

Cooperative Binding of the E2 Protein of Bovine Papillomavirus to Adjacent E2-Responsive Sequences

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The DNA-binding properties of purified full-length E2 protein from bovine papillomavirus type 1 have been investigated by utilizing a quantitative gel shift analysis. By using a recombinant baculovirus which expresses the E2 open reading frame from the polyhedrin promoter, the full-length E2 protein was synthesized in insect cells and purified to homogeneity by using an E2 binding site (ACCGN₄CGGT)-specific oligonucleotide column. The K_d of E2 binding to a 41-bp oligonucleotide containing a single binding site was found to be 2×10^{-11} M. When two binding sites were included on an oligonucleotide, cooperative binding to these sites by the E2 protein was observed. A cooperativity parameter of 8.5 was determined for E2 binding to two sites. An 86-amino-acid peptide encompassing the C terminus of the protein retains the ability to bind E2 binding sites with a K_d of 4×10^{-10} M but exhibits slight cooperativity of binding to two adjacent sites. A major determinant for cooperative binding of the full-length E2 protein is thus encoded by the N-terminal amino acids outside the minimal DNA binding domain.

The regulation of transcription of eukaryotic genes is mediated through the action of *trans*-acting proteins which bind to specific sequences in promoters and enhancers. Bovine papillomavirus type 1 (BPV-1) encodes a transcriptional regulatory protein, E2, which activates expression of papillomavirus genes by binding to the palindromic ACCG N₄CGGT sequence (2, 16, 17, 31, 39). Seventeen copies of this sequence are found in the BPV-1 genome (25), and binding to two adjacent sites is required to activate high-level enhancer function (16, 17, 38). The activity of the E2 *trans*-activator constitutes the major autoregulatory mechanism for papillomavirus expression (14, 15, 18, 34, 38).

The full-length 48-kDa E2 protein binds DNA as a dimer and consists of three functional domains (7, 12, 28, 29, 32). The N-terminal portion encodes an acidic domain which is necessary for transactivation, while the C terminus encodes sequences necessary for dimerization and sequence-specific DNA binding. The two domains are separated by an ~100-amino-acid "hinge" region of unknown function. Two smaller E2 proteins which lack the N-terminal activation domain are also synthesized during BPV-1 infections and play a major role in autoregulation (5, 20, 23). These truncated proteins are competent for specific DNA binding, but not transactivation, and therefore can repress E2-dependent transactivation. The antagonistic activity of the various positive and negative E2 regulatory proteins is thus important for the overall regulation of papillomavirus gene expression.

Although low-level activation of heterologous promoters is seen in *Saccharomyces cerevisiae* when a single binding site is present (22, 30, 40), two copies of an E2-responsive sequence are required for activity in mammalian cells (14, 15, 17, 39) and for high-level activation in *S. cerevisiae*. The synergistic effect of two or more E2 binding sites on transactivation of a heterologous promoter has been shown to

operate over distances of up to 2 kb (17, 38). Synergism occurs between the multiple E2-responsive sequences in the activation and autoregulation of several bovine papillomavirus promoters (18, 34). In general, the cooperative binding of transcriptional activators to multiple target sequences has been proposed as a possible explanation for a portion of their synergistic effects on transcription. Numerous transcriptional activators such as Oct-1 (33), Oct-2 (24), Gal 4 (11), estrogen receptor (3, 26), TEF-1 (6), and progesterone receptor (43) have been shown to bind cooperatively to

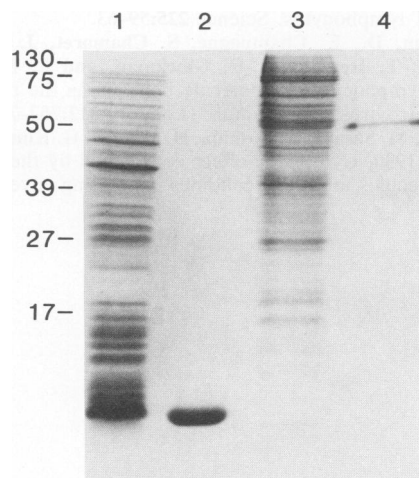


FIG. 1. Photograph of Coomassie blue-stained 7% sodium dodecyl sulfate-polyacrylamide gel of total protein extracts from 86-E2-expressing *E. coli*, vFE2-infected SF-9 cells, and purified full-length E2 and 86-E2 proteins. Lanes: 1, 86-E2-expressing *E. coli* extract; 2, purified 86-E2 protein; 3, vFE2-infected SF-9 cell extract; 4, purified E2 full-length protein.

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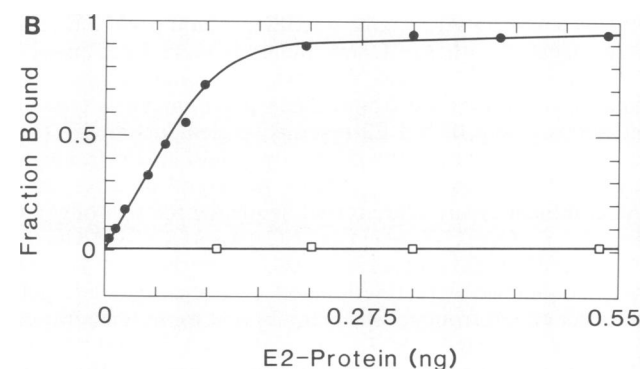
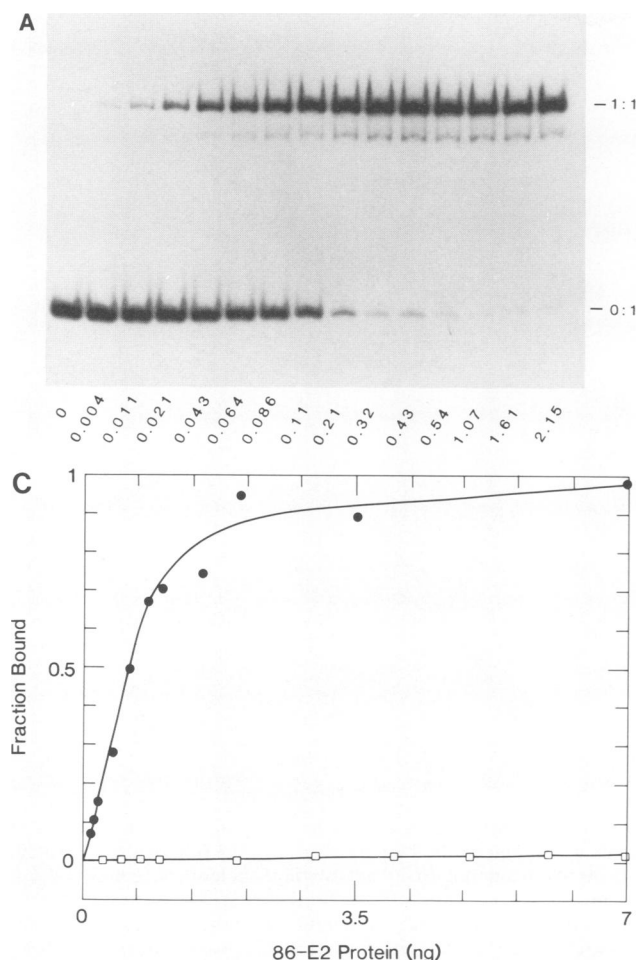


FIG. 2. Titration of ^{32}P -labeled 41-bp oligonucleotide containing a single E2 binding site and a 39-bp oligonucleotide lacking any E2-homologous sequences with full-length E2 protein and 86-E2 protein. (A) A fixed amount (0.025 ng) of 41-bp labeled oligonucleotide (4.5×10^{-11} M) was titrated in a 20- μl volume as described in the text. Numbers below the lanes are nanograms of active E2 protein (0.1 ng/20 μl = 5×10^{-11} M dimer). The shifted bands are identified as follows: 1:1, E2-oligonucleotide complex; and 0:1, free probe. The faint bands below the 1:1 complex correspond to the reactions of proteolytic cleavage products of E2 and never exceed 5% of total shifted bands. (B) Quantitative analysis of the gel shifts from panel A. Symbols: \bullet , single E2 binding site; \square , 1.1 ng of nonspecific 39-bp oligonucleotide. Quantitation of the shifted bands was performed with a Betascope 603, and the fraction of oligonucleotide shifted was calculated at each protein concentration. No dissociation of the complexes during electrophoresis was detected. The concentration of free protein at half-saturation was determined to be 10^{-11} M and corresponds to the dissociation constant, K_d . (C) Quantitation of the titration of 1.1 ng of 41-bp oligonucleotide (\bullet) or 39-bp nonspecific probe (\square) with increasing concentrations of 86-E2 protein. The concentration of the active protein at half saturation was determined to be 4×10^{-10} M.

adjacent binding sites, but similar characterization of the E2 protein remains incomplete.

Previous studies in *S. cerevisiae* have suggested that the binding of E2 molecules to multiple adjacent binding sites may involve a slight cooperativity. Using extracts of yeast cells expressing the E2 protein, Lambert et al. (22) observed by gel shift analysis that the fraction of complexes with proteins bound to two adjacent sites was greater than that predicted by purely random binding of protein to independent sites and was indicative of positive cooperativity. Cooperative binding by the E2 protein was also suggested in studies by Hirochika et al. in a filter binding assay, by using crude extracts of bacteria expressing the human papillomavirus type 11 E2 full-length protein (19).

Toward a more complete understanding of the interaction of the E2 protein with its target sequences, we have synthesized the BPV-1 full-length E2 protein with a baculovirus vector in insect cells and purified it to homogeneity (Fig. 1). The entire E2 open reading frame was first isolated from the bacterial expression vector pCOE2 (17) as a *Bam*HI fragment and cloned into the *Bam*HI site of the baculovirus transfer vector pVL106 (41) to form pVLE2. This vector encodes a polypeptide which includes the BPV-1 E2 open reading frame fused to four amino acids of the polyhedrin protein. A recombinant baculovirus was isolated after cotransfection of pVLE2 with wild-type baculovirus DNA into SF-9 insect cells, followed by plaque purification (41). A single isolate (vFE2) was used in all subsequent experi-

ments. Insect cells were infected with vFE2, harvested at 48 h postinfection, and lysed by Dounce homogenization in a solution containing 10 mM Tris (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, 10 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, and 1 μg of pepstatin per ml. The lysate was then adjusted to 30 mM Tris (pH 8.0), 5 mM MgCl_2 , 3.5 mM dithiothreitol, 12.5% (wt/vol) sucrose, 25% (vol/vol) glycerol, and 0.45 M $(\text{NH}_4)_2\text{SO}_4$ and stirred for 20 min at 4°C. After centrifugation (3,500 $\times g$ for 30 min), the supernatant was diluted 1.5 times with the buffer described above but without $(\text{NH}_4)_2\text{SO}_4$. The resulting 0.3 M salt solution was applied to a sequence-specific DNA affinity column prepared by the coupling of a multimerized 21-bp oligonucleotide containing the sequence ACCGN₄CGGT to CNBr-Sepharose (21). A major protein species of 48 kDa eluted at 0.5 M NaCl with yields ranging from 0.5 to 1 mg per 10^{10} cells (Fig. 1). The identity of the purified E2 peptide was confirmed by Western immunoblot analysis (data not shown), and protein concentration was determined by Bradford assay (Bio-Rad) by using bovine serum albumin (BSA) as a standard. In this baculovirus expression system, the E2 protein is phosphorylated with a pattern indistinguishable from that of mammalian cells and exhibits specific DNA binding (13a, 27).

We next used a quantitative gel shift assay (8, 9) to study the binding of purified full-length E2 protein to E2-responsive sequences. Plasmids containing either a single E2 binding site on a 41-bp *Bgl*II fragment or two adjacent E2 binding

sites on a 54-bp *Bgl*II fragment (p18IE2A and p18IE2M [13]) were digested with *Bgl*II, and the ends were filled in with [α - 32 P]dCTP and Klenow enzyme. The binding sites and flanking sequences are from human papillomavirus type 18 and act as strong BPV-1 E2-responsive elements in vivo (13, 42). The sequence of the 54-bp two-binding-site fragment described in Gius et al. (13) is incorrect because of a typographical error. The correct sequence for this oligonucleotide is GATCTGAGTGACCGAAAACGGTCGGGACCGAAAACGGTGTATATAAAAGA. The labeled 41- and 54-bp fragments were fractionated on polyacrylamide gels and soak eluted from gel slices for 24 h at room temperature in 50 mM Tris-HCl (pH 7.5)–50 mM NaCl–10 mM MgCl₂. Eluted probes were dialyzed and concentrated in the same buffer in a Centricon 10 (Amicon) ultrafiltration unit. The percent yield of purified probe was determined by running aliquots of crude labeling reaction and purified probe on a 6% polyacrylamide gel followed by quantitation on a Betascope 603 blot analyzer (Betagen, Waltham, Mass.) (4). The concentration of probe could then be accurately calculated on the basis of spectrophotometric quantitation of the original plasmid before digestion, the relative sizes of probe and plasmid, and the amount of radioactivity recovered. DNA-binding reactions with purified protein were performed in a solution containing 10 mM Tris (pH 7.5), 100 mM KCl, 2 mM dithiothreitol, 2 mM MgCl₂, 0.1 mg of BSA per ml, and 15% (vol/vol) glycerol with various amounts of probe and protein for 20 min at room temperature. Binding reaction mixtures were analyzed by 6% native polyacrylamide gel electrophoresis (30:1, acrylamide-bisacrylamide), as already described (8), and were followed by autoradiography and quantitation on the Betascope 603.

As a first characterization of the interaction of the E2 protein with its recognition site, we determined the dissociation constant of binding, K_d , by two methods. First, a fixed amount of purified E2 protein was titrated with increasing amounts of a 41-bp oligonucleotide containing one binding site (not shown). The binding was seen to increase to a plateau, from which the dissociation constant was determined as the free oligonucleotide concentration at half saturation. Values ranging from 1.5×10^{-11} to 3.2×10^{-11} M were obtained from such studies. The concentration of active E2 protein was determined from the plateau and was found to range from 33 to 53% (average, 43%) of that determined from the Bradford assay. This discrepancy may be the result of either an inaccuracy in the Bradford assay or inactivation of the protein perhaps resulting from oxidation (13a). We also determined the dissociation constant by titrating a fixed amount of oligonucleotide with increasing amounts of protein, and representative gel shifts and data plots are shown in Fig. 2A and B. Assuming the purified protein to be 43% active, the K_d of binding was determined to be 10^{-11} M. Five different titrations, performed at fixed protein or oligonucleotide concentrations, yielded dissociation constants ranging from 1×10^{-11} to 3.2×10^{-11} M with an average value of 1.9×10^{-11} M. A minor band, representing less than 5% of shifted probe, is one which we believe represents the binding of a degradation product of E2 (Fig. 2A). No binding was observed in a parallel experiment using a labeled 39-bp oligonucleotide lacking specific E2 binding sites obtained by digestion of pUC-19 with *Xma*I and *Hind*III restriction endonucleases (Fig. 2B).

We next investigated the binding of purified E2 protein to two adjacent binding sites separated by 4 nucleotides. A fixed amount (0.55 ng) of the labeled 54-bp two-binding-site probe was titrated with increasing amounts of protein. As

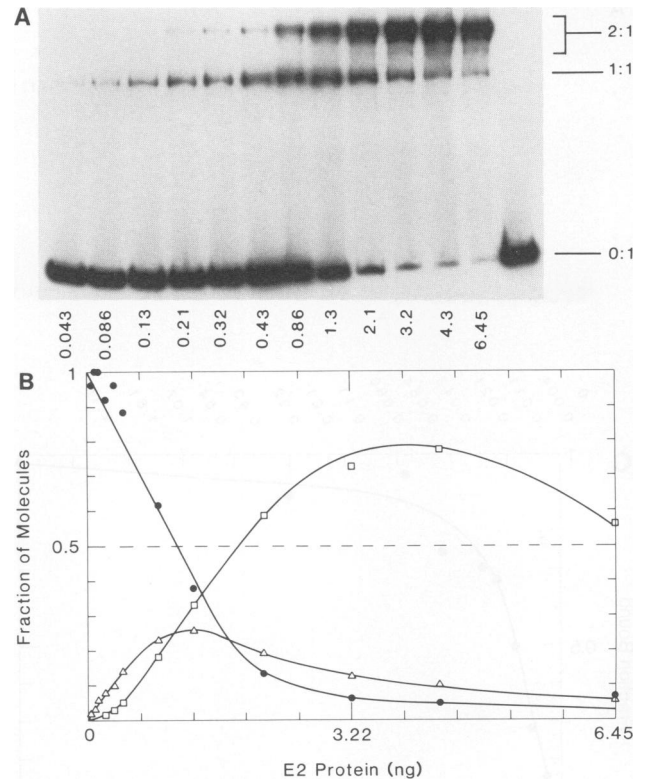
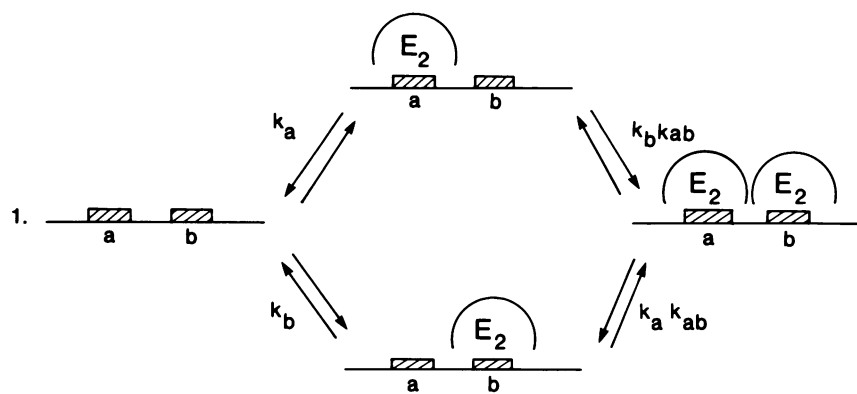


FIG. 3. Titration of the 32 P-labeled 54-bp oligonucleotide containing two adjacent E2 binding sites with the full-length E2 protein. (A) A fixed amount (0.55 ng) of oligonucleotide (1.4×10^{-9} M binding sites) was titrated in a 20- μ l volume, as described for the single-site oligonucleotide. Numbers below the lanes are nanograms of purified E2 protein. The shifted bands are identified as follows: 2:1, complexes with two E2 dimers bound; 1:1, complexes with one E2 dimer bound; and 0:1, free probe. Smaller shifted complexes due to proteolytic cleavage products are seen but never exceeded 5% of the total product. (B) Fractions of molecules are 2:1 complex (\square), 1:1 complex (\triangle), and free probe (\bullet). The decrease in signal seen in the 2:1 complexes at higher protein concentrations is believed to represent an artifact and is not observed in other experiments. By using equation 4 of Fig. 4, the cooperativity parameter for the binding of full-length E2 protein to adjacent binding sites was calculated to be 8.1.

expected, two principal shifts were observed, corresponding to occupancy of one (1:1) or two (2:1) sites (Fig. 3A), which is consistent with previous studies identifying these complexes (7). A graph of the fraction of the molecular species detected in the native gel as a function of increasing E2 protein concentration is shown in Fig. 3B.

In order to quantitatively analyze the binding of E2 protein, we have utilized the following analysis. The reaction of a binding protein with a small DNA fragment carrying two binding sites, a and b, is conveniently described by the fraction of DNA molecules carrying none (f_0), one (f_1), or two (f_2) proteins bound. The fractions can be expressed as a function of the association constants, k_a and k_b , the cooperativity parameter, k_{ab} , and the free protein concentration (Fig. 4). As seen experimentally in Fig. 3, the number of molecules with a single site occupied reaches a maximum and declines as protein concentration is increased and the second site is filled. When the fraction of complexes containing only a single occupied site reaches its maximum, the



$$2. \quad f_0 = \frac{1}{A}; \quad f_1 = \frac{(k_a + k_b) [P]}{A}; \quad f_2 = \frac{k_a k_b k_{ab} [P]^2}{A};$$

$$\text{where } A = (1 + (k_a + k_b) [P] + k_a k_b k_{ab} [P]^2).$$

$$3. \quad \text{At } \frac{df_1}{d[P]} = 0: \quad \begin{cases} f_{1\max} = \frac{k_a + k_b}{B}; \\ f_{2\max} = f_{0\max} = \frac{\sqrt{k_a k_b k_{ab}}}{B}; \end{cases}$$

$$\text{where } B = 2\sqrt{k_a k_b k_{ab}} + k_a + k_b.$$

$$4. \quad k_{ab} = \left(\frac{1}{f_{1\max}} - 1 \right)^2; \quad \text{if } k_a = k_b.$$

FIG. 4. Equations to calculate the ratio between the association constants for single and doubly bound E2 dimers. (1) Model used to describe binding of E2 to DNA containing two binding sites is shown. The association constants, k_a and k_b , represent the binding constants to sites a and b, while k_{ab} is the cooperativity parameter. (2) f_0 , f_1 , and f_2 are the fraction of oligonucleotides that are unbound, singly bound, and doubly bound, respectively, and $[P]$ is the concentration of free protein. (3) Values of f_1 , f_0 , and f_2 at the maximum value of f_1 ($df_1/d[P] = 0$). (4) The equation to calculate the cooperativity parameter is derived from equation 3, and the assumption is made that association constants are equal ($k_a = k_b$). Similar analyses have been performed for Oct-2 (24) and steroid hormone receptors (26, 43).

derivative of f_1 , df_1/dP , is 0. The value of f_1 at this point can be used to determine the cooperativity parameter (Fig. 4, equation 4). In the case of independent binding to adjacent sites, the cooperativity parameter, k_{ab} , is equal to 1. Substituting this value into equation 3, $f_{1\max}$ is found to be 0.5, while f_0 and f_2 are equal to 0.25 at this point. In the case of positive cooperativity ($k_{ab} > 1$), the value of $f_{1\max}$ is less than 0.5 and $f_{0(f1\max)} = f_{2(f1\max)}$ are above 0.25, while a value of $f_{1\max}$ greater than 0.5 with $f_{0(f1\max)} = f_{2(f1\max)} < 0.25$ indicates negative cooperativity.

We have used this analysis to quantitate the relative cooperativity of binding of E2 molecules to adjacent sites. As seen in Fig. 3, the fraction of oligonucleotides binding a single E2 dimer, f_1 , reaches a maximum value of 0.26 at an active E2 concentration of 0.7 nM, at which point $f_0 = f_2 = 0.36$. By the previous analysis, we calculate $k_{ab} = 8.1$, indicating that E2 binding to adjacent sites exhibits positive cooperativity. Three independent experiments yielded values ranging from 7.5 to 10.0, with an average of 8.5. These values for cooperativity parameters represent a lower limit, since we have made the assumption in our analysis that the association constants, k_a and k_b , are equal. If these association constants are not equal, the value of k_{ab} is greater. Similar values of cooperativity parameters have been found

for other site-specific DNA-binding proteins. The cooperativity coefficient measured for Oct-2 in binding to adjacent octamer sites was found to be approximately 10, while the cooperativity parameter for binding of bacteriophage λ cI repressor to the three operators of the λ Pr promoter is approximately 26 (1, 24).

In an attempt to localize the portion of the E2 protein responsible for cooperative binding, an 85-amino-acid C-terminal peptide corresponding to the minimal DNA binding domain of the E2 protein (26a) was expressed in bacteria using the T7-inducible expression vector pET8C (13a). This portion of E2 is not phosphorylated *in vivo* (27), so translation of this peptide in bacteria should mimic its synthesis in mammalian cells. To allow for translation initiation, an extra methionine was included at the N terminus of this peptide. The resulting 86-amino-acid protein (86-E2) was found to be soluble and was purified to homogeneity by sequential cation exchange and gel filtration chromatography (13a). The 86-E2 peptide retains the ability to dimerize and specifically bind E2-responsive sequences (13a). Three different titrations, each of which was done with either a fixed oligonucleotide concentration and increasing protein concentrations or the reverse experiment, resulted in calculated dissociation constants ranging from 2.9×10^{-10} to 6.0×10^{-10} M, with an

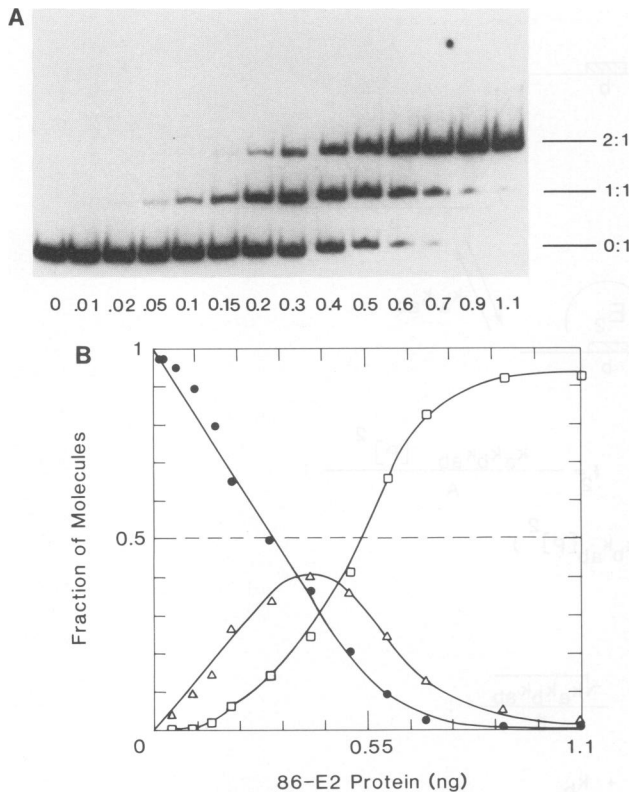


FIG. 5. Titration of binding of labeled 54-bp oligonucleotide containing two E2 binding sites with 86-E2 protein. (A) A fixed amount (0.55 ng) of oligonucleotide was titrated in a 20- μ l volume, as previously described. Numbers below are nanograms of purified protein. 2:1, Complex with two E2 dimers bound; 1:1, complexes with one E2 dimer bound; 0:1, free probe. Additional complexes are observed at the higher protein concentrations and are probably due to aggregation. (B) Titration curves for gel mobility shift experiment in panel A. The shifted bands are identified as follows: \square , 2:1 complex; \triangle , 1:1 complex; \bullet , free probe. The cooperativity coefficient (2.1) was calculated as shown in Fig. 4.

average of 4.3×10^{-10} M. A titration of 1.1 ng of a single-binding-site oligonucleotide with increasing amounts of the 86-E2 peptide (0.1 to 7 ng) is shown in Fig. 2C. No complexes were detected in a similar titration using 1.1 ng of a 39-bp oligonucleotide that did not contain E2 binding sites (Fig. 2C). Gel shift assays performed with the 86-E2 protein and the 54-bp oligonucleotide carrying two adjacent E2 binding sites resulted in two shifted bands (Fig. 5A) corresponding to single- and double-site occupancy. A graph of the fraction of bound and unbound species as a function of 86-E2 protein concentration is shown in Fig. 5. The maximum value for single-site occupancy is found to be 0.41 (Fig. 5B) with the values of f_1 and f_2 equal to 0.28. The lower limit value of k_{ab} for the 86-E2 protein was calculated to be 2.1 (see legend to Fig. 5B), which is suggestive of slight cooperativity of binding. In three separate experiments, values of the cooperativity parameter ranged from 1.6 to 2.1, with an average value of 1.9.

At high protein concentrations (Fig. 5A and data not shown), additional high-molecular-weight complexes appear in gel shift assays and may arise from aggregates of E2 proteins (13a). However, the analyses described in these studies were performed at lower concentrations of protein,

in which additional high-molecular-weight complexes were not observed.

Our studies suggest that at least a portion of the domain(s) responsible for cooperative binding of E2 molecules is located outside of the DNA binding domain. This region contains both the activation domain as well as a hinge region that separates the N terminus from the DNA binding and dimerization domains. Only minimal cooperativity in binding was observed with a peptide containing the minimal DNA binding and dimerization domains. The localization of the major cooperativity domain to amino acids external to the DNA binding domain contrasts with studies of the Oct-2 protein that showed this property to be encoded in the POU box DNA binding domain (24). The slight, but reproducible, cooperativity found in the 86-E2 protein suggests that a complex interaction of protein domains is responsible for the total cooperativity of binding of full-length E2 protein to its binding sites.

A balance between full-length E2 activator proteins and C-terminal repressor proteins regulates BPV expression in papillomavirus-transformed cells (5, 20, 23). It will be interesting to determine if, like the C-terminal 85-amino-acid peptide, the repressor proteins exhibit only low-level cooperativity in binding to adjacent sites. This would suggest that the initial binding of a full-length E2 protein to one binding site may facilitate the binding of a second full-length E2 dimer to the adjacent site. Such a mechanism may be particularly important for BPV gene regulation, since repressor molecules are more abundant than full-length proteins *in vivo* (20). Cooperativity may allow higher levels of transactivation at low levels of full-length E2 proteins. Cooperative binding of E2 molecules also allows control of transcription to be exerted over a narrower range of E2 concentrations than would occur with independent binding. Furthermore, the Oct-1 protein is thought to interact with the herpes simplex virus protein, α -TIF, and an additional cellular protein to cooperatively bind and regulate immediate-early viral promoters (10). It is possible that E2 interacts cooperatively with a cellular transcription factor in a similar fashion.

The E2 protein is capable of activating expression of the heterologous thymidine kinase or simian virus 40 early promoters 40- to 70-fold in transient assays in mammalian cells when two E2 binding sites are placed upstream of the promoter (13, 15, 17, 39, 42). The ability to activate is maintained even with a spacing of 60 bp between the two sites (37). In yeast cells, a similar 20- to 40-fold activation of expression from two binding sites has been observed (22, 30). Several models have been suggested to explain this synergistic action of multiple complexes of transcriptional activators. In one model, synergistic activation can result from cooperative binding of transactivators to enhancer binding sites. In an alternate model, factors bind independently to adjacent sites and then interact with an additional protein to provide synergistic activation (35). In this study, a cooperativity parameter of approximately 8.5 was observed for binding of E2 molecules to adjacent sites. While this cooperativity is significant, it may not be sufficient to explain the synergism observed when two E2 binding sites are present. If the sole source of synergism was a cooperativity in binding, cooperativity parameters at least 100-fold greater would be required to explain the extent of synergism observed (29a). Our studies with E2 show that additional mechanisms are probably involved in the synergistic activation of transcription by multiple E2-responsive sequences.

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