JOURNAL OF VIROLOGY, Oct. 1996, p. 7264–7269 Vol. 70, No. 10
0022-538X/96/$04.00+0

Transcriptional Activation Function Is Not Required for Stimulation of DNA Replication by Bovine Papillomavirus Type 1 E2

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Received 16 April 1996/Accepted 18 June 1996

Bovine papillomavirus type 1 replication was previously shown to require both the E1 initiator protein and the E2 transactivator protein. We show here that E1, in the absence of E2, is sufficient for low-level bovine papillomavirus type 1 DNA replication in C-33A cells. In addition, studies of genetically isolated E2 point mutants demonstrate that enhancement of replication by E2 does not require its transcriptional activation function. The uncoupling of the E2 functions suggests that stimulation of transcription and replication by enhancer proteins occurs via divergent mechanisms.

Many studies have shown that proteins which specifically activate transcription can also stimulate origins of DNA replication (reviewed in references 10 and 15). A requirement for both transcriptional activation and DNA-binding domains for this enhancement of replication in vivo has been reported (15). The role of transcription factors in DNA replication has frequently been examined with polyomavirus (Py) and simian virus 40 (SV40) as model systems. In SV40 and Py, the only viral protein required for replication is its large tumor antigen (T-ag), which binds DNA specifically and initiates unwinding of the viral origin (5). The papillomaviruses (PVs) differ from the lytic SV40 and Py in that their genome is maintained as a stable nuclear plasmid under strict copy number control (3, 11, 18). The PVs encode a protein, E1, which shares functional and primary sequence homology with the T-ag proteins (9). Like T-ag, E1 alone can efficiently induce DNA synthesis in vitro (4), and at limiting E1 levels, the E2 enhancer protein stimulates E1-dependent in vitro replication 10- to 20-fold (19, 40). In contrast to in vitro studies, a strict requirement for both the E1 and E2 proteins for BPV-1 DNA replication in vivo has been reported (34, 35). The differences between the in vitro and in vivo requirements may in part be due to relief of nucleosome repression by E2; in fact, E2 has been shown to counteract chromatin repression of bovine PV type 1 (BPV-1) replication in vitro (20).

The long control region of the PV genome is a noncoding region containing viral promoters and the origin of replication. The minimal BPV-1 origin includes an imperfect inverted repeat element specifically recognized by BPV-1 E1 (24, 38), and an E2 binding site (34). The E2 protein coordinates expression of viral genes and replication of the PV genome (for review, see reference 23). BPV-1 E2 contains a 220-residue transactivation domain separated by approximately 100 amino acids from a C-terminal DNA-binding and -dimerization domain (DBD) (12). An internally initiated repressor protein (E2TR) lacks much of the transactivation domain and does not stimulate replication (35, 39) or transcription (17), demonstrating the requirement for the transactivation domain in both functions.

The results of replication studies involving the E2 transactivation domain (TAD) parallel findings with Py and SV40 which demonstrate that, in vivo, stimulation of DNA replication by cellular transcriptional regulators requires their transactivation domains (15, 36, 39). Most Py studies demonstrate that replication enhancement and transcriptional activation are coupled processes (1, 2, 26, 33, 37). However, a recent study questions this conclusion (14). The findings that the same site-specific DNA-binding proteins enhance both transcription and replication have prompted the notion that common mechanisms mediate these two processes. To investigate this hypothesis, we have used the BPV-1 system to examine the requirements for both transcription and replication enhancement. Unlike earlier studies of BPV-1 replication, we find that the E1 protein alone can support low-level replication in vivo. E2 dramatically enhances E1-driven replication, defining E2 as a factor which stimulates basal replication as well as transcription. In this regard, E2 behaves similarly to cellular transcription factors which stimulate basal levels of transcription and replication at viral and yeast promoters and origins. In addition, we studied a series of E2 proteins containing point mutations in the transactivation domain. These E2 mutants were isolated with a yeast-based genetic screen designed to identify amino acids necessary for transcriptional activation. A subset of these E2 mutants was found to stimulate wild-type levels of E1-dependent replication despite being completely defective for transcriptional activation. These results are consistent with a model in which enhancer proteins stimulate both transcription and replication through a common first step, such as chromatin clearing, and diverge at subsequent steps which could involve the binding of cellular coactivating and/or coreplicating proteins.

The BPV-1 E1 protein alone can drive replication in vivo. Previous data have shown that both the E1 and E2 proteins were required for BPV-1 replication in vivo, and yet the E1 protein alone is sufficient for in vitro replication (4, 40). The discrepancy between the in vitro and in vivo requirements is not well understood. One possibility is that E2, like cellular transcription factors which stimulate Py and SV40 replication, enhances low-level E1-driven replication. During the course of our experiments to examine the role of E2 in E1-directed replication, low levels of DNA replication were repeatedly detected when higher levels of E1 plasmid were transfected in the absence of E2. To test whether the E1 protein alone could direct replication in vivo, increasing amounts of the BPV-1 E1-expressing plasmid pCGEAg (35) were cotransfected with

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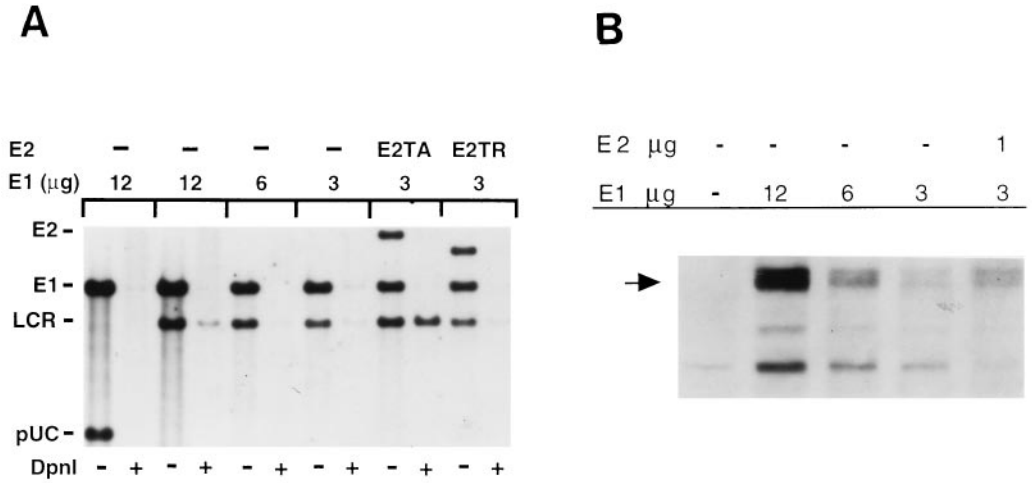

FIG. 1. E1 supports the replication of a BPV-1 origin plasmid in the absence of E2. (A) Southern blot. C-33A cells were cotransfected with either origin-negative plasmid pUC19 (first two lanes) or origin plasmid pUCLCR-BPV (all other lanes) along with 12, 6, or 3 μg of E1-expressing plasmid pCGEAg in the absence of E2 plasmid (shown in the left eight lanes) or in the presence of plasmids expressing either wild-type E2 or the truncated E2 repressor form (E2TR). The positions of bands corresponding to pCGEAg (E1), pCGE2b (E2), pUC19 (pUC), and pUCLCR-BPV (long control region [LCR]) are indicated to the left. (B) Western blot. Duplicate plates of transfected C-33A cells corresponding to the center four pairs of lanes in panel A plus a mock-transfected negative control were harvested in denaturing buffer and analyzed for E1 protein by immunoblotting with an E1 peptide-specific antibody. The position of the full-length E1 proteins is indicated by the arrow. Lower-molecular-weight bands are degradation products of full-length E1.

the origin-containing plasmid pUCLCR-BPV (32) by calcium phosphate coprecipitation (8) into the human PV-negative human cervical carcinoma cell line C-33A. Transfection efficiencies ranged from 15 to 30%. Hirt extracts (16) containing low-molecular-weight DNA were prepared 3 days posttransfection, and the DNA was digested with *Eco*RI to linearize the plasmids. Half of each sample was digested with *Dpn*I to remove unreplicated, methylated input DNA (27). The DNA samples were analyzed by agarose gel electrophoresis and Southern blot hybridization with a random-primed, digoxigenin-dUTP-labeled pUC19 probe and chemiluminescent detection (Genius system; Boehringer-Mannheim) (29). Replication of the pUCLCR-BPV origin plasmid was detected only at the highest amounts of input plasmid pCGEAg (12 μg), as evidenced by the *Dpn*I-resistant band corresponding to pUCLCR-BPV in lanes treated with *Dpn*I (Fig. 1A). This E1-directed replication was specific for the BPV-1 origin, because pUC19 did not replicate under identical conditions. The replication seen with 12 μg of pCGEAg transfected corresponds to increased expression of E1 protein in the cell as determined by immunoblotting (Fig. 1B). These results demonstrate that the BPV-1 E1 protein alone is sufficient for DNA replication in vivo. Under conditions in which replication was not detectable with E1 alone (i.e., 3 μg of pCGEAg), cotransfection of wild-type BPV E2 markedly stimulated replication as expected (Fig. 1A). This enhancement of E1-directed replication was specific for full-length E2, because the E2 repressor form (E2TR) was unable to stimulate replication (Fig. 1A).

Replication and transcription properties of E2 transactivation domain mutants. Previous studies and the results presented in Fig. 1A demonstrate a requirement for the E2 transactivation domain in the stimulation of E1-directed replication (36, 39, 40). Under conditions in which replication by E1 alone was not detectable, we tested the replication activity of a series of 20 E2 mutants containing single-amino-acid substitutions with substitutions in the N-terminal activation domain. The isolation and characterization of these mutants in yeast cells are described elsewhere (6). The majority of these E2 mutants were isolated from a library of random E2 mutations with phenotypic screening in *Saccharomyces cerevisiae* to identify transcriptionally defective E2 proteins. This was done in order to enhance the isolation of mutants specifically defective for transcription and not replication. This class of mutants may be underrepresented if only conserved residues of E2 were targeted for directed mutagenesis. Three mutants with changes D-24→A, K-25→E, and A-46→E (D24A, K25E, and A46E, respectively) were isolated from the library without phenotypic screening in yeast cells. Transactivation domain point mutations were transferred into the pCGE2b vector for expression from the cytomegalovirus promoter. The pCGE2b plasmid was modified from pCGE2 (35) to contain a *Bam*HI site in the polylinker and encodes the wild-type methionine at codon 161 of BPV-1 E2 (nucleotide 3089). One microgram of the pCGE2b expression plasmid containing either wild-type or mutant E2 was cotransfected with 3 μg of pCGEAg and 500 ng of pUCLCR-BPV into the C-33A cell line. Titration experiments determined that these conditions were optimal for replication (31a). The replication phenotypes of the E2 mutants ranged from completely defective to wild type (Fig. 2). E2 mutants Q66R, W92R, P106S, and W145R were unable to stimulate replication of the origin-containing plasmid. Mutants Q15H, F87S, and W99C induced low but consistently detectable levels of replication. Mutants L31P, Y32H, S93P, N127Y, and R208G exhibited slightly decreased levels of replication compared with that of the wild type, while mutants D24A, K25E, E39G, A46E, W130R, E176G, R179G, and S181F reproducibly demonstrated wild-type levels of E1-dependent replication.

To study the relationship between transcription and replication functions in E2, the series of E2 TAD point mutations described above were tested for their ability to activate transcription. For these studies, C-33A cells were transfected with 100 ng of the E2 expression plasmid and 1 μg of reporter plasmid (pMJG1) by calcium phosphate coprecipitation. Titration experiments determined these amounts to be optimal for transcriptional activation in C-33A cells (unpublished data). The pMJG1 reporter was made by insertion of three E2 binding sites (ACCGAATTCGGT) into the *Hin*dIII-*Nde*I sites up-

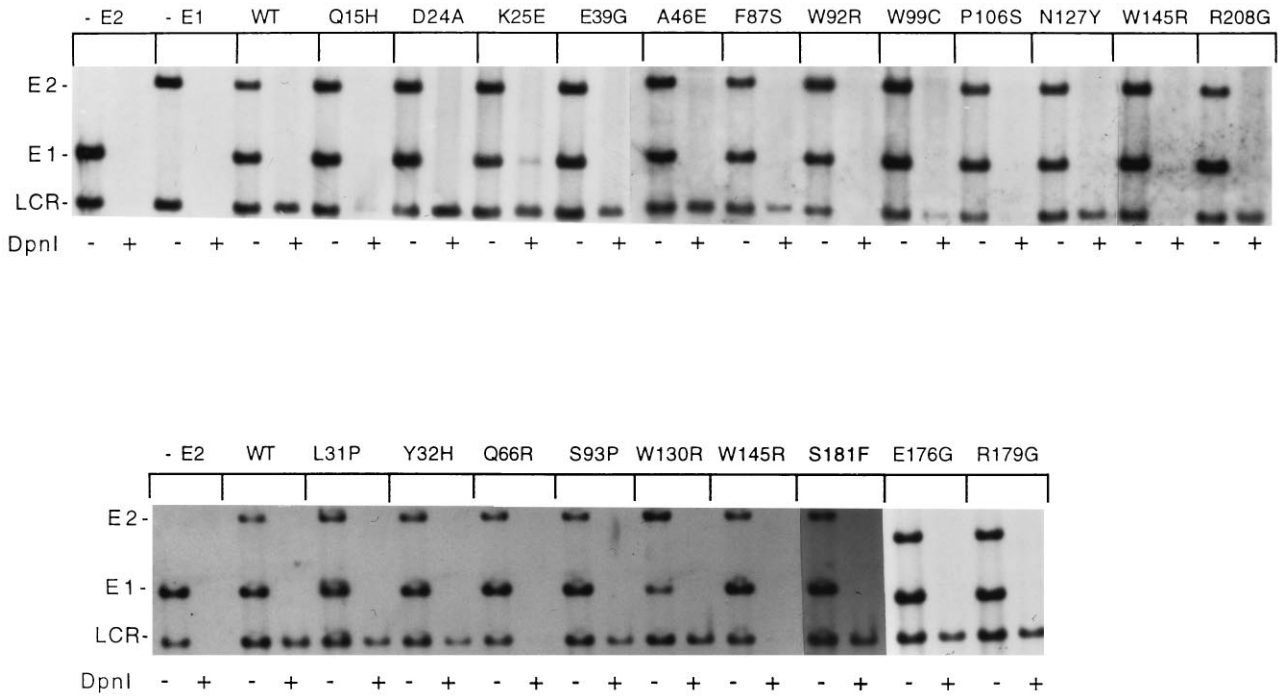

FIG. 2. Replication enhancement phenotypes of the E2 transactivation domain mutants. C-33A cells were cotransfected with plasmids pUCLCR-BPV (origin), pCGEAg (expressing E1), and pCGE2b expressing either wild-type (WT) or mutant E2 proteins by calcium phosphate coprecipitation. Cells were harvested 3 days after transfection. Control lanes include pCGEAg transfected with the origin-containing plasmid (-E2) and the pCGE2b wild type transfected with the origin plasmid (-E1). The positions of bands corresponding to the E1 and E2 expression plasmids and origin plasmid (long control region [LCR]) are indicated to the left.

stream of the thymidine kinase promoter elements and human growth hormone gene in pTKGH (Nichols Institute, San Juan Capistrano, Calif.). Medium was assayed for human growth hormone at day 2 posttransfection with the Human Growth Hormone-Transient Gene Expression Kit according to instructions from the manufacturer (Nichols Institute). The transcriptional activation properties of these 20 E2 point mutants are summarized in Fig. 3. The majority of these phenotypically selected E2 mutants (Q15H, L31P, Y32H, E39G, Q66R, F87S, W92R, S93P, W99C, P106S, W145R, S181F, and R208G) showed activity ~10% or less of the level of wild-type activity. Mutants N127Y, E176G, and R179G retained ~40% of the level of wild-type activity, while mutants D24A, K25E, A46E, and W130R demonstrated greater than 50% of the level of wild-type activity. Expression of all 20 E2 mutant proteins in C-33A cells was confirmed by immunoblot analyses. A representative blot of nine E2 mutants which were defective for either transcriptional activation, stimulation of replication, or both is shown in Fig. 4. Thus, the inability of these mutants to enhance either transcription or replication was not due to lack of synthesis or the rapid degradation of the mutant proteins.

Comparison of E2 transcription and replication functions. Our studies of the transcription and replication activities of E2 TAD mutants identified two classes of mutants: those in which stimulation of transcription and replication were similarly affected or coupled (Table 1, top) and a second class in which these functions were uncoupled (Table 1, bottom). The first group includes E2 mutants which retain the ability to stimulate transcription and replication as well as mutants that are defective for both functions (Table 1), in agreement with earlier studies of the E2 TAD (7, 36, 39). The second group of E2 mutants were defective for transcriptional activation but stimulated replication (Table 1). Notably, three of these uncoupled mutants were within amino acids 31 to 39, and three were within amino acids 87 to 99. Two other uncoupled mutants in the distal region of the activation domain, S181F and R208G, also showed high-level stimulation of replication. Because these mutants were isolated on the basis of a transcriptionally defective phenotype, their ability to stimulate replication implies that their defect is based on an inability to interact with transcription-specific cellular factors and may be useful for the identification of the components of this E2 pathway. The uncoupled phenotype of the E-39→G (glycine) mutant is particularly interesting. This glutamic acid (E) residue is absolutely conserved in the coding sequence of all published papillomavirus E2 genes, indicating that it may be crucial to one or more functions of the E2 protein. Two other groups have recently described mutations at this residue that resulted in proteins with different phenotypes. In one study, a mutant containing a conservative substitution of E-39 to aspartic acid (D) was found to be highly defective for both transcriptional activation and replication (7). The corresponding glutamic acid of HPV-16 E2 was mutated to alanine and was found to be active for transcriptional activation but defective for replication (28), a phenotype opposite that of the BPV-1 E39G mutant reported here. The specific residue substituted for glutamic acid 39 in these studies may explain the different phenotypes observed. Our genetic screen isolated a mutant containing a W-92→R substitution that was defective for transcription and for replication. In a study employing site-directed mutagenesis, a W-92→F (phenylalanine) substitution resulted in a protein that retained significant activity in both transcription and replication assays (7). The corresponding tryptophan residue of HPV-16 E2 was substituted with alanine, resulting in a protein with reduced activity in both assays (28). The differences in the

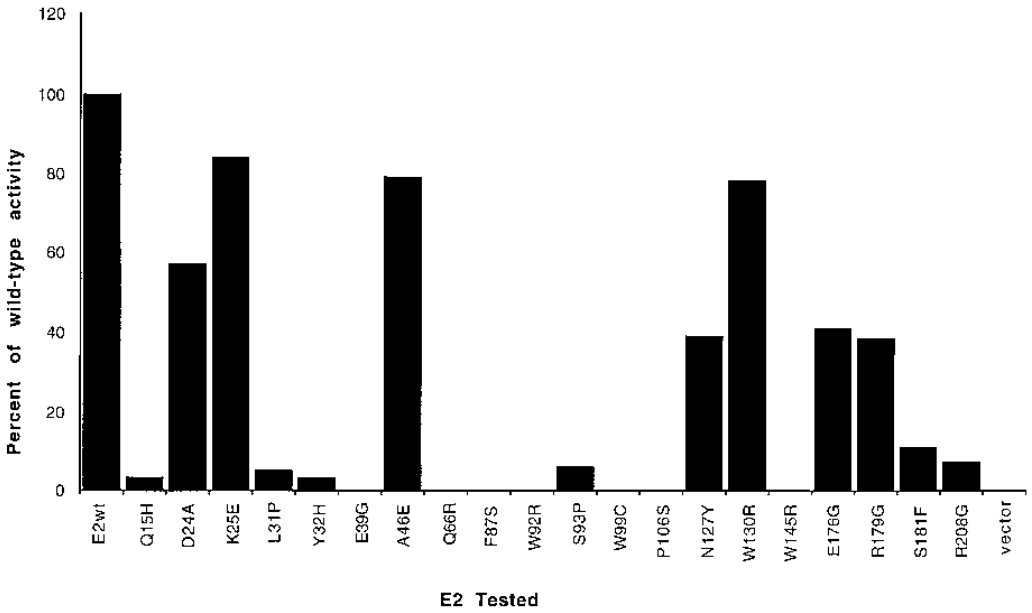

FIG. 3. Transcriptional enhancement phenotypes of the E2 transactivation domain mutants. C-33A cells were cotransfected with either pCG vector (control) or plasmids expressing E2 wild-type (E2wt) or E2 mutant coding sequences along with an E2-dependent human growth hormone reporter. Two days posttransfection, medium was removed and assayed for human growth hormone. The plasmids transfected are indicated on the horizontal axis. Transcriptional activities are expressed as percentages of wild-type E2 activity and represent three separate experiments.

results described above are likely due to the severity of amino acid substitutions at residue W-92.

Several systems have provided evidence for functional overlap between transcription and replication. ARS elements from *S. cerevisiae*, chromosomal origins in mouse embryos, and origins from DNA viruses require activator proteins for efficient DNA replication in vivo (15). The mechanism by which a transcription factor stimulates replication has not been determined, but evolutionary conservation is evident, since yeast transcription proteins such as GAL4 can stimulate eukaryotic viral origins (1, 2). Analogous to cellular transcription factors which stimulate replication, E2 possesses a TAD and a DNA-binding domain which serve to activate transcription from viral promoters and to stimulate replication from the PV origin. Unlike the cellular enhancers of replication, E2 has an additional function in directly binding to its specific initiator protein E1. As the in vitro replication studies demonstrated (4, 40), we find that BPV E1 alone, like the T-ag proteins of SV40 and Py, is able to initiate low-level DNA synthesis in vivo and that the E2 protein enhances this E1-driven replication. This result is consistent with findings that the human PV type 1a and human PV type 16 E1 proteins are able to support low-level replication of their respective origin plasmids when expressed to high levels in C-33A cells (13, 28). Immunoblots of E1 suggest that cotransfection with BPV E2 may result in increased E1 protein levels (Fig. 1B). However, this slight increase in E1 does not account for the high degree of replication observed with E1 and E2 cotransfections (Fig. 1A), suggesting that activities other than stabilization or increased expression of E1 by the E2 protein are operative. At least three distinct activities of E2 have been proposed to participate in the stimulation of replication: alleviation of nucleosome repression (20), enhancement of E1 binding to its specific DNA-binding site (22, 25, 30, 31), and interaction with cellular replication factors such as replication protein A (19). A combination of these activities likely participates in replication enhancement.

Consistent with studies of enhancer proteins in Py, Epstein-Barr virus, and SV40, deletion mutations in the transactivation domain of E2 abolish both transcription and replication functions (36, 39). Nine of 20 genetically screened point mutations spanning the E2 TAD described here resulted in proteins with phenotypes dissociating the functions of transcription and replication, with one mutant (E39G) maintaining full replication activity while being completely defective for transcriptional activation. The other eight mutants were highly defective for transcriptional activation while retaining significant replication activity.

Our data demonstrate that the transcription and replication enhancement functions of the BPV-1 E2 protein can be uncoupled. We suggest a model in which the first step is common to both transcription and replication functions and requires the E2 TAD. This may involve the process of nucleosome clearing, relieving chromatin-mediated repression of both transcription and replication. Interestingly, the chromatin-clearing SWI-

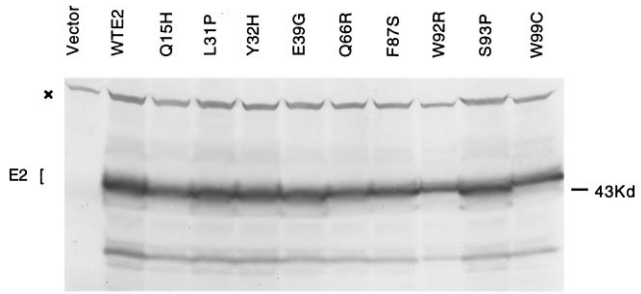

FIG. 4. E2 mutants encode stable protein. Five micrograms of either pCGb vector or pCGE2b expressing wild-type E2 (WTE2) or mutant E2 coding sequence was transfected into C-33A cells. Equal volumes of cell extract were loaded, and E2 proteins, indicated by the bracket, were detected by immunoblotting (25a). A cellular protein which cross-reacts with the E2 antibody is indicated by **x**.

TABLE 1. Comparison of replication and transcriptional activation functions of E2 point mutants[a]

E2 tested[b]	Transcriptional activation	DNA replication
Group 1		
Wild type E2	+++	+++
D24A	++	+++
K25E	++	+++
A46E	+++	+++
Q66R	−	−
W92R	−	−
P106S	−	−
N127Y	+	++
W130R	++	+++
W145R	−	−
E176G	+	+++
R179G	+	+++
Group 2		
Q15H	−	+
L31P	−	++
Y32H	−	++
E39G	−	+++
F87S	−	+
S93P	−	++
W99C	−	+
S181F	−	+++
R208G	−	++

[a] Transactivation activities were designated as follows: −, approximately less than 10% of wild type activity; +, ++, and +++, 10 to 50%, 50 to 90%, and wild-type levels of activity, respectively. Levels of replication were approximated by examination of at least three independent replication experiments. Transcriptional activation levels were taken from Fig. 3.

[b] Group 1 includes E2 mutants which retain the ability to stimulate transcription and replication as well as mutants defective for both functions. Group 2 is made up of mutants that were defective for transcriptional activation but that stimulated replication. See text for more details.

SNF complex has been shown to be necessary for replication enhancement of a yeast ARS element by the GAL4 transcriptional activation domain (27a). As shown previously, the requirement for the E2 TAD cannot be fulfilled by the VP16 TAD (36), suggesting there is an additional function(s) of the E2 TAD in replication. This additional function is not only E1 binding, since E2 has been shown to stimulate replication of the Py origin in the absence of the E1 protein (26). Also, recent work by Chow and coworkers indicates that both the BPV-1 and human PV-11 E2 proteins are required for a step in preinitiation complex formation which occurs after stabilization of E1 binding to the origin (21). Since the functions of transcription and replication can be separated or uncoupled by mutations in the E2 TAD, a subsequent step in the regulation of these functions must discriminate between transcription and replication. This step might involve interactions of the E2 TAD (and DNA binding and dimerization domain) with cellular cofactors required specifically for transcription or replication.

The first two authors contributed equally to this work.

We thank Peter Bullock for critical reading of the manuscript, Arne Stenlund for plasmids pCGE2 and pCGEAg, and Saleem Khan for plasmid pUCLCR-BPV.

This work was supported by NIH RO1 CA58376. M.J.G. was supported in part by NIH T32 A107422.

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