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trans Activation by the Bovine Papillomavirus E2 Protein in *Saccharomyces cerevisiae*

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The papillomavirus E2 protein functions as an enhancer-binding factor to promote transcription in mammalian cells. We found that one copy of the E2 binding site acted as an E2 protein-dependent upstream activating sequence in *Saccharomyces cerevisiae*. Additional copies of the binding motif further augmented transcription. These results imply that the E2 protein functionally interacts with highly conserved transcriptional elements.

The E2 enhancer affords a model for discriminating the cellular requirements for gene expression. The bovine papillomavirus (BPV) E2 open reading frame encodes a 48-kilodalton (kDa) enhancer-binding protein that stimulates viral transcription in mammalian cells (13, 18, 33). The protein mimics the modular structure of bacteriophage, yeast, and several eucaryotic transcriptional regulators. Previous genetic analyses have demonstrated that the N-terminal region (amino acids 1 to 210) is required for enhancement and that the C terminus (amino acids 286 to 410) recognizes the E2 binding site $ACCN_6GGT$ (1, 3, 5, 7, 14, 15, 27–29). These domains are separated by a variable length of residues that are not shared among the papillomaviruses.

Although conformational changes may occur in the DNA after binding by E2 (26), current evidence supports the role for structural features of the N-terminal portion of E2 in *trans* activation. This region stimulates transcriptionally active mammalian promoters in the absence of the DNA-binding domain, presumably by cooperating with other proteins that initiate the transcription process (14). Studies by several investigators have demonstrated that *trans*-activation domains often include amphipathic helices with negatively charged residues exposed on one surface (17, 30), and similar structures are predicted for the E2 protein (7, 17). Using chimeric proteins encoding a specific DNA-binding domain molecularly linked to an acidic activating region, it has been inferred that at least some transcriptional mechanisms are shared in yeast, mammal, plant, and insect cells (2, 6, 9, 12, 20, 23–25, 30, 31, 34).

To determine whether transcriptional factors with which the N-terminal region of the E2 protein presumably interacts are conserved in lower eucaryotes, we tested the ability of the E2 proteins to modulate transcription in *Saccharomyces cerevisiae*. Protein-protein associations are difficult to functionally assess in mammalian cells, and because yeast cells can be more easily manipulated genetically, we believe that this system can be exploited to identify and isolate the evolutionarily conserved transcription elements that function with E2.

Synthetic oligonucleotides containing the functional E2 binding site were cloned into the *Xho*I site at position −178 of the reporter plasmid pLGΔ178, an upstream activating sequence (UAS)-deleted derivative of pLGΔ312 (10). Blunt-end oligonucleotides 5′-GAGAACCGTCTTCGGTGCTC-3′ were ligated, digested with *Xho*I, religated, and inserted into the unique *Xho*I site of the UAS-deleted vector pLGΔ178. Construction pBY-1 contains the sequence 5′-CCCTCGAG A ACCG TCTT CGGT GCTCGCAG-3′, in which the first nucleotides represent the *Sma*I-*Xho*I fusion in pLGΔ178 and the E2 binding site is underlined. Since there was no 3′ *Xho*I site, an additional E2 motif was inserted into pBY-1. This resulted in pBY-2, which has two motifs 5′-CCCTCGAGA GC ACCG AAGA CGGT TCTCGAGA ACCG TCTT CGGT GCTCGCAG-3′. pBY-4 contains four sets of the oligonucleotides as 5′-CCCTCGAGAGC ACCG AAGA CGGT CGGT TCTCGAG ACCG TCTT CGGT GCTCGAGA ACCG TCTT CGGT GCTGAGC ACCG AAGA CGGT TCTCTCG AG-3′. Therefore, clone pBY-1 contains a single E2 binding site, and clone pBY-2 contains two copies and pBY-4 contains four copies of the $ACCGN_4CGGT$ motif (Table 1). In some experiments, the *URA* selection gene in these reporter vectors was inactivated by digestion with *Stu*I and replaced with the *LEU2* gene from plasmid CV-13.

All E2 proteins were expressed from the construction pKP-15, a derivative of SD5-ATG (11; called C2 in reference 8), which contains the *GAL* UAS, *CYC1* TATA elements, 2μ yeast replication origin, and *URA* gene for selection (Table 1). The BPV E2 gene was isolated as a 1.8-kilobase *Bam*HI fragment from the bacterial expression vector pCO-E2 (15) and ligated into the *Bam*HI site of pKP-15. The first ATG in this clone, called pYE2, is the initial methionine codon at the N terminus of the E2 open reading frame. pYE2-R was constructed by using synthetic oligonucleotides to convert the *Nco*I site at nucleotide 3089 in BPV to a *Bam*HI site. The oligonucleotides, $\begin{matrix}5'\text{-GATCCTTTGCCGCCAC-}3'\\ \text{GAAACGGCGGTGGTAC}\end{matrix}$ were ligated to the *Nco*I site at nucleotide 3089, the E2 gene was released after digestion with *Bam*HI (nucleotide 4450), the fragment was inserted into pKP-15, and the orientation was confirmed. The C-terminal DNA-binding domain was deleted between nucleotides 3455 to 3881 by digestion of pYE2 with *Kpn*I and *Bst*XI; the ends were made blunt with T4 DNA polymerase and religated.

Yeast transformation was performed by the lithium acetate method (19). Yeast strain BGW1-7a (HATα *leu2-3 leu2-112 his4-519 ade1-100 ura3-52*) was grown in yeast extract-peptone-dextrose medium. Transformants were selected on minimal medium (YMM) plates with the appropriate supplements for *LEU* and/or *URA* gene selection and

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TABLE 1. Constructions used

Plasmid[a]	UAS	Gene expressed
pLGΔ178	None	*lacZ*
pBY-1	One E2 binding site	*lacZ*
pBY-2	Two E2 binding sites	*lacZ*
pBY-4	Four E2 binding sites	*lacZ*
pLGΔ312	*CYC*	*lacZ*
PLGSD5	*GAL*	*lacZ*
pKP15	*GAL*	None
pYE2	*GAL*	E2 amino acids 1–410
pYE2-KB	*GAL*	E2 amino acids 1–282
pYE2-R	*GAL*	E2 amino acids 162–410

[a] All contain the *CYC* TATA elements in the promoter and the 2μ yeast replication origin.

amplified in YMM with 2% glucose. In experiments using galactose induction of E2, the cells were pelleted, washed twice with water, and suspended in YMM with 2% galactose for 4 to 8 h before being assayed. In some experiments, yeast cells were seeded directly into selective medium with galactose for 6 h. The β-galactosidase (β-gal) assay was performed as described by Guarente (8).

We first confirmed production of the E2 protein in yeast cells selected for the plasmids containing the *URA* gene and the inducible E2 construct by immunoblot. The predicted 48-kDa band was observed in YMM-galactose-stimulated cells but not in yeast cells grown in glucose or with the E2 gene in the opposite orientation (data not shown). The quantity of E2 increased during 18 h of galactose induction, but after about 8 h smaller E2 peptides, presumably from proteolytic degradation, were observed. The vector containing pYE2-R was shown to synthesize the carboxy-terminal 30 kDa of the E2 open reading frame, and pYE2-KB yeast produced the predicted 34-kDa peptide.

The ability of the full-length E2 protein to activate transcription was then tested. Yeast cells were selected for the E2 protein expression vector via the *URA* gene and the E2 binding site reporter constructs via the *LEU* gene by growth in leucine- and uracil-deficient media. Transformants were grown in glucose (which repressed E2 protein expression) and then switched to galactose for 6 h before assays for β-gal activity. Insertion of the single E2 motif present in pBY-1 resulted in a seven- to eightfold increase in β-gal expression that depended on the presence of E2 (Table 2). Furthermore, a direct correlation between the number of E2 binding sites and the level of β-gal production was found (Table 2). With two motifs in pBY-2, E2 protein-dependent upstream activation of the *CYC1* promoter was 40 times the basal activity and was four- to fivefold higher than with a single binding site. The tandem of four E2 elements in pBY-4 demonstrated an additional threefold increase over that found with two E2 binding sites. In YMM-glucose, all of the E2 binding sites clones produced basal quantities of β-gal (data not shown). In the absence of E2 protein, increasing the number of E2 binding sites did not promote expression of β-gal in galactose (Table 2), indicating that the E2 binding elements were not activated by an endogenous factor in *S. cerevisiae*. Cotransformation of the UAS-deleted parental plasmid pLGΔ178 with pYE2 did not result in β-gal expression (Table 3). These results demonstrate that E2 binding sites function as an E2 protein-dependent UAS in *S. cerevisiae*, presumably mediated by interaction of the virus-encoded protein with yeast transcriptional factors.

In addition to activation by full-length E2 protein, DNA-binding-defective E2 mutants have been shown to stimulate

TABLE 2. Effects on transcription of the E2 enhancer in *S. cerevisiae*

Yeast reporter	E2 vector	β-Gal activity[a]
pBY-1		2.4
pBY-2		2.0
pBY-4		2.4
pBY-1	pYE2	16.6
pBY-2	pYE2	77.0
pBY-4	pYE2	206.4
pBY-1	pYE2-KB	2.4
pBY-2	pYE2-KB	2.6
pBY-4	pYE2-KB	2.6
pBY-1	pYE2-R	3.0
pBY-2	pYE2-R	3.2
pBY-4	pYE2-R	2.9

[a] Activity of yeast cells transformed with the E2 binding site reporter series and E2 proteins. Expression is in β-gal units (optical density at 420 nm × 1,000/optical density at 600 nm, corrected for time). For each yeast reporter, three independent colonies were grown and evaluated for β-gal activity. Results are averages of two experiments.

heterogenous enhancers (14). We wished to determine whether E2 had this activity in yeast cells, an activity that could easily be monitored because the basal activity of these constructions is very low. Therefore, cotransformations were performed with the *lacZ* reporters and pYE2-KB, in which the C-terminal DNA-binding domain was deleted (Table 1). Results of the cotransformation experiments using the UAS-deleted indicator plasmids are shown in Table 2. Yeast cells containing the vectors with one, two, or four E2 binding sites demonstrated no increase in β-gal expression in the presence of pYE2-KB.

These results confirmed that the DNA-binding domain of E2 is required for *trans* activation of the E2 enhancer. To test for stimulation from a transcriptional unit containing an active UAS, plasmid pLGΔ312, which contains a *CYC* UAS prior to the *lacZ* gene (Table 1), was used. When cells were transformed with pYE2 and grown in galactose, there was an average twofold stimulation of β-gal expression over that of pLGΔ312 alone in galactose (Table 3). This result suggested that E2 can induce weak effects on a yeast transcriptional unit when it includes a functioning UAS.

We also tested the requirement of the entire N-terminal region of E2 for *trans* activation in yeast cells. pYE2-R encoded a functional DNA-binding domain but lacked the first 18 kDa of the E2 and E2-KB proteins. Cotransformation of clone pYE2-R with the E2 binding site reporter series demonstrated no significant change from the base-line production of β-gal (Table 2). These results imply that the DNA-binding domain in E2-R, along with the C-terminal portion of the E2 activating region, is not sufficient for transcriptional enhancement.

In this study, we demonstrated that the E2 enhancer functions as a UAS in *S. cerevisiae* in a manner conditionally

TABLE 3. Effects of E2 protein on reporter constructions without E2 binding sites

Yeast reporter	E2 vector	β-Gal activity[a]
pLGΔ178		0
pLGΔ178	pYE2	0
pLGΔ312		78
pLGΔ312	pYE2	159
pLGSD5		1,060
pLGSD5	pYE2	1,120

[a] Assays were performed as for Table 1.

dependent on the authentic BPV E2 protein. Binding of the E2 protein to DNA (by pYE2-R, for example) was not sufficient for activation, as has also been demonstrated in mammalian cell assays (4, 22). Several copies of the E2 motif provided a stronger activation signal in both systems. Four tandem E2 binding sites provided an activation signal about 20% as active as that of the *GAL* UAS, one of the strongest regulatory elements in *S. cerevisiae* (Table 3). It has been reported by several investigators that a single E2 motif does not act as an enhancer in mammalian cells (15, 16, 32). In our experiments, the single binding site present in pBY-1 provided E2-dependent upstream activation. It is unclear whether this was due to a primary difference between the transcriptional machinery operative in these systems, to potential plasmid-derived contributions in the constructions used, or to the high levels of E2 protein in the yeast cells. A trivial explanation is that the yeast promoter contains a cryptic, functional E2 binding site. There is a sequence ($ACCN_7GGT$) in the *URA* gene 5′ to the E2 binding sites that may contribute to the level of expression of *lacZ* in PBY-1. The *CYC* UAS itself has no E2 consensus binding sequence.

These data indicate that the E2 binding sites can act as a UAS upon expression of a full-length E2 protein and therefore strongly suggest that E2 functionally interacts with a highly conserved factor of eucaryotic transcription. The power of yeast genetics can now be applied to the molecular identification of functionally significant factors that interact with the E2 protein.

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ADDENDUM

While this report was in preparation, Lambert et al. reported E2-dependent *trans* activation in *S. cerevisiae* (21).

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