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Cooperative DNA Binding of the Bovine Papillomavirus E2 Transcriptional Activator Is Antagonized by Truncated E2 Polypeptides

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Cooperative DNA binding of the bovine papillomavirus type 1 (BPV-1) E2 transcriptional activator (E2-TA) is thought to play a role in the transcriptional synergism of multiple E2-responsive DNA elements (J. Ham, N. Dostatni, J.-M. Gauthier, and M. Yaniv, Trends Biochem. Sci. 16:440–444, 1991). Binding-equilibrium considerations show that such involvement is unlikely, thereby suggesting that the E2-TA cooperative capacity may have evolved to play other, different roles. The role of cooperative interactions in the antagonistic activity of BPV-1-positive and BPV-1-negative E2 regulatory proteins was investigated by an in vitro quantitative gel shift assay. Viral repressor E2-TR, a truncated peptide encompassing the activator DNA-binding domain, possesses a small but measurable cooperative capacity. Furthermore, the minimal E2 DNA-binding domain interacts with the activator in a positive, heterocooperative manner. As a result, the in vitro competition of full-length and truncated E2 peptides appears to be (macroscopically) noncooperative. This heterocooperative effect is probably dominant in latently infected G_0-G_1 cells, in which repressor E2-TR is 10- to 20-fold more abundant than the activator. The data are discussed considering the possible role of homo- and heterocooperative DNA binding in E2-conditional gene expression.

Interaction of multiple sequence-specific DNA-binding activator molecules with transcriptional promoters and enhancers results in the synergic activation of gene expression (45). Multimerized DNA-binding sites, in either a homologous or heterologous combination, elicit a transcriptional response that is more than additive with respect to single sites (8, 12, 30–32, 34, 41, 42, 46, 47, 52). Duplication of a binding site can induce a dramatic stimulation of gene expression; additional sites do not necessarily cooperate in a strong, synergistic fashion (8). These phenomena are a general feature of the most thoroughly studied activators, such as Gal4 (8, 14); Oct-1 (42) and Oct-2 (28); the estrogen (34), glucocorticoid (22, 46, 47, 52), and progesterone receptors (55); the transcriptional enhancer factor 1 (TEF-1) (11); the activating transcription factor (ATF) (32); the upstream stimulatory factor (USF) (31); and the bovine papillomavirus type 1 (BPV-1) E2 transcriptional activator (E2-TA) (13, 16, 19, 23, 25, 30, 48, 49, 54).

There is evidence that transcriptional synergism may be caused by the cooperative binding of transcriptional activators to DNA (28, 34, 45, 55). Other observations contradict this conclusion (4, 8, 32, 43). Another possible explanation for transcriptional synergism is that multiple activator molecules assembled at DNA regulatory sequences are specifically recognized by an additional factor (8, 32, 45). These mechanisms are not necessarily mutually exclusive. Finally, a model has been proposed in which the binding of a TATA factor may be dramatically stabilized by "sampling" several activator molecules bound to DNA sequences in a tandem array (45).

Transcriptional synergism of multimerized, homologous binding sites is generally measured by comparing the expression of a reporter gene in cells transfected with single-site versus multisite templates. Cooperative DNA binding is thought to determine a synergistic activation of gene expression by inducing an additional degree of template saturation by the activator. This hypothesis requires that a fraction of the single-site template be free under the conditions used for the transfection experiments (8). If the single-site template has already been saturated by the activator, transfection of a multisite template would not elicit any additional level of gene expression. A similar prediction has been verified for yeast activator Gal4 and mammalian factor ATF, which, in an in vitro assay, have proved to synergize independently of cooperative binding (8, 32).

Recently we measured, by a quantitative gel retardation assay, the cooperativity parameter of the BPV-1 homodimer activator E2-TA (2, 15, 19, 40, 51) for two adjacent DNA-binding sites separated by 1.5 helix turns (37). This parameter was estimated to be 8.5. In vivo, the same DNA fragment is able to enhance the expression of a reporter gene by 50-fold with respect to a single binding site (16). Such in vitro and in vivo measurements make it possible to evaluate the role of E2 cooperative DNA binding in transcriptional synergy.

How strong must E2 cooperative interactions be to account for the synergic effect observed in vivo? E2-TA binding to a DNA fragment containing one or two adjacent binding sites (a and b) is described in Fig. 1A. The efficiency of cooperative DNA binding can be evaluated by comparing the fraction of single-site template with one bound protein, ϕ_1, with the fraction of two-site template with at least one bound protein, ϕ_2. The fractions can be expressed as a function of the association constant, K, the cooperativity parameter, K_{ab}, and the free protein concentration. Since the DNA fragment used to measure the E2-TA cooperative and synergistic capacity carries two identical copies of the

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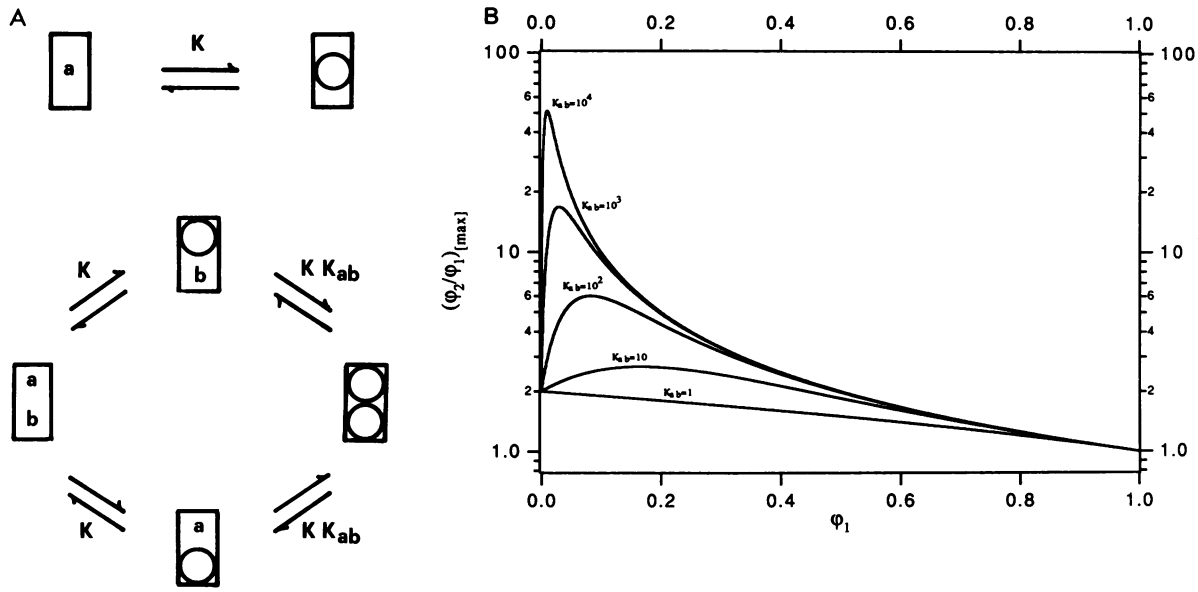

FIG. 1. Binding of the E2 activator to single-site and two-site templates. (A) Protein interaction with DNA templates carrying two copies of an E2-responsive palindrome. K is the association constant for the binding sequence, and K_{ab} is the cooperativity parameter. (B) Semilogarithmic plot of equation 3. The graph was obtained with a personal computer, allowing ϕ_1 to vary from 0 to 1. At $\phi_1 = 0$, $\phi_2/\phi_{1[max]} = 2$; this limit value reflects the fact that ϕ_2/ϕ_1 is calculated as an upper estimate.

palindrome ACCGAAAACGGT (16, 37), the two binding sites are here assumed to have similar affinities (however, as a result of flanking nucleotides, this is not always the case) (29). Positive cooperativity occurs for $K_{ab} > 1$, negative cooperativity occurs for $K_{ab} < 1$, and independent binding occurs for $K_{ab} = 1$ (1). Fractions ϕ_1 and ϕ_2 are described by the following expressions:

$$\phi_1 = KP/(1 + KP) \quad (1)$$

$$\phi_2 = (2KP + K^2K_{ab}P^2)/(1 + 2KP + K^2K_{ab}P^2) \quad (2)$$

where P is the concentration of free protein. An appropriate way to compare the two fractions is by using their ratio, ϕ_2/ϕ_1. From equation 1, one can derive $P = \phi_1/K(1 - \phi_1)$. Substituting this expression in equation 2 and assuming that the free protein concentration is not decreased by cooperative, enhanced binding to DNA, an upper limit for the ratio of the two fractions can be calculated by the expression

$$\phi_2/\phi_{1[max]} = [2 + (K_{ab} - 2)\phi_1]/[1 + (K_{ab} - 1)\phi_1^2] \quad (3)$$

which is independent of the in vivo protein affinity and concentration.

The hypothesis of cooperative synergism requires that fraction $\phi_2/\phi_{1[max]}$ be >1, and this can occur only if the single-site template is partially free. However, a protein reservoir must be present and, via cooperative interactions, "loaded" onto the template present in the nuclei of the cells transfected with the two-site plasmid. Hence, single-site template and activator concentrations must be well below the dissociation constant. Alternatively, a considerable amount of activator must be bound to nonspecific targets or to competing transcriptional units. Activator interaction with a multisite template is enhanced by cooperative binding, but, similarly to the single-site template, this is antagonized by low concentrations or strong competition. Transcriptional synergism is therefore expected to be maximal at some (low) fractional saturation of the single-site template but must decrease below this point.

This is illustrated by the graph shown in Fig. 1B, which has been calculated by varying ϕ_1 from 0 to 1 and K_{ab} from 1 to 10^4. According to the hypothesis of cooperative synergism, $\phi_2/\phi_{1[max]}$ reaches a maximum at a low single-site template saturation. Higher values for $\phi_2/\phi_{1[max]}$ are elicited by higher cooperativity parameters, but, as predicted, this happens at a lower fractional saturation of the single-site template. When ϕ_1 approaches zero, synergism is weaker, consistent with negligible affinity or with strong competition by nonspecific targets. At $\phi_1 = 1$, synergism is impossible, since cooperative interactions cannot further increase the fractional saturation of the template. At this point, $\phi_2/\phi_{1[max]} = 1$.

According to this analysis, a cooperativity parameter of 10^4 is required to increase the active template fraction by the 50-fold corresponding to the synergic activation observed in vivo (Fig. 1B) (16). This is more than 10^3 times the experimental parameter determined for the BPV-1 E2 activator in vitro. Furthermore, this represents only a lower estimate, since the ϕ_2/ϕ_1 ratio has been calculated as an upper limit. Hence, it is unlikely that cooperative DNA binding is involved in E2 transcriptional synergism. These results show that the E2-TA cooperative capacity may have evolved to play other, different roles.

Although this analysis applies particularly well to the BPV-1 activator E2-TA, it can be extended to other eukaryotic transcriptional regulators with similar cooperative and synergic capacities, such as the estrogen receptor (34). Other transcription factors, such as Oct-2, have cooperativity parameters more compatible with the extent of transcriptional synergism observed in vivo (28).

These calculations were performed by assuming that the two-site DNA template does not display, in addition to cooperative site interaction, an overall affinity higher than that of the single-site template. Evidence that this is the case

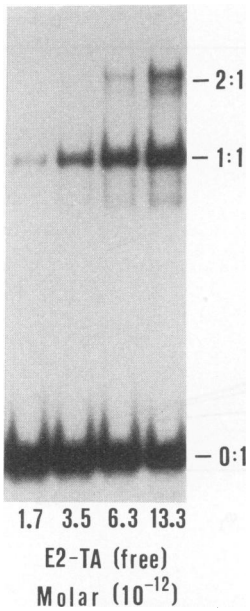

FIG. 2. Determination of the macroscopic association constant $(K_a + K_b)/2$ for E2-TA binding to the two-binding-site DNA fragment. A fixed amount (0.011 ng) of DNA was incubated in a 20-μl volume (1.4×10^{-11} M) with increasing amounts of purified E2-TA protein. The autoradiographic bands are identified as follows: 2:1, probe with two bound activator molecules; 1:1, probe with one bound activator molecule; 0:1, free probe. Complexes and unbound probe were quantitated on the Betascope 603, and the free protein concentration (active dimer) was calculated from the total amount of E2-TA present in the reactions. Total protein activity was determined as the average of titration experiments (37). Faint bands are full-length E2 protein proteolytic cleavage products and were included in the quantitations. The association constant was determined from equation 4. Calculated values were (from left to right) 1.06×10^{10} M^{-1}, 1.72×10^{10} M^{-1}, 2.35×10^{10} M^{-1}, and 2.02×10^{10} M^{-1} (average, 1.78×10^{10} M^{-1}).

is now presented. The association constant (K_a) of the single-site 41-bp DNA fragment was previously determined to be 5.3×10^{10} M^{-1} (37). The overall association constant for the two-binding-site DNA fragment $[(K_a + K_b)/2]$ was now determined. The DNA probe was purified, labeled, and quantified as described previously (37). The full-length E2 peptide used in these experiments is the same one that was used in a previous study, and purification and binding properties were described in that study (37). Different amounts of E2-TA protein were incubated with a fixed probe concentration, and complexes were resolved by gel shift as previously described (37). Unlike experiments performed to calculate cooperativity parameters, these experiments were done at low concentrations; hence, E2-TA binding was nonstoichiometric. DNA complexes and free probe were quantified on a Betascope 603 blot analyzer (Betagen, Waltham, Mass.) (3) as previously described (37) (Fig. 2). The concentration of unbound E2-TA was determined as the total protein present in the binding reactions minus the bound protein. The overall association constant $(K_a + K_b)/2$ was calculated as a function of unbound protein, free probe, and complexes with a single E2-TA molecule bound, as shown in equation 4.

$$(K_a + K_b)/2 = [\text{complex 1:1}]/2[\text{DNA}]_{\text{free}}[\text{E2-TA}]_{\text{free}} \quad (4)$$

Values ranging from 1.1×10^{10} to 2.4×10^{10} M^{-1} (average, 1.8×10^{10} M^{-1}) were obtained. In another, independent experiment, values ranging from 2.2×10^{10} to 3.4×10^{10} M^{-1} (average, 3×10^{10} M^{-1}) were observed. Hence, the two DNA fragments display similar affinities for the E2 activator.

An important target for the BPV-1 activator E2-TA is a transcriptional enhancer, $E2RE_1$, contained within the long control region of the viral genome. $E2RE_1$ activates transcription from promoters p_{89} and p_{7940} in an E2-dependent manner and contains two pairs of adjacent E2-responsive elements which are indispensable for its transcriptional activity (48, 50). In addition to activator E2-TA, BPV-1 encodes two transcriptional repressors, E2-TR and E8/E2. These peptides lack the E2-regulatory domain but retain the ability to interact with target DNA sequences, thereby competing with the E2 activator for specific DNA binding (9, 20, 27). A possible function for E2-TA cooperative DNA binding could be to antagonize the interaction of E2 repressors with the viral enhancer $E2RE_1$. In fact, unlike activator E2-TA, E2 truncated peptides fail to loop DNA sequences with an E2-responsive motif at each end (23), suggesting that the E2 repressors may not possess cooperativity capacities comparable to that of the full-length protein. This conclusion is further supported by the observation that an 86-amino-acid peptide (86-E2), encompassing the minimal DNA-binding domain, binds to adjacent sites with a cooperativity parameter of 1.9 (37).

To verify this hypothesis, the BPV-1 repressor E2-TR was synthesized by infecting *Spodoptera frugiperda* Sf-9 cells with a recombinant baculovirus (53). BPV-1 sequences encoding the viral repressor (nucleotides 3098 to 4450) were isolated with *Bam*HI endonuclease from plasmid pYE2-R (38), a gift of E. J. Androphy, Tufts University School of Medicine, Boston, Mass., and inserted in plasmid pBlueBac (Invitrogen, San Diego, Calif.), whose cloning site was replaced by a *Bgl*II site. Following Blue-gal-plaque purification (Maxbac, baculovirus expression system, manual version 1.4; Invitrogen), insect cells were infected with a single isolate and the protein was purified to homogeneity by affinity chromatography (data not shown) as previously described for the BPV-1 E2 transcriptional activator (37).

The binding of the repressor to adjacent binding sites was then investigated, as previously described for E2-TA (37), by titrating a fixed amount of the two-binding-site DNA fragment with increasing amounts of the protein. As observed for the transcriptional activator, binding of the repressor to the oligonucleotide yielded two principal shifts corresponding to the occupancy of one or two sites (Fig. 3). A faint band, which may correspond to an E2 repressor degradation product, was also detected. The cooperativity parameter was calculated by the maximum of one-site occupancy, as previously described (37). The graph in Fig. 3 was obtained by quantitation on the Betascope analyzer, and it shows that the fraction of singly bound DNA reached a maximum value of 0.37 at an active protein concentration of 0.46 nM (dimer). In five different experiments, performed at different fixed amounts of the 41-bp oligonucleotide, values ranging from 0.36 to 0.38 were obtained and the cooperativity parameter was calculated as ranging from 2.7 to 3.2 (average, 2.9). This is close to the cooperativity parameter of the 86-E2 C-terminal peptide containing the minimal DNA-binding domain. These data show that a major determinant for E2 cooperative DNA binding is encompassed within the regulatory N-terminal domain and that cooperative binding of the full-length E2 protein may be relevant for the extent of competitive inhibition by repressor E2-TR.

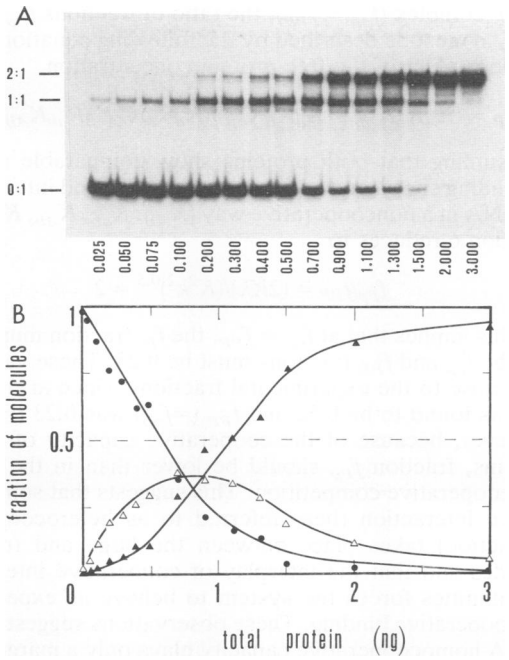

FIG. 3. (A) Titration of the ^{32}P-labeled 54-bp two-binding-site oligonucleotide with repressor E2-TR. A fixed amount (0.55 ng) of oligonucleotide (1.4×10^{-9} M binding sites) was incubated in a 20-μl volume with increasing amounts of the repressor. Numbers below the lanes are nanograms of purified proteins (Bradford determination). The autoradiographic bands are identified as follows: 2:1, probe with two bound repressor molecules; 1:1, probe with one bound repressor molecule; 0:1, free probe. Minor complexes due to proteolytic products never exceeded 4% of the total bound protein. (B) Quantitation of the gel shift. Fractions of molecules are free probe (●), complex 1:1 (△), and complex 2:1 (▲). The maximum of the single-site binding isotherm is 0.37; the cooperativity parameter (2.9) was calculated as previously described (37). This value represents a macroscopic estimate, since we have made the assumption in our analysis that the two binding sites have equal affinities. If the affinities are not equal, the cooperativity parameter is higher. The same assumption was previously made for E2-TA and 86-E2 proteins (37).

In vivo, the E2 polypeptides bind to adjacent E2-responsive elements in a competitive manner. To gain a more complete understanding of the role of E2 cooperative DNA binding in viral gene expression, we performed competition experiments with truncated and full-length peptides and the 54-bp two-site probe. Because of their similar electrophoretic mobilities, experiments with the E2 activator and E2-TR repressor yielded little information. At lower acrylamide concentrations or at lower acrylamide/bisacrylamide ratios, the complexes were better resolved; however, dissociation phenomena occurred, preventing a quantitative analysis of the binding isotherms. A quantitative analysis of the competitive binding was indeed possible with 86-E2, whose binding properties have been described previously (37). No additional bands of intermediate mobility corresponding to heterodimeric E2-TA/86-E2 molecules were observed in specific experiments performed by mixing the two proteins with the 41-bp *Bgl*II DNA fragment containing a single E2-binding site (37) (data not shown). Hence, as observed with other truncated E2 polypeptides (35), E2 full-length and truncated homodimers were stable under the conditions used.

Interaction of the full-length and truncated peptides with the two-binding-site DNA probe is expected to generate six different radioactive bands, which can be identified by nondenaturing gel electrophoresis. These six bands correspond to the probe with the following bound proteins: (i) none, (ii) one large (P), (iii) one truncated (p), (iv) two large (PP), (v) two truncated (pp), and (vi) one large and one truncated (Pp).

Theoretical binding equilibria, association constants, and cooperativity parameters for the interaction of the two proteins with the DNA are shown in Fig. 4A. On the basis of the binding equilibria, the fractions of DNA molecules with the various combination of bound proteins can be expressed as a function of the affinities of the large and truncated peptides for binding sites a and b, the cooperativity parameters for the interaction of the proteins with the adjacent sites, and the free-protein concentrations (equation 5). Equilibrium constants are as follows: K_a and K_b, association constants for the binding of truncated molecules to sites a and b; K_A and K_B, association constants for the binding of full-length molecules to sites a and b; K_{ab} and K_{AB}, cooperativity parameters for truncated and full-length proteins, respectively; and K_{Ab} and K_{aB}, heterocooperativity parameters for large and truncated molecules interacting with adjacent binding sites. The latter parameters are considered to be potentially different because of the asymmetric structure of the two heterocomplexes. According to these binding equilibria, the fractions of DNA molecules with the various combinations of bound proteins can be described by the expressions

$$\begin{aligned} f_0 &= 1/D \\ f_p &= K_1 p/D \\ f_{pp} &= K_2 p^2/D \\ f_P &= K_3 P/D \\ f_{PP} &= K_4 P^2/D \\ f_{Pp} &= K_5 Pp/D \\ D &= 1 + K_1 p + K_2 p^2 + K_3 P + K_4 P^2 + K_5 Pp \end{aligned} \qquad (5)$$

where p and P are the concentrations of nonbound truncated and large proteins and macroscopic constants K_1 to K_5 are functions of the association constants and cooperativity parameters: $K_1 = K_a + K_b$; $K_2 = K_a K_b K_{ab}$; $K_3 = K_A + K_B$; $K_4 = K_A K_B K_{AB}$; $K_5 = K_A K_b K_{Ab} + K_a K_B K_{aB}$.

In one experiment performed by titrating a fixed amount of the oligonucleotide with a mixture of the two proteins (whose ratio was constant), all the expected bands were identified (data not shown). A second experiment was performed by incubating the oligonucleotide with a saturating concentration of 86-E2 in the presence of increasing amounts of the full-length E2 protein (Fig. 4B). Three principal complexes were detected, corresponding to the doubly bound probe in the three possible combinations (pp, Pp, and PP). Radioactive complexes were quantified, and binding isotherms were determined. As the full-length E2 protein was added to the binding mixture, the pp complex concentration decreased, the Pp complex concentration reached a maximum, and the PP complex concentration increased (Fig. 4C).

From equation 5 it is possible to calculate that, when the concentration of the pp complex equals the concentration of

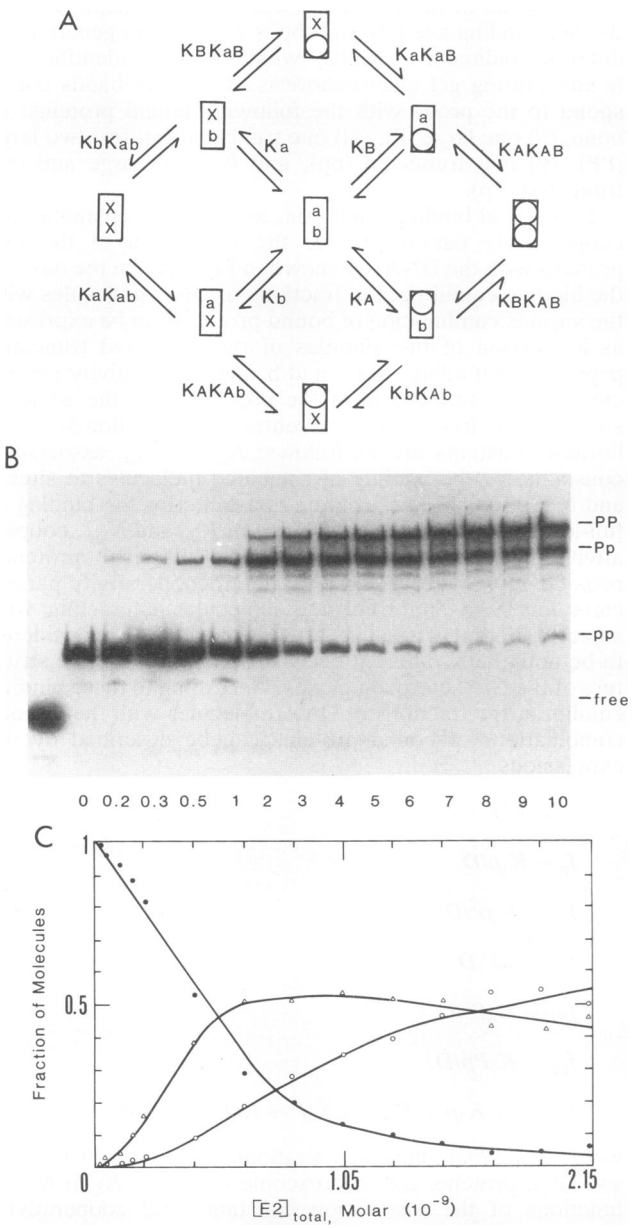

FIG. 4. Competition between the full-length E2 protein and the 86-amino-acid C-terminal peptide encompassing the minimal E2 DNA-binding domain. (A) Binding equilibria of large and truncated E2 peptides interacting with the two-binding-site DNA probe. Symbols: ○, large protein; ×, truncated protein. Equilibrium constants are defined in the text. (B) A fixed amount (0.275 ng) of oligonucleotide (7×10^{-10} M binding sites) was incubated in a 20-μl volume with 5 ng of the 86-amino-acid peptide (10^{-8} M active dimer) and with increasing amounts of the full-length E2 protein (from 4.3×10^{-11} to 2.15×10^{-9} M active dimer). Numbers below the lanes are nanograms of full-length E2 protein (Bradford determination). Faint bands are due to full-length E2 protein proteolytic cleavage products and correspond to about 5% of the bound proteins. The autoradiographic bands are identified as follows: PP, doubly bound full-length E2 protein; Pp, heterocomplex; pp, doubly bound truncated peptide. Active protein concentrations are the averages of the titration experiments (37). (C) Quantitation of the gel shift assay shown in panel B. Symbols: ●, pp complex; ○, PP complex; △, Pp complex. Proteolytic product bands were included in the quantitation.

the PP complex ($f_{pp} = f_{PP}$), the ratio of fractions f_{Pp} and f_{PP} (f_{Pp}/f_{PP}) must be described by the following equation, which is independent of the free-protein concentration.

$$f_{Pp}/f_{PP} = (K_A K_b K_{Ab} + K_a K_B K_{aB})/(K_a K_b K_A K_B K_{ab} K_{AB})^{0.5} \quad (6)$$

Assuming that both proteins show comparable affinities for binding sites a and b ($K_A = K_B$, $k_a = k_b$) and interact with the DNA in a noncooperative way (K_{AB}, K_{ab}, K_{Ab}, $K_{aB} = 1$), equation 6 reduces to

$$f_{Pp}/f_{PP} = (2Kk)/(K^2k^2)^{0.5} = 2 \quad (7)$$

and this implies that at $f_{pp} = f_{PP}$, the f_{Pp} fraction must be 0.5 and the f_{pp} and f_{PP} fractions must be 0.25. These values are very close to the experimental fractions, since at this point f_{Pp} was found to be 0.52 and f_{PP} (=f_{pp}) was 0.23 (Fig. 4C). However, because of the cooperative capacity of the two proteins, fraction f_{Pp} should be lower than in the case of non-cooperative competition. This suggests that some cooperative interaction (here referred to as heterocooperative interaction) takes place between the large and truncated peptides and that the interplay of cooperative interactions and affinities forces the system to behave as expected for noncooperative binding. These observations suggest that the E2-TA homocooperative capacity plays only a marginal role in the activation of viral enhancer $E2RE_1$ under conditions of competitive binding.

A heterocooperative parameter for the interaction between the E2 full-length and truncated peptides could be determined by substituting the experimental values for cooperativity parameters K_{AB} (8.5) and K_{ab} (1.9) and for fractions f_{Pp} (=0.52) and f_{PP} (=0.23) in equation 6:

$$(K_A K_b K_{Ab} + K_a K_B K_{aB})/(K_a K_b K_A K_B)^{0.5} = 9.1 \quad (8)$$

Assuming that the two proteins display similar affinities for binding sites a and b ($K_A = K_B$; $k_a = k_b$), an overall, macroscopic heterocooperativity parameter ($K_{aB} + K_{Ab}$) is calculated from equation 8, which reduces to ($K_{aB} + K_{Ab}$) = 9.1. The average value for the heterocooperative binding of the full-length and truncated E2 polypeptides can thus be determined to be 4.5. Such an average estimate is higher than the cooperativity parameter for both the minimal DNA-binding domain peptide and repressor E2-TR. This suggests that the E2 regulatory domain may (i) interact with the C-terminal domain of an adjacent, truncated molecule or (ii) induce a DNA conformational transition, eventually affecting DNA bending caused by its C-terminal domain (6, 39), facilitating the binding of an adjacent, truncated peptide. These results show that cooperative DNA binding can be a complex phenomenon. Indeed, binding energies involved in the interaction of macromolecular domains are not additive (21). In this respect, to consider transcriptional regulators to be modular proteins whose domains are strictly responsible for separate functions could be a useful but sometimes imprecise approximation.

An additional experiment performed by incubating the DNA probe with a saturating concentration of the full-length E2 protein in the presence of increasing amounts of the 86-amino-acid peptide gave similar results, with an overall heterocooperativity parameter ($K_{Ab} + K_{aB}$) of 8.1 (data not shown).

In transformed cells latently infected with BPV-1, repressors E2-TR and E8/E2 are significantly more abundant than are E2 activator molecules (20). Viral gene expression in these cells is thought to be essentially down-regulated by

truncated E2 peptides, and mutations in the BPV-1 E2-TR and E8/E2 proteins have a complex, pleiotropic effect (26).

Little is known about the relative affinities of the E2 polypeptides for specific and nonspecific DNA sequences. However, these parameters must play an important role in the competitive binding of the E2 transcriptional modulators. For instance, binding of E2 repressors to adjacent sites would be negligible, assuming that their specific affinities are low relative to the affinity of activator E2-TA. Furthermore, nonspecific, competing DNA sequences could titrate E2-TR, E8/E2, and E2-TA with different efficiencies. Despite the predominance of E2 repressors, the cooperative interaction of the E2 activator with pairs of E2-responsive DNA elements might, under these conditions, be barely affected by the truncated peptides. Therefore, specific and nonspecific DNA-binding constants were determined for both the activator E2-TA and repressor E2-TR.

The affinity of the BPV-1 repressor E2-TR for the E2-responsive palindrome ACCG(N_4)CGGT was measured, in a similar way as was previously done for the activator E2-TA (37), by incubating the purified protein with an oligonucleotide containing one specific binding site. A 41-bp DNA fragment containing one copy of the palindromic sequence ACCGAAAACGGT was isolated from plasmid p18IE2M (16), labeled with [α-^{32}P]dCTP, quantitated, and used in gel retardation experiments as described previously (37). The protein and the oligonucleotide were incubated at different concentrations, and the binding isotherms were determined by quantitation on the Betascope 603. Four different titrations, performed at fixed protein or oligonucleotide concentrations, yielded dissociation constants ranging from 1.28×10^{-11} to 1.74×10^{-11} M, with an average value of 1.5×10^{-11} M (data not shown). The dissociation constant of the full-length E2 protein for the same DNA fragment was previously found to be 1.9×10^{-11} M (37).

The affinities of the E2 activator and repressor for nonspecific DNA sequences were measured next. Cultures of C-127 mouse fibroblasts were harvested, and total cellular DNA was isolated by proteinase K digestion in the presence of sodium dodecyl sulfate, digested with RNase, phenol-chloroform extracted, and extensively dialyzed. The dialyzed DNA was further purified of high-molecular-weight contaminants by repeated concentration in a Centricon 30 ultrafiltration unit (Amicon, Beverly, Mass.). Commercial salmon sperm DNA (Sigma, St. Louis, Mo.) and plasmids p18IE2M (16) and pA10CAT2 (24) were similarly purified; their concentrations were determined by densitometric measurement at 260 nm. A fixed amount of the 41-bp oligonucleotide (4×10^{-11} M) was incubated with E2-TA or E2-TR in the presence of increasing amounts of the unrelated macromolecular DNAs (Fig. 5). Assuming that pseudo-sites are negligible with respect to the bulk of unrelated sequences (36), the dissociation constant for nonspecific DNA binding, K_d, is given by the following expression (equation 9), originally developed by Lin and Riggs (33) for the *Escherichia coli lac* repressor.

$$K_d = 2K_sD_{0.5}/(2P_t - E_t - 2K_s), \quad (9)$$

where K_s is the dissociation constant for the specific DNA binding, $D_{0.5}$ is the concentration of nonspecific sites required to reduce the fractional saturation of the oligonucleotide to 0.5; and P_t and E_t are the absolute protein and specific site concentrations, respectively.

As for the *E. coli lac* repressor, the various competitor DNAs yielded different dissociation constants. Plasmid p18IE2M, containing one specific E2-binding motif, was very effective in inhibiting the specific binding of both the full-length and the truncated E2 proteins. The other competitor DNAs displayed variable degrees of inhibition. According to equation 9, the nonspecific dissociation constant of the full-length E2 protein was calculated to range from 5×10^{-7} to 1×10^{-5} M and the E2 repressor K_d was found to range from 2.5×10^{-7} to 1×10^{-5} M (Table 1). Hence, the relative affinities of the two peptides for the unrelated macromolecular DNAs are comparable, ruling out nucleosome-free DNA as a major determinant in E2 competition in vivo.

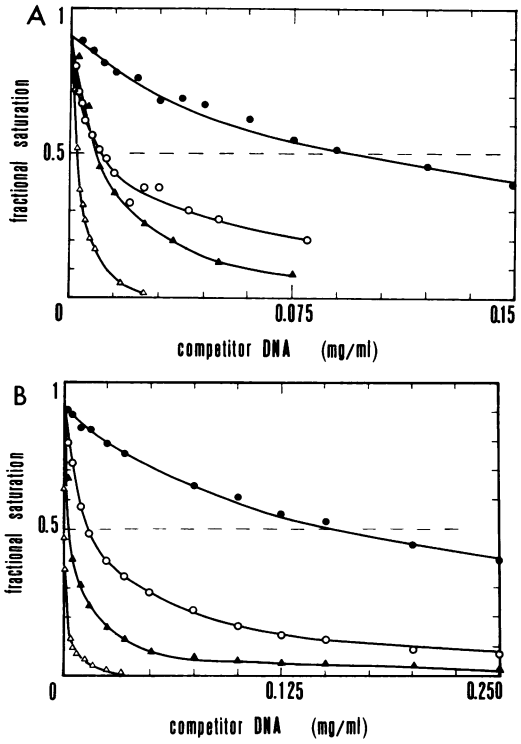

FIG. 5. Competition of E2 activator- and repressor-specific DNA binding by nonspecific, macromolecular DNAs. Symbols: ●, c-127 DNA; ○, salmon sperm DNA; ▲, plasmid pA10CAT2; △, plasmid p18IE2M. The dissociation constant for nonspecific DNA binding, K_d, can be determined by an expression originally developed for the *E. coli lac* repressor (33). (A) E2 activator binding. Competitions with plasmids pA10CAT2 and p18IE2M were performed in a 20-μl volume by incubating 30 ng of the ^{32}P-labeled 41-bp oligonucleotide containing one copy of the palindromc ACCGAAAACGGT sequence (6×10^{-11} M binding sites) with 2.8 ng (Bradford determination) of the full-length E2 protein (6×10^{-10} M active dimer). Competitions with salmon sperm and c-127 DNAs were performed in a similar manner, but the concentrations of specific binding site and full-length E2 protein were 4×10^{-11} and 3×10^{-10} M (active dimer), respectively. The protein was added to the binding reaction mixture last. (B) E2 repressor binding. All competitions were performed as for the full-length E2 protein, with 4×10^{-11} mol of specific DNA-binding site per liter and 3.45×10^{-10} mol of repressor (active dimer) per liter. Active-protein concentrations are averages of titration experiments (data not shown) (37).

These results confirm the suggestion that E2-TA cooperative DNA binding is efficiently antagonized by the E2 repressors both in vitro and in latently infected cells.

Does E2 cooperative DNA binding play any role in BPV-1 gene expression? It has been shown that the full-length E2 protein is not capable of transactivating adjacent E2-respon-

TABLE 1. K_d values for the E2 activator and repressor

DNA	Activator K_d (mol/liter)	Repressor K_d (mol/liter)
Salmon sperm	1.2×10^{-6} ($D_{0.5}$ = 1.50×10^{-5})	1.0×10^{-6} ($D_{0.5}$ = 2.05×10^{-5})
c-127	1.0×10^{-5} ($D_{0.5}$ = 1.35×10^{-4})	1.1×10^{-5} ($D_{0.5}$ = 2.24×10^{-4})
pA10CAT2	5.0×10^{-7} ($D_{0.5}$ = 1.25×10^{-5})	2.5×10^{-7} ($D_{0.5}$ = 5.2×10^{-6})

sive elements cloned upstream of a promoter containing a TATA box alone. Once binding sites for cellular factors are included between the TATA box and the E2-binding sites, the template becomes responsive to the protein (18). At least two E2-binding sites are required for this transcriptional activation. Similar evidence was obtained for human papillomaviruses types 16, 18, and 33 (13). Those studies suggested that the active form of the E2 activator may consist of two adjacent dimers (a "functional E2 tetramer" [13]) interacting with some additional factor. The present finding that cooperative binding cannot account for the E2 transcriptional synergy supports this conclusion. However, our study suggests possible roles for E2 cooperative DNA binding.

The E2 polypeptides form heterodimers through their carboxy-terminal DNA-binding domain (35, 44). Experiments performed with truncated E2 peptides competent for dimerization but not for DNA binding suggested that heterodimerization of full-length and truncated E2 proteins is a major mechanism for transcriptional repression (5). Those studies also suggested that the full-length E2 activator is not competent for *trans*-activation when present in a heterodimeric form with E2-TR. Repressor E2-TR is significantly more abundant than both E2-TA and E8/E2 in BPV-1 transformed cells at the G_0 and early G_1 phases of the cell cycle (56). In those cells, E2-TR homodimers must predominate over the E2 heterodimeric forms, and this suggests that E2-conditional gene expression may be regulated essentially by the interplay of E2-TA and E2-TR homodimers under conditions of nonproductive viral infection. The present in vitro analysis could apply to this in vivo situation.

According to the cooperative capacities of E2 proteins, E2-TA homocooperative binding to adjacent sites would be antagonized by heterocooperative interactions. Under conditions of competitive binding, a fraction of the templates would have one site occupied by E2-TA and the other occupied by E2-TR. Nonadjacent sites could display a similar behavior, since heterocooperative interactions may enable distantly bound E2-TA and E2-TR molecules to loop intertwining DNA sequences as described for E2-TA homodimers (23). In contrast, the low E2-TR homocooperative capacity would not favor the occupancy of adjacent sites by E2-TR molecules.

There is no absolute stereoalignment constraint for E2 transcriptional synergy, and synergic activation is observed when adjacent E2-binding sites are on the opposite faces of the DNA double helix (13, 48). It has been suggested that this could reflect the ability of the E2 "hinge" region to wrap around the DNA, enabling the transactivating domains of two adjacent E2-TA molecules to interact in the proper way (15, 48). The flexible E2 hinge region could similarly facilitate the heterocooperative interaction of the *trans*-activating domain of an activator molecule with the DNA-binding domain of a truncated, adjacent peptide. Since E2-TR homocooperativity probably reflects the interactions of E2 DNA-binding domains, occupancy of adjacent sites by E2-TR would be further discouraged by site misalignment.

These observations suggest that heterocooperative interactions could determine a primed state with half of a functional E2 tetramer already in place. Such a "committed" state could be relevant for the transcriptional regulation of BPV-1 promoters during the dynamic changes of E2 protein concentrations through the cell cycle (56).

Truncated and full-length E2 proteins are present in comparable amounts in BPV-1-transformed cells during late G_1, S, M, and G_2 phases or in giant cells permissive for viral DNA amplification induced by growth arrest of transformed cultures (7, 56). It is conceivable that, under these conditions, binding and cooperative properties of the E2 heterodimeric molecules would predominate over homodimeric cooperative capacities. These properties should therefore be fully elucidated for a complete understanding of BPV-1 biology.

The present study has been conducted in vitro with purified proteins and naked DNA. An important determinant of gene expression in living cells is the presence of nucleosomes on assembled chromatin. It has been suggested that binding of transcriptional activators to DNA may mediate nucleosome displacement in vivo (reviewed in reference 17). One example is the reversible displacement of a nucleosome from the mouse mammary tumor virus long terminal repeat associated with the binding of glucocorticoid receptor, transcription factor NF1, and TFIID to their cognate DNA sequences (10, 57). E2 proteins could be involved in a similar mechanism along the BPV-1 noncoding region, and homo- and heterocooperative interactions might be a part of the energetic balance involved in nucleosome displacement (or alteration) in the viral life cycle.

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