25.27 ± 4.70 | 13.65 |
|        |               | Rev-VP16 | 70.00 ± 1.41 | 37.83 |
|        |               | GAL4-VP16 + Rev-VP16 | 567.50 ± 41.7 | 306.75 |
| B. pG6RE | 2.55 ± 0.14 | GAL4-VP16N | 25.43 ± 3.06 | 9.97 |
|        |               | Rev-VP16N | 14.10 ± 3.95 | 5.52 |
|        |               | GAL4-VP16N + Rev-VP16N | 307.84 ± 12.8 | 120.72 |
|        |               | GAL4-VP16C | 11.77 ± 0.73 | 4.61 |
|        |               | Rev-VP16C | 3.55 ± 0.19 | 1.39 |
|        |               | GAL4-VP16C + Rev-VP16C | 12.61 ± 1.85 | 4.94 |
|        |               | GAL4-VP16N + Rev-VP16C | 25.51 ± 4.17 | 10.00 |

a HIV-1 LTR reporter plasmid which contains 6 GAL4 DNA-binding sites placed upstream of the HIV-1 promoter and RRE (SIIB) RNA sequence downstream of the transcription start site.
b Basal CAT activity of target reporter plasmid in the absence of effector plasmids is expressed as the ratio of formation of [3H]-labeled chloramphenicol acetyltransferase (cpm/min). Carrier DNA (pGEM-7Zf(+) Promega) was used to keep the total amount of DNA transfected constant for all transfections.
c CAT activities of target plasmids in the presence of effector plasmids.
d Fold-trans-activation was calculated by dividing CAT activities from cotransfections with target and effector plasmids over those obtained with target plasmids alone (column 4 over column 2). Values provided are from one set of transfections performed in duplicate. Standard errors of the mean for each transfection are given.
FIG. 2. The minimal activation domain of VP16 targeted simultaneously to RNA and DNA is sufficient for synergistic activation of transcription. A, schematic drawing of the effector plasmids coding for truncated hybrid fusions of VP16 with either GAL4 (1-147) or Rev used in cotransfections with the reporter, pG6RRE. The activation domain of VP16 (amino acids 413–490) has been divided into two independent activation domains, VP16N (proximal amino acids 413–452) and VP16C (distal amino acids 453–490). GAL4-VP16N and GAL4-VP16C are fusion’s expressing the GAL4 (1–147) DNA-binding domain fused to VP16N and VP16C, respectively, while Rev-VP16N and Rev-VP16C regionsofVP16,respectively,while GAL4-VP16C are fusion’s expressing the GAL4 (1–147) DNA-binding domain fused to VP16N and VP16C regions of VP16, respectively, while Rev-VP16N and Rev-VP16C fusion’s express the Rev RNA-binding protein fused to VP16N and VP16C, respectively. B, trans-activation resulting from targeting truncated VP16 fusion derivatives simultaneously to RNA and DNA in pG6RRE reporter in Jurkat cells. The basal activity of pG6RRE as measured by CAT activity is 2.55 cpm/min. Reporter CAT activity data expressed as fold-trans-activation indicates that RNA-bound VP16N (Rev-VP16N) can synergize with DNA-bound VP16N (GAL4-VP16N) and that the N region contains all the determinants for synergism. RNA and DNA-bound VP16C (GAL4-VP16C and Rev-VP16C) did not synergize indicating that the synergism seen for VP16N is a specific event. Similar results were also obtained with CV1 and Hela cell lines. The data presented above are representative of three transfections, each done in duplicate.

Our analysis indicates that VP16 when tethered to RNA can synergize with upstream DNA-bound VP16 to activate transcription potently. Tethering the “functionally defective” minimal activation domain of VP16 mutated at critical residue 442 (F442P) via RNA and a wild-type minimal activation domain via DNA allowed us to uncover a second pathway for VP16 function, mediated by this minimal activation domain, hitherto unknown. This feature is distinct from its known function in initiation in that it does not require the participation of the critical residue Phe442. Our studies suggest that VP16 activates transcription by a sequence of at least two events. As shown in the model in Fig. 4, the first event involves both the proximal (N) and distal (C) regions of VP16 activation domain interacting with components of the basal transcription initiation machinery at the promoter to stimulate rates of initiation. The N region may stimulate initiation by contacts with TFIIB and TFIIID, and for which the participation of the aromatic residue at 442 is critical (Lin and Green, 1991, Ingles et al., 1991), while the C region may interact with TAF(s) in the TFIIID complex (Goodrich et al., 1993; Klemm et al., 1995), leading to a stable initiation complex. VP16 has also been reported to bind in vitro to the human coactivator PC4 and the critical residue at 442 was found to be important for this interaction. Since PC4 directly binds TFIIA (or a TBP-TFIIA complex), it is suggested that PC4 mediates functional interactions between VP16 and TFIIA (or a TBP-TFIIA complex) (Ge and Roeder, 1991). Next, we hypothesize, once a stable initiation complex is assembled, the N region of VP16 activation domain may interact either directly or indirectly with target(s) in the transcription complex, modifying the complex to increase its processivity and for this function the aromatic residue at 442, found to be critical for initiation, is dispensable. The dispensability of this critical residue may suggest that the targets mediating this additional activity of VP16 may not include TFIIID, TFIIB, or PC4, since interaction of these components with VP16 is disrupted by this mutation. The ability of VP16 to contact both general transcription factors involved in stimulating initiation and factor(s) modulating the processivity of chain elongation by RNA polymerase II would result in the potent activation characteristic of VP16. We suggest that the reason this postinitiation activity could not be detected in DNA targeting studies is that mutation of Phe442 abolishes VP16’s initiation function and consequently assembly of stable initiation complexes upon which VP16 could act to increase processivity. Tethering VP16 to RNA resulted in a large induction of transcription from the HIV-1 promoter even though multiple VP16 activation domains were tethered to DNA upstream of the promoter. We suggest that tethering VP16 to the 5’ end of nascent RNA in close spatial proximity to the transcription start site may have allowed for a more efficient targeting of VP16 to the nascent elongating polymerase complexes initiated from the promoter and consequently for VP16 to exert its postinitiation processive activity leading to high overall rates of
transcription. Interestingly, the HIV-1 Tat protein which is an extremely potent viral trans-activator activates transcription from the HIV-1 promoter via interaction with the nascent TAR RNA element located at the 5' end of all HIV transcripts, and it has been shown by both in vivo and in vitro studies that Tat acts as an elongation factor and can also stimulate transcription initiation (see Zhou and Sharp (1995) and references therein). However, the mutated Tat protein tethered to DNA did not have any effects on the basal activity of pG6RRE. Rev-VIP16m442 or Rev-VIP16m442 fusions did not alter the basal expression of pG6RRE, individually but synergized with an intact VP16 activation domain targeted to upstream DNA (Fig. 3). The low levels of basal activity obtained with our modified HIV-1 LTR reporter, pG6RRE, is consistent with previous findings from several laboratories that the HIV-1 LTR promoter, in the absence of activator, is characterized by the production of low levels of productive full-length RNA. Phillip Sharp and colleagues have proposed that these RNA species are generated from the more productive class of polymerase elongation complexes that initiate from the HIV promoter (Marciniak and Sharp, 1991). We suggest that these more productive elongating polymerase complexes derived from the HIV promoter in the absence of activator may not be responsive any further to the processive activity of VP16. The processive function of VP16 specifically acts on the transcription initiation complexes stimulated from the promoter by the intact VP16 activation domain requiring critical residue 442, modifying it to increase its processivity resulting in increased rates of transcription.

It is generally believed that transcriptional synergy exhibited by VP16 on DNA may be a result of it interacting with more than one target affecting multiple steps involved in assembly of preinitiation complex. This hypothesis is supported by in vitro studies demonstrating GAL4-VP16 interactions with multiple basal transcription factors. However, transcriptional activation by RNA polymerase II is a multistep process, where each step may serve as a potential control point for activators and for transcriptional synergism. Transcriptional activation begins with assembly of a preinitiation complex followed by ATP-dependent events including melting of the DNA at the transcription start site to give an open complex and phosphorylation of the CTD (C-terminal domain) of RNA polymerase II. Next is the promoter clearance step resulting in the transition of the initiation complex to elongation complex and synthesis of phosphodiester bonds of the nascent RNA transcript followed by chain elongation (reviewed in Herschlag...
Synergistic activation of transcription by mutant and wild-type minimal transcriptional activation domain of VP16

| Target | Basal CAT activity | Effectors | Trans-activated CAT activities | Fold-trans-activation |
|--------|-------------------|-----------|--------------------------------|-----------------------|
| A. pG6RRE | 3.01 ± 0.95 | GAL4 + Rev | 2.92 ± 0.39 | 0.97 |
| | | GAL4-VP16 | 70.80 ± 6.22 | 30.54 |
| | | Rev-VP16m442 | 10.88 ± 1.15 | 36.31 |
| | | GAL4-VP16 + Rev-VP16m442 | 401.68 ± 16.8 | 1334.44 |
| B. pG6RRE | 2.55 ± 0.35 | GAL4 + Rev | 3.40 ± 0.28 | 1.33 |
| | | GAL4-VP16N | 25.98 ± 3.51 | 10.18 |
| | | Rev-VP16Nm442 | 3.22 ± 0.10 | 1.26 |
| | | GAL4-VP16N + Rev-VP16Nm442 | 48.48 ± 9.72 | 34.60 |
| | | GAL4-VP16Nm442 | 2.97 ± 0.19 | 1.16 |
| | | Rev-VP16N | 13.85 ± 3.60 | 5.43 |
| | | Rev-VP16Nm442 + Rev-VP16N | 4.04 ± 2.09 | 8.01 |
| | | SW22 | 11.8 ± 1.39 | 4.62 |
| | | SW22 + Rev-VP16Nm442 | 35.05 ± 3.09 | 13.74 |

**Note:**

a HIV-1 LTR reporter plasmid which contains 6 GAL4 DNA-binding sites placed upstream of the HIV-1 promoter and RRE (SLIIB) RNA sequence downstream of the transcription start site.

b Basal CAT activity of target reporter plasmid in the absence of effector plasmids is expressed as the rate of formation of 3H-labeled acetyl chloramphenicol transferase (cpm/min). Carrier DNA (pGEM-7Zf(+), Promega) was used to keep the total amount of DNA transfected constant for all transfections.

c CAT activities of target plasmids in the presence of effector plasmids.

d Fold-trans-activation was calculated by dividing CAT activities from cotransfections with target and effector plasmids over those obtained with target plasmids alone (column 4 over column 2). Values provided are from one set of transfections performed in duplicate. Standard errors of the mean for each transfection are given.

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**Fig. 4. Model for transcriptional activation by VP16.** Analysis of synergistic activation features of VP16 acidic activation domain when targeted simultaneously to RNA downstream of the start site and to DNA upstream of the promoter suggest that VP16 in addition to stimulating rates of transcription initiation also plays a role in postinitiation control of transcriptional activation. We hypothesize that VP16 activates transcription by a sequence of at least two events. First, the activation domain of VP16 interacts with basal components of the transcription initiation machinery via both its proximal N region (amino acids 413–453) and the distal C region (amino acids 454–490) to stimulate rates of transcription initiation from the promoter. This function of VP16 involves the participation of the critical aromatic residue at position 442 within the N region of its activation domain. Mutation of Phe442 to proline (m442) drastically affects the initiation step and in context of the minimal activation domain (N region) abolishes transcription. Next, distinct determinant(s) within the same 41-amino acid N region of VP16 activation domain required for initiation, may interact with target(s) in the transcription complex at a postinitiation step to increase rates of transcription resulting in potent activation characteristic of VP16. Phe442 critical for initiation is dispensable for this postinitiation function.

that mutation of critical residue 442 within the VP16 activation domain which drastically affects transcriptional activity in vivo led to a defect in open complex formation in in vitro studies (Jiang et al., 1994). This finding is consistent with the previous studies showing that mutation at residue 442 (F442P) disrupts VP16's interaction with general transcription factors TBP, TFIIIB, and PC4, all of which act at steps preceding open complex formation. Since the additional transcriptional activity of VP16 which we have uncovered in our synergy assay does not require the participation of the critical residue at 442, we suggest that this new activity of VP16 acts at a step that follows open complex formation. For example, VP16 could facilitate the steps leading to CTD phosphorylation which allows uncoupling of polymerase II from the promoter (reviewed in Eck et al., 1994)). Alternatively, this postinitiation activity of VP16 might act at the promoter clearance stage. It has been shown that TFIIIE and TFIIH are not required for initiation, but are necessary for promoter clearance (Maxon et al., 1994; Goodrich and Tijan, 1994) and may be potential targets for the postinitiation activity of VP16. Of the two, TFIIE, may be a more likely candidate since interaction of VP16 with TFIIH has been shown to require residue 442 (Xiao et al., 1994). We suggest that the transcriptional synergism obtained when VP16 is tethered to multiple DNA-binding sites is a result of both the action of VP16 on multiple steps leading to preinitiation complex formation, via interactions with multiple targets, as well as on a postinitiation step(s) leading to overall potent levels of mRNA synthesis. However, our experimental approach has limitations and cannot determine the exact stage of the transcription process at which the proposed postinitiation activity of VP16 functions. We are currently examining the residue(s) within the N region of the VP16 activation domain which is(are) responsible for the postinitiation activity detected in our in vivo analysis of VP16 transcriptional activation, the stage in transcription at which it acts, and the cellular targets with which this region interacts using biochemical approaches. Our studies may lead to an understanding of the mechanism by which the herpes simplex virus VP16 protein triggers activation of the viral immediate early genes upon interaction with host factors, Oct-1, and host cell factor protein (reviewed in Thompson and McKnight, 1992)). Finally, a dual role in both

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transcription initiation and postinitiation may be a general property of other potent viral and cellular activators.

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