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Viral transactivators specifically target distinct cellular protein kinases that phosphorylate the RNA polymerase II C-terminal domain

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ABSTRACT

Phosphorylation of the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II has been implicated as an important step in transcriptional regulation. Previously, we reported that a cellular CTD kinase, TAK, is targeted by the human immunodeficiency virus transactivator Tat. In the present study, we analyzed several other transactivators for the ability to interact with CTD kinases *in vitro*. The adenovirus E1A and herpes simplex virus VP16 proteins, but not other transactivators tested, were found to associate with a cellular kinase activity that hyperphosphorylates the CTD. The interaction is dependent upon a functional activation domain of E1A or VP16, suggesting that the interaction with a CTD kinase is relevant for the transactivation function of these proteins. The CTD kinase activities that interact with E1A and VP16 are related to each other but distinct from TAK. The Tat-, E1A- and VP16-associated CTD kinase activities detected in our assay also appear unrelated to MO15, the catalytic component of the CTD kinase activity of the general transcription factor TFIIH. Thus, this study has identified a novel interaction between viral transactivators and a cellular CTD kinase and suggests that at least two CTD kinases may mediate responses to viral transactivators.

INTRODUCTION

The study of cellular factors targeted by viral transactivators can provide insights into fundamental mechanisms of transcriptional regulation. Viral transactivators have been shown to interact with general transcription factors and other proteins involved in activated gene expression. Three particularly potent transactivators are the human immunodeficiency virus (HIV) Tat protein, the adenovirus E1A protein, and the herpes simplex virus VP16 protein. The mechanism of transcriptional activation by these proteins has been studied extensively but is still not completely understood. E1A and VP16 are considered typical transactivators because their *cis*-acting targets are DNA elements located upstream of the site of initiation of transcription. Tat is unique in that it interacts directly with an RNA element, TAR, that is located 3′ to the transcriptional start site. Although their *cis*-acting targets differ, it has been suggested that Tat acts through a mechanism similar to DNA-dependent activators. First, Tat transactivates transcription when targeted to an upstream DNA-binding site (1,2). Secondly, VP16 functions when targeted to a promoter-proximal RNA element (3). Thirdly, Tat, E1A and VP16 have all been shown to stimulate transcriptional elongation, in addition to effects on initiation (4–9).

Transactivators typically contain two domains that are important for their transactivation function: an activation domain, the region of the protein that interacts with cellular factors involved in transcriptional stimulation and a DNA (or RNA)-targeting domain that is required for binding of the transactivator to its *cis*-acting target. For the Tat proteins of HIV, the transactivation function resides in the amino-terminal region [a.a. 1–48 for HIV type 1 (HIV-1) Tat and a.a. 1–77 for HIV type 2 (HIV-2) Tat] (1,2,10,11), while a highly basic region near the center of the protein directs binding to TAR RNA (12,13). E1A contains three regions known as CR1, CR2 and CR3 that are highly conserved among different adenovirus serotypes (14). CR1 and CR2 are involved in the transforming functions of E1A through an association with cellular proteins including the retinoblastoma (pRB), p107 and p300 proteins (15–17). CR3 is required for the transactivation function of E1A and contains the activation domain (a.a. 140–180) and a region that directs E1A to the promoter through interactions with host factors (18). The activation domain of VP16 (a.a. 413–490) is located at the extreme C-terminus, while the N-terminal region interacts with cellular factors that direct VP16 to the promoter (19–21). The VP16 activation domain has been shown to be comprised of two subdomains that appear to possess distinct functions in transactivation (22,23). Mutational analysis has defined amino acid residues 413–456 as the minimal activation domain (20), although the C-terminal region contributes to full transactivation function (22,23).

Much effort has been directed at identifying cellular factors that interact with the activation domains of these viral transactivators. The activation domains of Tat, E1A and VP16 have all been shown to interact with the TATA-binding protein (TBP), a component of TFIID (24–26). The VP16 activation domain has been reported to associate with two additional general transcrip-

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tion factors, TFIIB and TFIIH (27,28), as well as a TBP-associated factor that serves as a co-activator (29). Recently, we reported that the activation domains of the Tat proteins of HIV-1 and HIV-2 (Tat-1 and Tat-2, respectively) specifically interact *in vitro* with a cellular protein kinase, TAK (Tat-associated kinase), and that TAK hyperphosphorylates the C-terminal domain (CTD) of the largest subunit of RNA polymerase (RNAP) II (30). The CTD contains an unusual, highly repetitive structure consisting of tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (31,32). Two forms of RNAP II exist *in vivo* which differ with respect to phosphorylation of the CTD. The underphosphorylated IIA form preferentially enters into the pre-initiation complex, while the hyperphosphorylated IIO form is associated with actively elongating complexes (33–36). Phosphorylation of the CTD has been proposed to regulate transcription initiation, activation and elongation (34–40). The association of Tat with a CTD kinase suggests a model in which Tat functions by recruiting or activating a CTD kinase which modifies the CTD, resulting in a more processive transcription complex (30).

In this study, we examined whether other viral or cellular transactivators associate with CTD kinases. The activation domains of E1A and VP16, but not other viral or cellular transactivators tested, were found to specifically interact with a cellular CTD kinase *in vitro*. The interaction with a CTD kinase was dependent on a functional activation domain of E1A and VP16, suggesting that the interaction is biologically relevant for the transactivation function of these proteins. The E1A- and VP16-associated kinases appear related to each other but distinct from TAK. The Tat-, E1A- and VP16-associated kinase activities described here also appear to be unrelated to MO15, the catalytic component of general transcription factor TFIIH, which is known to possess CTD kinase activity (41–44). These results suggest that multiple CTD kinases exist which can specifically interact with some viral transactivators.

MATERIALS AND METHODS

Plasmids

For expression of the E1A activation domain as a GST-fusion, the region encoding amino acid residues 140–180 was PCR amplified from plasmid pLE1A (45), which encodes the adenovirus type 5 E1A gene. The 5′ and 3′ amplimers used were 5′-GCGCGGATCCGAGGAGTTTGTGTTGATTATGTGG-3′ and 5′-GCGCGAATTCTTAGCCACAGGTCCTCATATAGC -3′, respectively, which contained *Bam*HI or *Eco*RI sites (underlined). The PCR reactions were performed using 10 ng template, 1 µm each of the 5′ and 3′ amplimers, 200 µm dNTPs, 10 U Vent DNA polymerase (New England BioLabs) and subjected to 30 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 45 s in a DNA thermal cycler (Perkin Elmer). Following amplification, the product was digested with restriction enzymes *Bam*HI and *Eco*RI and ligated with pGEX-2T (Pharmacia) digested with the same enzymes. The E1A GST-C174S expression plasmid encodes a substitution of serine for cysteine at position 174 and was generated using the primer 5′-GCGCGAATTCTTAGCCACAGGTCCTCATTGGAAAGCGAACA-3′ in combination with the 5′ primer used to generate the wild-type CR3 clone. PCR amplification and cloning into pGEX-2T was performed as for the wild-type GST-E1A construct. The sequences of GST-E1A and GST-C174S were confirmed by di-deoxy sequencing using Sequenase (US Biochemical).

For expression of the VP16 activation domain as a GST-fusion, the region encoding amino acid residues 413–490 was PCR amplified from plasmid pSG424-VP16 (46). The 5′ and 3′ amplimers were 5′-GCGCGGATCCGCACCACCGACCGATGTCA-3′ and 5′-GCGCGAATTCCTATCCACCGTCTCGTC-3′. PCR reactions and cloning into pGEX-2T were performed as described above. The VP16 double-mutant, F442S + F475A, was generated by PCR mutagenesis of the GST-VP16 template. First, GST-F442S, a construct containing a Phe→Ser mutation at position 442 was generated. An internal primer with the sequence 5′-GCGCGCATGCCGACGCGCTAGACGATCCGATCTGGAC-3′, which encompasses the *Sph*I site (underlined) and changes the codon at amino acid position 442 from T*T*C to T*C*C (underlined), was used in combination with the 3′ VP16 amplimer in a PCR amplification as described above. The product was digested with the restriction enzymes *Sph*I + *Eco*RI and then ligated with the GST-VP16 plasmid digested with the same enzymes, so that the wild-type sequence was replaced by the mutation at position 442. This construct was then used as a template (1 ng) in a three-primer PCR strategy to mutate phenylalanine 475 to alanine using the primer 5′-CCGACTTCGAGGCTGAGCAGATGTT-3′ in combination with the VP16 3′ amplimer using the same cycling parameters as described above (40 cycles). The amplified fragment was gel purified and used as a mega-primer (47) in a second PCR reaction, in which 50% of the initial fragment yield was used in combination with the 5′ VP16 amplimer to generate a product containing the double mutations. The isolated fragment was digested with *Eco*RI + *Bam*HI and ligated with pGEX-2T. The sequences of GST-VP16 constructs were confirmed by di-deoxy sequencing using Sequenase (US Biochemical). The constructs used to express GST fusions of VP16NC, VP16N, VP16N442 and VP16C have been described (29).

The plasmids used to express GST fusions of the HIV Tat proteins, Tat-1 (a.a. 1–48) (48Δ) and Tat-2 (a.a. 1–99) have been described previously (48,49). The GST-C59A expression construct is identical to the GST-Tat-2 construct except that it encodes a substitution of alanine for cysteine at position 59 (50). The constructs used to express GST fusions of p53 (a.a. 1–73) (51) and Sp1 (a.a. 83–621) (52) have also been described. The construct used to express a GST fusion of the HTLV-1 Tax protein (full length) was kindly provided by Paul Lindholm (Medical College of Wisconsin, Milwaukee, WI) and the GST-EBNA2 activation domain (a.a. 437–477) construct was the generous gift of Paul Ling (Baylor College of Medicine, Houston, TX).

Preparation of bacterially expressed fusion proteins

The activation domains fused to GST were expressed and purified as described previously (48,49). The murine CTD fused to GST was expressed and purified as described by Peterson *et al.* (53).

Preparation of HeLa cell nuclear extracts

HeLa cell nuclear extracts were prepared from frozen cell pellets (Cellex Biosciences, Inc., Minneapolis, MN) as described (54). Prior to use in *in vitro* binding and kinase assays, nuclear extracts were treated with 100 U/ml DNase and 50 µg/ml RNase for 10 min at 37°C to reduce non-specific background bands.

Purification of kinase activities

HeLa cell nuclear extracts were dialyzed against 50 vol Buffer D (50 mM Tris–HCl pH 8.0, 10% glycerol, 0.2 mM EDTA, 0.5 mM EDTA and 0.5 mM PMSF) + 50 mM KCl. Typically, a nuclear extract prepared from frozen cell pellets obtained from 20 l of HeLa cells (175 mg total protein) was applied to an 8 ml Phosphocellulose P11 column (Whatman) pre-equilibrated with at least 10 column volumes of Buffer D + 50 mM KCl. The 50 mM KCl flowthrough was collected at a flow rate of ~0.5 ml/min and the column was washed with three to four column volumes of Buffer D + 50 mM KCl. The majority of TAK activity and all of the E1AK and VP16K activity was eluted with three column volumes of Buffer D + 0.5 M KCl. The eluate (40 mg) was dialyzed against 100 vol Buffer D + 50 mM KCl and then applied to a Resource Q column (6 ml, Pharmacia) in the same buffer. The column was washed with Buffer D + 50 mM KCl and proteins eluted with a 120 ml linear gradient of 50–525 mM KCl at a flow rate of 4 ml/min and 4 ml fractions were collected. For some of the experiments described here, the peaks of TAK activity (eluted at 90–240 mM KCl; 2.9 mg protein) and E1AK and VP16K (eluted at 270–300 mM KCl; 0.8 mg protein) were further purified and concentrated by heparin–agarose chromatography. The active peaks from the Resource Q column were pooled, adjusted to 150 mM KCl by dilution and applied to a heparin–agarose column (5 ml, BioRad) in Buffer D + 150 mM KCl. The column was washed with the same buffer and proteins eluted with a 20 ml linear gradient of 150–750 mM KCl for TAK and 150 mM–1 M KCl for E1AK/VP16K at a flow rate of 0.5 ml/min. The peak of TAK and E1AK/VP16K activities eluted at 370–520 mM KCl and 430–490 mM KCl, respectively. All procedures were performed at 4°C. At each stage of the purification, aliquots of fractions were diluted to ≤ 200 mM KCl, nuclease-treated (see above), and assayed for Tat-2-, E1A- and VP16- associated CTD kinase activity.

Binding of kinase activities to GST-fusion proteins

GST or GST-fusion proteins (0.5 µg) were bound to 10 µl of glutathione–Sepharose beads (50% slurry) pre-equilibrated in EBC buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% NP-40 and 5 mM DTT) by incubation for 15 min at 4°C with gentle rocking. The beads were pelleted and washed two times with EBC buffer containing 0.075% SDS, followed by one wash with EBC buffer. The GST-Tat beads were subsequently incubated with HeLa nuclear extracts or partially purified kinase preparations for 60 min at 4°C with rocking. The resulting complexes were washed two to three times with EBC buffer containing 0.03% SDS, followed by one wash with Tat kinase buffer (TKB/Mg) (50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$ and 5 mM DTT).

CTD phosphorylation assay

The CTD kinase assays were performed by adding 50 µl of a mix containing TKB/Mg, 2.5 mM $MnCl_2$, 200 ng GST-CTD, 5 µM ATP and 5 µCi [γ-^{32}P]ATP (NEN, 3000 Ci/mmol) to the bead complexes and incubating for 60 min at room temperature. The complexes were pelleted briefly, denatured in Laemmli sample buffer, and resolved by 9% SDS–polyacrylamide gel electrophoresis (PAGE) unless stated otherwise. Autoradiography was performed at –70°C in the presence of an intensifying screen.

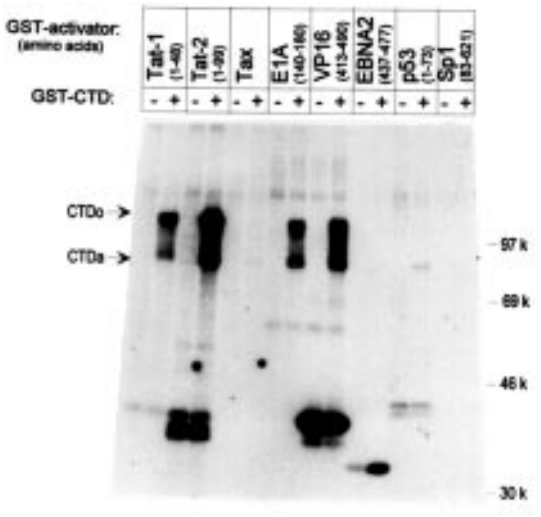

Figure 1. Interaction of activation domains with CTD kinases. The activation domains of viral and cellular transactivators comprising the indicated amino acid residues fused to GST were incubated with a HeLa cell nuclear extract and phosphorylation assays performed as described in Materials and Methods in the absence (–) or presence (+) of GST-CTD. These proteins were all expressed at levels at least equivalent to wild-type Tat-2 as detected by Coomassie staining. The migration of the underphosphorylated CTDa and hyperphosphorylated CTDo forms is indicated. The migration of molecular standards (in kilodaltons) is shown at the right.

Western blot analysis

Proteins complexed to fusion beads under conditions described above were separated by SDS–PAGE and transferred to nitrocellulose (55). The blots were probed with an anti-p62 monoclonal antibody (56) kindly provided by L. Zawel and D. Reinberg or an anti-MO15 rabbit polyclonal antibody (57), the generous gift of J. P. Tassan and E. Nigg. Following incubation with an appropriate secondary antibody conjugated to horseradish peroxidase, immunoreactive proteins were detected using enhanced chemoluminescence (Kirkegaard and Perry).

RESULTS

E1A and VP16 specifically interact with a CTD kinase

Recently, we showed that the Tat proteins of HIV-1 and HIV-2 interact *in vitro* with TAK, a cellular protein kinase that hyperphosphorylates the CTD of RNAP II (30). We proposed that Tat might function by recruiting a CTD kinase to the transcription complex, which modifies the CTD resulting in a more processive elongation complex. Because other transcriptional activators might act by a similar mechanism, a number of viral and cellular activation domains were expressed in bacteria as fusions with glutathione S-transferase (GST) and tested for their ability to interact with a CTD kinase activity *in vitro* (Fig. 1). Following incubation of the purified GST-activator fusions with a HeLa cell nuclear extract, the bead complexes were washed extensively and incubated with a GST-CTD substrate under kinase reaction conditions (see Materials and Methods). As reported previously, the Tat proteins of HIV-1 and HIV-2 interact with a kinase activity that hyperphosphorylates the recombinant CTD and gives rise to

the slower migrating form, CTDo (30). Tat-2, but not Tat-1 serves as a substrate of the associated kinase; an unidentified 42 kDa protein is also a substrate of TAK (30,48). The transactivator protein Tax of human T-cell leukemia virus type 1, the EBNA2 protein of Epstein–Barr virus, and cellular transactivator proteins p53 and Sp1 failed to interact with a CTD kinase activity in this assay, although EBNA2 and p53 were phosphorylated in this assay. Interestingly, the activation domains of the adenovirus E1A protein and the herpes simplex virus VP16 protein interacted with a CTD kinase. VP16 also became phosphorylated. Like Tat, E1A and VP16 are strong transcriptional activators that have been shown to stimulate elongation, as well as initiation (4–9).

To determine whether the interaction of E1A and VP16 with a CTD kinase is specific for a functional activation domain, we tested E1A and VP16 proteins that contain point mutations within the activation domain that have previously been shown to be defective for transactivation *in vivo*. A substitution of serine for cysteine at amino acid residue 174 within conserved region 3 of the E1A protein has been shown to abolish the transactivation function of E1A (58). Kinase activity that hyperphosphorylates the CTD was not detected using this mutant (Fig. 2A). This indicates that the E1A-associated kinase activity is dependent on a functional activation domain of E1A. Similarly, no CTD kinase activity was detected using a mutant, F442S+F475A, which contains an amino acid substitution in each of the two subdomains of the VP16 activation domain (Fig. 2B). The phenylalanine at position 442 has previously been shown to be critical for the transactivation function of VP16 (59). While a single substitution of the phenylalanine at position 475 alone had little effect on the activity of VP16, when combined with a substitution at position 442, the double mutant displayed significantly reduced transactivation activity (22). Therefore the presence of an associated CTD kinase activity correlates with the transcriptional activity of E1A and VP16. These results do not distinguish whether the activation domains of these proteins are required for the interaction of a CTD kinase with E1A and VP16 or whether they might be required for activation of the associated kinase activities. However, depletion experiments shown below (Fig. 4) imply that the activation domain mutants do not bind a CTD kinase activity.

The two subdomains of the VP16 activation domain have been suggested to function by distinct mechanisms and can independently stimulate transcription in an *in vitro* system (22,29). To determine which region(s) is required for the interaction with a CTD kinase, we tested GST-fusion proteins that contained only the N- (a.a. 412–456) or C-terminal (a.a. 452–490) portion of the VP16 activation domain (Fig. 2C). Neither subdomain alone detectably interacted with a CTD kinase, suggesting that the entire transactivation region of VP16 is required for a stable interaction with a CTD kinase. This result is consistent with the observation that the entire activation domain of VP16 is required for optimal transactivation (22,23). For convenience, we will refer to the E1A-associated kinase as E1AK and the VP16-associated kinase as VP16K.

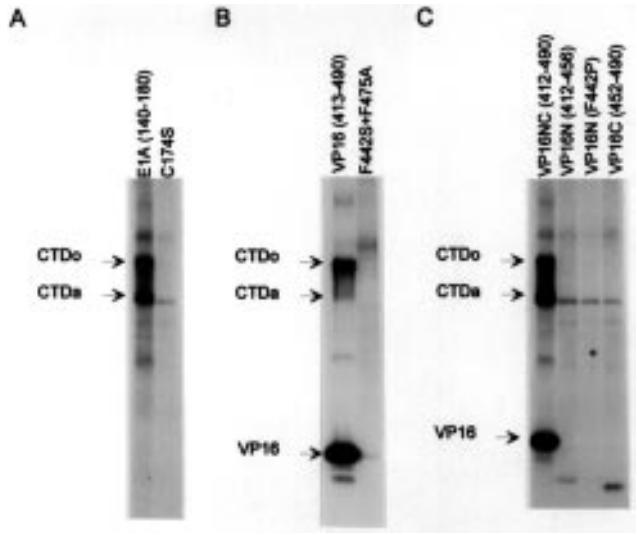

Figure 2. Interaction of E1A and VP16 with a CTD kinase is specific for a functional activation domain. (**A**) GST fusions of the wild-type E1A activation domain comprising amino acid residues 140–180 or a mutant construct comprising the same amino acid residues except for a substitution of serine for cysteine at residue 174 were incubated with a HeLa cell nuclear extract and a CTD phosphorylation assay was performed as described in Materials and Methods. (**B**) GST fusions of the wild-type VP16 activation domain comprising amino acid residues 413–490 or a mutant construct comprising the same amino acid residues except for substitution of serine for phenylalanine at residue 442 and alanine for phenylalanine at residue 475 were assayed as described in (A). (**C**) GST fusions of VP16 comprising the indicated amino acid residues were assayed as described in (A). VP16N (F442P) is identical to VP16N except for a substitution of proline for phenylalanine at residue 442. The migration of CTDo, CTDa and phosphorylated VP16 is indicated.

The E1A- and VP16-associated kinase is not related to TAK

To determine whether the E1AK and VP16K might be related to each other or to TAK, these activities were partially purified and characterized. HeLa cell nuclear extracts were passed over a phosphocellulose P11 column (see Materials and Methods for details). The 0.5 M KCl eluate was collected and then applied to a Resource Q column. Fractions were assayed for CTD kinase activity using GST-Tat-2, GST-E1A or GST-VP16 to selectively purify the associated kinase activity (Fig. 3). E1AK and VP16K co-eluted, but eluted at a higher salt concentration than TAK, suggesting that the E1AK and VP16K are distinct from TAK.

Next the substrate specificity, sensitivity to the nucleoside analog DRB (dichloro-1-β-D-ribofuranosylbenzimidazole), and nucleotide donor specificity of the kinase activities were compared (summarized in Table 1). Activities associated with all three viral proteins phosphorylated the CTD and did not phosphorylate histone H1. As reported previously, TAK is sensitive to low concentrations of DRB (30), an inhibitor of RNAP II transcription (60,61). Using partially purified kinase preparations (see legend to Table 1), TAK was found to be sensitive to DRB at a >10-fold lower concentration than either E1AK or VP16K. Nucleotide donor specificity in kinase reactions also differed between TAK and E1AK or VP16K: CTDo phosphorylation by TAK was competed efficiently by unlabeled ATP and less efficiently by dATP or GTP, while CTDo phosphorylation by E1AK and VP16K was competed efficiently by ATP and dATP but not GTP. Results of this analysis suggest that E1AK and VP16K are distinct from TAK but possibly related to each other.

Table 1. Comparison of the Tat-, E1A- and VP16-associated kinase activities

	Tat-associated kinase (TAK)	E1A-associated kinase (E1AK)	VP16-associated kinase (VP16K)
Substrates:[a]			
CTD	+	+	+
histone H1	–	–	–
Elution from Resource Q[b]	90–240 mM	270–300 mM	270–300 mM
DRB sensitivity (50% I.C.)[c]	<2 μM	20 μM	20 μM
NTP competition:[d]			
ATP	++	++	++
dATP	+	++	++
GTP	+	–	–
CTP	–	–	–

[a]Amounts of substrates added to standard kinase reaction mixtures were 200 ng GST-CTD and 2.5 μg histone H1. The histone H1 phosphorylation assay was performed using a HeLa cell nuclear extract; CTD phosphorylation assays were performed with nuclear extracts, as well as partially purified preparations. For the CTD reactions, + indicates the presence of the hyperphosphorylated CTDo form.
[b]HeLa cell nuclear extracts were passed over a phosphocellulose P11 column and the 0.5 M KCl eluate was then applied to a Resource Q column under conditions described in Materials and Methods. The concentration of KCl required to elute the various kinase activities is indicated.
[c]Concentration of DRB required for 50% inhibition of CTDo phosphorylation under standard kinase assay conditions. The heparin–agarose peaks of the respective kinase activities were used as the source of the kinase (see Materials and Methods). CTD phosphorylation assays were performed using 0, 2, 10 and 50 μM final concentrations of DRB. Quantitation of SDS–PAGE analysis was performed using a Betagen Betascope scanner.
[d]The ability of excess radioinert nucleotides (1 mM) to compete the [γ-^{32}P]ATP-dependent reaction. The heparin–agarose peaks were used as the source of kinase activity for these reactions. ++ indicates complete inhibition of CTDo phosphorylation and + indicates partial inhibition.

To compare further E1AK, VP16K and TAK, we used GST-Tat-2 to deplete a HeLa cell nuclear extract of TAK activity. As a control, we used a mutant of Tat-2, GST-C59A, which contains a substitution of the conserved cysteine at position 59 to alanine. This mutation largely abolishes Tat-2 transactivation *in vivo* and this GST-Tat-2 mutant does not bind TAK *in vitro* (50). As shown in Figure 4A, depletion of TAK with GST-Tat-2, but not C59A, greatly diminishes Tat-2-associated CTD kinase activity (lanes 1 and 2). Depletion with GST-Tat-2 had no effect on the CTD kinase activity associated with VP16 or E1A. The converse experiment was performed using GST-VP16 or the mutant F442S+F475A to deplete VP16K activity from HeLa cell nuclear extracts (Fig. 4B). VP16, but not the mutant, efficiently depleted not only VP16-associated kinase activity but also E1A-associated kinase activity. TAK activity was similar in the extracts depleted with the wild-type or mutant VP16 fusion proteins. Together, these results indicate that E1AK and VP16K are related to each other but distinct from TAK.

The E1A-, VP16- and Tat-associated kinases are unlikely to be related to TFIIH/MO15

The general transcription factor TFIIH has been shown to phosphorylate the CTD (62). Recently, it was demonstrated that MO15, the catalytic subunit of the cdk-activating kinase (CAK), is a component of TFIIH and contains the CTD kinase activity (41–44). To establish whether TFIIH/MO15 is the CTD kinase associated with Tat, E1A or VP16, we investigated whether the p62 subunit of TFIIH or the p40/MO15 subunit of CAK was associated with E1A, VP16 or Tat. GST fusions of E1A, VP16 or Tat absorbed to glutathione–Sepharose beads were incubated with a HeLa cell nuclear extract and the bead complexes were washed extensively under conditions used to assay kinase activity. The presence of p62 or MO15 was analyzed by Western blotting (Fig. 5). Neither p62 nor p40/MO15 was detected in the E1A, VP16 or Tat complexes, although both p62 and MO15 were readily detectable in the nuclear extract. It should be noted that 50-fold (Fig. 5A) or 10-fold (Fig. 5B) more nuclear extract was used for binding to the GST-fusion proteins than for the nuclear extract control lane (N.E.). In a separate experiment, no MO15 was detected by Western blot analysis in VP16 complexes when 100-fold more nuclear extract was used for binding to the GST-fusion proteins than for the nuclear extract control lane (not shown). However, since the affinity of the activators for the CTD kinase is not known, we cannot rule out the possibility that MO15 is associated with these complexes at a level that is undetectable by Western blot analysis.

To further address this question, we measured the levels of p40/MO15 in the extracts depleted of TAK or VP16K (used in Fig. 4). As seen in Figure 5C, levels of p40/MO15 were similar in pairs of extracts depleted with Tat-2 or the C59A mutant and VP16 or the F442S+F475A mutant. This contrasts with the dramatic difference in CTD kinase activity observed between these extracts depleted with either wild-type or mutant Tat-2 or VP16 (Fig. 4). This result suggests that p40/MO15 does not bind to Tat-2 or VP16 under the conditions used here. We further failed to detect binding of labeled CAK subunits to E1A, VP16 or Tat or the presence of CAK activity in E1A, VP16 or Tat complexes using a cdk2 phosphorylation assay (63). In addition, we found

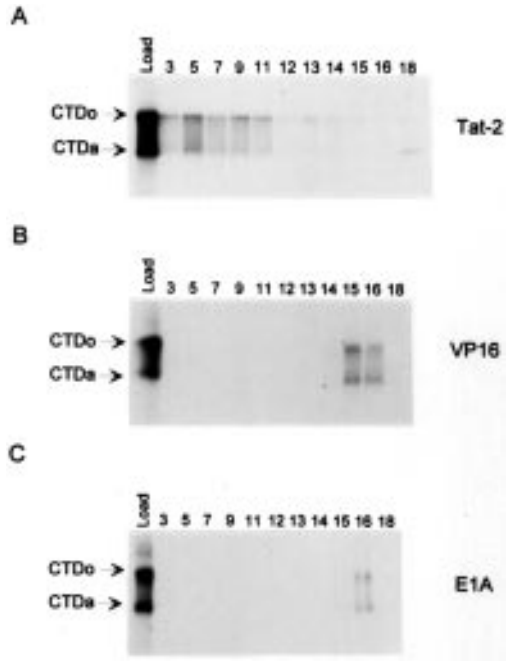

Figure 3. Fractionation of CTD kinase activity by Resource Q chromatography. The phosphocellulose 0.5 M KCl eluate was passed over a Resource Q column under conditions described in Materials and Methods. Aliquots (25 µl) of the phosphocellulose 0.5 M KCl eluate (load) or selected fractions were diluted 10-fold in EBC buffer and incubated with GST-Tat-2 (**A**), GST-VP16 (**B**) or GST-E1A (**C**) bound to glutathione–Sepharose beads. CTD phosphorylation assays were performed as described in Materials and Methods. TAK activity eluted largely in fractions 3–11, VP16K in fractions 15 and 16, and E1AK mostly in fraction 16, although some activity is seen in fraction 15 on a longer exposure of the gel shown here.

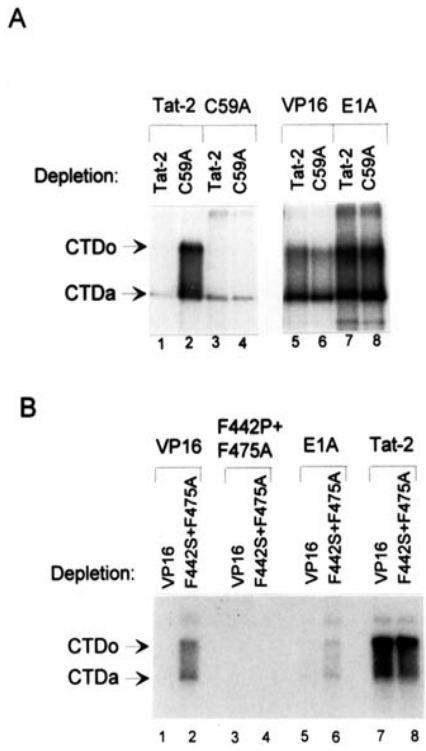

Figure 4. Depletion of CTD kinase activity. (**A**) HeLa cell nuclear extract was incubated with 2 µg GST-Tat-2 or GST-C59A absorbed to glutathione beads for 60 min at 4°C. The beads were pelleted and the supernatant was collected and incubated twice more with fresh GST-Tat-2 or GST-C59A (2 µg) beads for 30 min each. Extracts depleted with Tat-2 or C59A, as indicated, were then used for binding to the indicated GST fusions under standard conditions (see Materials and Methods). Lanes 1–4 were exposed for 4 h, lanes 5–8 for 16 h. (**B**) Depletions were performed as in (A) using 2 µg of GST-VP16 or the mutant F442S+F475A. Exposure time was 3 h.

that the p62 subunit of TFIIH was not detected in the fractions containing E1AK, VP16K or TAK activity but rather eluted largely in the flowthrough fraction of the Resource Q column (64). Therefore, it appears that E1AK, VP16K or TAK are not related to TFIIH/MO15 and may be novel kinase activities.

DISCUSSION

Cellular CTD kinases are selectively targeted by E1A, VP16 and Tat

We have shown here that the viral transactivators E1A and VP16 specifically target a cellular CTD kinase *in vitro*. Our previous work demonstrated that Tat also interacts with a CTD kinase *in vitro* (30), and recent results suggest that Tat specifically interacts with a CTD kinase *in vivo* (50). Other viral and cellular transactivators tested failed to interact with a CTD kinase in our *in vitro* assay (Fig. 1). We cannot rule out the possibility that Tax, EBNA2, p53 or Sp1 interact with a CTD kinase *in vivo*, since it is conceivable that the activation domains of these proteins expressed in bacteria are not capable of interacting with a CTD kinase *in vitro* due to improper folding, lack of post-translational modifications, or the absence of regions outside of the activation domain that could stabilize an interaction with a CTD kinase. However, at least in the case of p53 and Sp1, the activation domains fused GST have been shown to interact with other cellular factors *in vitro* (28,51,52). It is interesting that although VP16, p53 and EBNA2 are all classified as acidic activators, the interaction with a CTD kinase does not appear specific for acidic activators in general. In fact the activation domains of Tat and E1A are not highly acidic. The interaction of E1A, VP16 and Tat with a CTD kinase implies that some strong transactivators selectively target CTD kinases.

Existence of multiple CTD kinases that associate with viral transactivators

Although the activation domains of E1A, VP16 and Tat associate with a CTD kinase, it appears that the kinase activities that associate with E1A and VP16 are unrelated to TAK (Figs 3 and 4, Table 1). The kinase activities described here are also apparently distinct from the MO15/TFIIH kinase (Fig. 5). Using GST-VP16 as an affinity matrix, Greenblatt and colleagues have demonstrated an interaction of the VP16 and p53 activation domains with the p62 subunit of TFIIH (28). The interaction between VP16 and TFIIH has recently been confirmed by Bentley and colleagues (65). Although we did not detect an interaction between VP16 and TFIIH in our assays, we do not wish to imply that the TFIIH–VP16 interaction does not occur. Our results suggest that VP16, but not p53, can selectively target a distinct CTD kinase activity that was not previously detected. Since the experiments by the Greenblatt and Bentley labs and our own lab were done in different ways, our interpretation of these

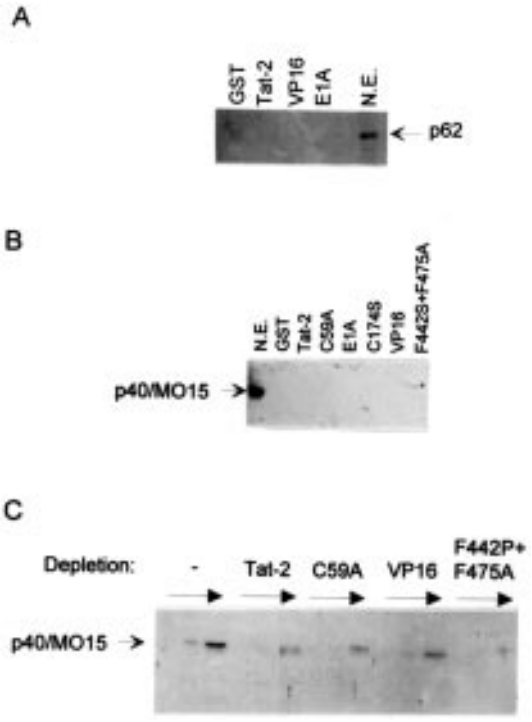

Figure 5. (A, B) TFIIH p62 subunit and MO15 are not detected in Tat-2, E1A or VP16 complexes. GST fusions of the indicated proteins were incubated with a HeLa cell nuclear under conditions described in Materials and Methods. Western blots were performed using an antibody directed against the p62 subunit of TFIIH (**A**) or p40/MO15 (**B**). HeLa cell nuclear extract was loaded in the lane marked N.E. In (A), 50-fold more nuclear extract was used per binding reaction than loaded onto the gel in the lane N.E.; in (B), 10-fold more nuclear extract was used. (**C**) TAK- or VP16K-depletion does not affect p40/MO15 levels. HeLa nuclear extracts were depleted using GST-Tat-2, GST-C59A, GST-VP16 or GST-F442S+F475A as described in Figure 4. Either 2, 6 or 18 µl of a HeLa nuclear extract or the indicated depleted extract was loaded onto a 9% SDS–polyacrylamide gel. The Western blot was performed using an antibody directed against p40/MO15. The extracts used here are from the same experiment as shown in Figure 4A.

results is that VP16 can bind both to TFIIH and another CTD kinase and that the kinase activity which is detected is dependent on the conditions used for binding. It is not unreasonable to suggest that VP16 can associate with more than one CTD kinase, as it is known that VP16 can bind to several components of the transcription complex in addition to TFIIH, such as TBP and TFIIB (24,27); this may be a reason that VP16 is such a powerful activator. Furthermore, the yeast RNAP II holoenzyme has been shown to contain at least two CTD kinases (66).

E1A is known to associate indirectly with the cell cycle regulated kinase cdk2 (67,68). Cdk2 and the related cdc2 kinase have been shown to possess CTD kinase activity (30,69). It is unlikely that the E1A-associated kinase activity described here is cdk2 for several reasons. First, cdk2 bound to a cyclin regulatory subunit phosphorylates histone H1 (67,70–73), whereas the E1A-associated kinase described here does not. Secondly, the association of cdk2 with E1A is thought to occur through an interaction with the E1A-associated p107 and p130 proteins, which interact with cdk2 independently of E1A and are substrates of cdk2 (68,72,74,75). Genetic analysis and peptide mapping studies have shown that the CR1 and CR2 regions of E1A are necessary and sufficient for the stable interaction of p107 and p130 with E1A (76–78); the GST-E1A construct used in our *in vitro* assays lacks these regions and contains only a portion of the CR3 region. Thirdly, the E1A-associated CTD kinase activity is not inhibited *in vitro* by p21 (63), an inhibitor of cdk2 (79–81). Therefore this study has identified a novel interaction of E1A and VP16 with a CTD kinase.

Role of CTD kinases in transcription

The role of CTD phosphorylation in transcription is not yet clear. Since the underphosphorylated form of RNAP II is present in pre-initiation complexes while the hyperphosphorylated form is associated with actively elongating complexes, it has been proposed that phosphorylation of the CTD regulates assembly of the pre-initiation complex, release of RNAP II from the promoter, and progression of the elongation complex (34–40). The existence of multiple mammalian CTD kinase activities (30,53,62,69,82,83) suggests that CTD kinases might function at different points in the transcription process, at different times during the cell cycle, or in conjunction with different activators. For example, some CTD kinases might exert their effects predominantly at the stages of pre-initiation complex assembly, initiation of transcription, or promoter clearance, while others may influence events associated with transcript elongation, such as the rate of elongation or the processivity of the elongation complex.

Based on the correlation between the effect of Tat, VP16 and E1A on transcription elongation and their interaction with a CTD kinase, it is tempting to speculate that TAK and VP16K/E1AK may belong to a subclass of CTD kinases that act to increase the processivity of elongation. Interaction of transactivators with other CTD kinases, such as TFIIH/MO15, may mediate effects at earlier stages in the transcription cycle. Identification and functional analyses of the E1A-, VP16- and Tat-associated CTD kinases will be required to determine their roles in transcription.

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REFERENCES

1 Kamine,J., Subramanian,T. and Chinnadurai,G. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8510–8514.
2 Southgate,C.D. and Green,M.R. (1991) *Genes Dev.* **5**, 2496–2507.
3 Tiley,L.S., Madore,S.J., Malim,M.H. and Cullen,B.R. (1992) *Genes Dev.* **6**, 2077–2087.
4 Kao,S.Y., Calman,A.F., Luciw,P.A. and Peterlin,B.M. (1987) *Nature*, **330**, 489–493.
5 Laspia,M.F., Rice,A.P. and Mathews,M.B. (1989) *Cell*, **59**, 283–292.
6 Laspia,M.F., Rice,A.P. and Mathews,M.B. (1990) *Genes Dev.* **4**, 2397–2408.
7 Feinberg,M.B., Baltimore,D. and Frankel,A.D. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 4045–4049.
8 Marciniak,R.A. and Sharp,P.A. (1991) *EMBO J.* **10**, 4189–4196.
9 Yankulov,K., Blau,J., Purton,T., Roberts,S. and Bentley,D.L. (1994) *Cell*, **77**, 749–759.

10 Selby,M.J. and Peterlin,B.M. (1990) *Cell*, **62**, 769–776.
11 Rhim,H. and Rice,A.P. (1994) *Nucleic Acids Res.* **22**, 4405–4413.
12 Roy,S., Delling,U., Chen,C.-H., Rosen,C.A. and Sonenberg,N. (1990) *Genes Dev.* **5**, 1365–1373.
13 Calnan,B.J., Biancalana,S., Hudson,D. and Frankel,A.D. (1991) *Genes Dev.* **5**, 201–210.
14 Moran,E. and Mathews,M.B. (1987) *Cell*, **48**, 177–178.
15 Harlow,E., Whyte,P., Franza,B.R.,Jr. and Schley,C. (1986) *Mol. Cell. Biol.* **6**, 1579–1589.
16 Whyte,P., Buchkovich,K.J., Horowitz,J.M., Friend,S.H., Raybuck,M., Weinberg,R.A. and Harlow,E. (1988) *Nature*, **334**, 124–129.
17 Dyson,N. and Harlow,E. (1992) *Cancer Surveys*, **12**, 161–195.
18 Lillie,J.W. and Green,M.R. (1989) *Nature*, **338**, 39–44.
19 Sadowski,I., Ma,J., Triezenberg,S. and Ptashne,M. (1988) *Nature*, **335**, 563–564.
20 Triezenberg,S.J., Kingsbury,R.C. and McKnight,S.L. (1988) *Genes Dev.* **2**, 718–729.
21 Cousens,D.J., Greaves,R., Goding,C.R. and O'Hare,P. (1989) *EMBO J.* **8**, 2237–2342.
22 Regier,J.L., Shen,F. and Triezenberg,S.J. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 883–887.
23 Walker,S., Greaves,R. and O'Hare,P. (1993) *Mol. Cell. Biol.* **13**, 5233–5244.
24 Stringer,K.F., Ingles,C.J. and Greenblatt,J. (1990) *Nature*, **345**, 783–786.
25 Lee,W.S., Kao,C.C., Bryant,G.O., Liu,X. and Berk,A.J. (1991) *Cell*, **67**, 365–376.
26 Kashanchi,F., Piras,G., Radonovich,M.F., Duvall,J.F., Fattaey,A., Chiang,C.M., Roeder,R.G. and Brady,J.N. (1994) *Nature*, **367**, 295–299.
27 Lin,Y.-S. and Green,M.R. (1991) *Cell*, **64**, 971–981.
28 Xiao,H., Pearson,A., Coulombe,B., Truant,R., Zhang,S., Regier,J.L., Triezenberg,S.J., Reinberg,D., Flores,O., Ingles,C.J. and Greenblatt,J. (1994) *Mol. Cell. Biol.* **14**, 7013–7024.
29 Goodrich,J.A., Hoey,T., Thut,C.J., Admon,A. and Tjian,R. (1993) *Cell*, **75**, 519–530.
30 Herrmann,C.H. and Rice,A.P. (1995) *J. Virol.* **69**, 1612–1620.
31 Corden,J.L., Cadena,D.L., Ahearn,J.M.,Jr. and Dahmus,M.E. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7934–7938.
32 Allison,L.A. and Ingles,C.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 2794–2798.
33 Cadena,D.L. and Dahmus,M.E. (1987) *J. Biol. Chem.* **262**, 12468–12474.
34 Laybourn,P.J. and Dahmus,M.E. (1989) *J. Biol. Chem.* **264**, 6693–6698.
35 O'Brien,T., Hardin,S., Greenleaf,A. and Lis,J.T. (1994) *Nature*, **370**, 75–77.
36 Dahmus,M.E. (1994) *Prog. Nucl. Acid Res. Mol. Biol.* **48**, 143–179.
37 Lu,H., Flores,O., Weinmann,R. and Reinberg,D. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 10004–10008.
38 Spencer,C.A. and Groudine,M. (1990) *Oncogene*, **5**, 777–785.
39 Marshall,N.F. and Price,D.H. (1992) *Mol. Cell. Biol.* **12**, 2078–2090.
40 Kang,M.E. and Dahmus,M.E. (1993) *J. Biol. Chem.* **268**, 25033–25040.
41 Feaver,W.J., Svejstrup,J.Q., Henry,N.L. and Kornberg,R.D. (1994) *Cell*, **79**, 1103–1109.
42 Roy,R., Adamczewski,J.P., Seroz,T., Vermeulen,W., Tassan,J.P., Schaeffer,L., Nigg,E.A., Hoeijmakers,J.H. and Egly,J.M. (1994) *Cell*, **79**, 1093–1101.
43 Serizawa,H., Makela,T.P., Conaway,J.W., Conaway,R.C., Weinberg,R.A. and Young,R.A. (1995) *Nature*, **374**, 280–282.
44 Shiekhattar,R., Mermelstein,F., Fisher,R.P., Drapkin,R., Dynlacht,B., Wessling,H.C., Morgan,D.O. and Reinberg,D. (1995) *Nature*, **374**, 283–287.
45 Dery,C.V., Herrmann,C.H. and Mathews,M.B. (1987) *Oncogene*, **2**, 15–23.
46 Berger,S.L., Cress,W.D., Cress,A., Triezenberg,S.J. and Guarente,L. (1990) *Cell*, **61**, 1199–1208.
47 Aiyar,A. and Leis,J. (1993) *Biotechniques*, **14**, 366–369.
48 Herrmann,C.H. and Rice,A.P. (1993) *Virology*, **197**, 601–608.
49 Rhim,H., Echetebu,C.O., Herrmann,C.H. and Rice,A.P. (1994) *J. Acq. Imm. Defic. Syn.* **7**, 1116–1121.
50 Yang,X., Herrmann,C.H. and Rice,A.P. (1995) (UnPublished).
51 Li,R. and Botchan,M.R. (1993) *Cell*, **73**, 1207–1221.
52 Sif,S. and Gilmore,T.D. (1994) *J. Virol.* **68**, 7131–7138.
53 Peterson,S.R., Dvir,A., Anderson,C.W. and Dynan,W.S. (1992) *Genes Dev.* **6**, 426–438.
54 Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
55 Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
56 Fischer,L., Gerard,M., Chalut,C., Lutz,Y., Humbert,S., Kanno,M. and Chambon,P. (1992) *Science*, **257**, 1392–1395.
57 Tassan,J.P., Schultz,S.J., Bartek,J. and Nigg,E.A. (1994) *J. Cell Biol.* **127**, 467–478.
58 Geisberg,J.V., Lee,W.S., Berk,A.J. and Ricciardi,R.P. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 2488–2492.
59 Cress,W.D. and Triezenberg,S.J. (1991) *Science*, **251**, 87–90.
60 Fraser,N.W., Sehgal,P.B. and Darnell,J.E. (1978) *Nature*, **272**, 590–593.
61 Sehgal,P.B., Derman,E., Molloy,G.R., Tamm,I. and Darnell,J.E. (1976) *Science*, **194**, 431–433.
62 Lu,H., Zawel,L., Fisher,L., Egly,J.M. and Reinberg,D. (1992) *Nature*, **358**, 641–645.
63 Herrmann,C.H., Harper,J.W. and Rice,A.P. (1995) (Unpublished)
64 Herrmann,C.H. and Rice,A.P. (1995) (Unpublished)
65 Yankulov,K., Yamashita,K., Roy,R., Egly,J.-M. and Bentley,D.L. (1995) *J. Biol. Chem.* **270**, 23922–23925.
66 Liao,S.M., Zhang,J., Jeffery,D.A., Koleske,A.J., Thompson,C.M., Chao,D.M., Viljoen,M., van Vuuren,H.J. and Young,R.A. (1995) *Nature*, **374**, 193–196.
67 Tsai,L.H., Harlow,E. and Meyerson,M. (1991) *Nature*, **353**, 174–177.
68 Faha,B., Harlow,E. and Lees,E. (1993) *J. Virol.* **67**, 2456–2465.
69 Cisek,L.J. and Corden,J.L. (1989) *Nature*, **339**, 679–684.
70 Giordano,A., Whyte,P., Harlow,E., Franza,B.R.,Jr., Beach,D. and Draetta,G. (1989) *Cell*, **58**, 981–990.
71 Pines,J. and Hunter,T. (1990) *Nature*, **346**, 760–763.
72 Herrmann,C.H., Su,L.K. and Harlow,E. (1991) *J. Virol.* **65**, 5848–5859.
73 Rosenblatt,J., Gu,Y. and Morgan,D.O. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2824–2828.
74 Ewen,M.E., Faha,B., Harlow,E. and Livingston,D.M. (1992) *Science*, **255**, 85–87.
75 Faha,B., Ewen,M.E., Tsai,L.H., Livingston,D.M. and Harlow,E. (1992) *Science*, **255**, 87–90.
76 Dyson,N., Guida,P., Munger,K. and Harlow,E. (1992) *J. Virol.* **66**, 6893–6902.
77 Giordano,A., McCall,C., Whyte,P. and Franza,B.R.,Jr. (1991) *Oncogene*, **6**, 481–485.
78 Wang,H.G., Rikitake,Y., Carter,M.C., Yaciuk,P., Abraham,S.E., Zerler,B. and Moran,E. (1993) *J. Virol.* **67**, 476–488.
79 Gu,Y., Turck,C.W. and Morgan,D.O. (1993) *Nature*, **366**, 707–710.
80 Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) *Cell*, **75**, 805–816.
81 Xiong,Y., Hannon,G.J., Zhang,H., Casso,D., Kobayashi,R. and Beach,D. (1993) *Nature*, **366**, 701–704.
82 Payne,J.M. and Dahmus,M.E. (1993) *J. Biol. Chem.* **268**, 80–87.
83 Baskaran,R., Dahmus,M.E. and Wang,J.Y. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 11167–11171.