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Sequences Flanking the Core DNA-Binding Domain of Bovine Papillomavirus Type 1 E2 Contribute to DNA-Binding Function

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We have compared a series of molecular constructs that contain the minimal DNA-binding and dimerization domain of bovine papillomavirus type 1 (BPV-1) E2 alone or this binding domain plus the adjacent 16 or 40 amino acids to test the role of the flanking sequences in E2 function. The presence of these sequences resulted in an up to eightfold increase in the affinity of E2 for its target DNA and stabilized the protein against denaturation both in the absence of DNA and in the form of DNA-protein complexes. In addition, an aspartic acid-to-tyrosine mutation within the flanking region blocked DNA binding and function. These data demonstrate that sequences flanking the core domain contribute to E2 function and are, in fact, an integral part of the DNA-binding domain of BPV-1 E2.

The papillomavirus E2 protein serves two pivotal functions in the viral life cycle: regulation of viral gene expression and replication of the viral DNA genome. The 410-amino-acid (aa) bovine papillomavirus type 1 (BPV-1) E2 protein contains an amino-terminal region that mediates transcriptional activation and a carboxy-terminal DNA-binding and dimerization domain. These domains are separated by approximately 100 aa of unknown function (7). E2 coordinates viral gene expression by binding specifically to the palindrome $ACCGN_4CGGT$ found in papillomavirus genomes (2, 8, 18, 21). The carboxy-terminal 85 aa of BPV-1 E2 (aa 325 to 410) are sufficient for sequence-specific DNA binding and dimerization (10, 12, 14, 20). A high-resolution crystal structure for this minimal core region complexed with DNA revealed that the DNA-binding domain is folded into a dimeric β-barrel (9). Across the surface of the barrel, each E2 monomer contributes a short α helix (aa 336 to 342) which lies in the major groove of DNA, making specific contacts with the DNA (9). Data presented here demonstrate that sequences N terminal to the minimal, or core, BPV-1 E2 DNA-binding region influence DNA-binding affinity, protein and complex stability, and function. The data highlight the integral role this region plays in E2 structure and function.

Binding affinity correlates with length of C-terminal peptides. To examine the role of the flanking sequences in E2 function, C-terminal peptides with lengths of 127, 103, and 87 aa were expressed with the pET-8C vector in the bacterial strain pLYS-S (22). These proteins each contain methionine-valine at their N terminus as a result of cloning. Thus, E2-87 refers to the sequence Met-Val followed by the E2 85-aa core binding domain from the C terminus of E2, and E2-103 and E2-127 contain Met-Val followed by 16 or 40 aa, respectively, from the adjacent flanking region plus the core binding domain. E2 proteins were purified from *Escherichia coli* with S-Sepharose and gel filtration as previously described (6, 16, 20). Protein concentrations were estimated from the A_{280} with extinction coefficients of 0.99 (E2-87), 0.82 (E2-103), and 0.67 (E2-127). For quantitative assessment of sequence-specific DNA-binding affinity, serial dilutions of purified E2 protein were added to 20-μl binding reaction mixtures containing 0.1 ng of radiolabeled E2 binding site probe. The proportion of E2 protein bound to DNA was determined by electrophoretic mobility shift assay and densitometry (6). As shown in Fig. 1, the 127-aa form of the E2 protein bound this DNA probe with eightfold higher affinity than the core 87-residue form did (K_d, 0.9 versus 7.0 nM, respectively). The 103-aa E2 protein demonstrated intermediate affinity relative to the 127- and 87-residue forms (K_d, 2.5 nM). These results demonstrate that although the C-terminal residues spanning aa 325 to 410 of the core domain are capable of high-affinity DNA binding and dimerization, the region including residues 285 to 325 directly contributes to the function of this domain. The differences in the apparent affinity constants for E2-127 and E2-87 resemble those previously reported for full-length E2 and E2-87 (16).

Flanking sequences stabilize E2 structure. In addition to increasing the affinity for DNA, the flanking sequences also stabilized E2 against denaturation by urea both in the absence of DNA and in the form of protein-DNA complexes. E2 proteins were incubated with a ^{32}P-labeled DNA probe and subjected to gel shift analysis in a polyacrylamide gel containing a gradient of urea from 0 to 8 M perpendicular to the direction of electrophoresis (6). Samples containing E2 87-, 103-, or 127-aa peptides were each analyzed at 10 different urea concentrations across the gel (Fig. 2). The appearance of free probe (indicated by the asterisk in Fig. 2) shows disruption of the 87-aa E2-DNA complex at a concentration of 5 M urea. In contrast, the 103- and 127-aa E2-DNA complexes were stable in 8 M urea. For these peptides, the slower-mobility E2-DNA complexes were observed at all urea concentrations tested. The dramatic differences in the susceptibility of E2-87, compared with E2-103, and E-127 to denaturation highlight the stabilizing effect of the flanking region on the E2-DNA complex.

Differences in the stability of E2-87 with and without additional flanking sequences were also observed in the absence of DNA by monitoring unfolding or dimer dissociation with urea. In these studies, denaturation was monitored by changes in fluorescence. Changes in the spectrum of E2-87 were apparent with as little as 0.5 M urea (Fig. 3), and 50% of the product was

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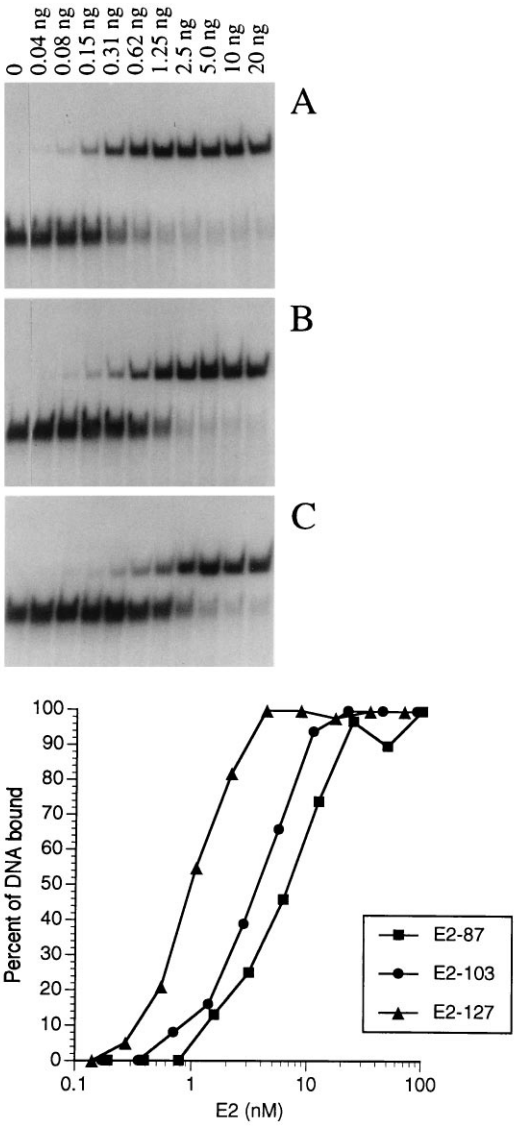

FIG. 1. DNA-binding affinity of C-terminal proteins. Electrophoretic mobility shift assay of E2-127 (A), E2-103 (B), and E2-87 (C) C-terminal peptides with a probe containing one E2-binding site previously described (6). Complexes were resolved on 10% polyacrylamide gels. Samples contained 0.1 ng of target DNA and 100 ng of dATP carrier DNA. The amounts of E2 protein added to each reaction mixture are indicated at the top of the figure. The results of these DNA-binding assays are graphed at the bottom as the percentage of DNA bound as a function of E2 input protein.

denatured with 2 M urea. The presence of flanking sequences resulted in a dramatic shift in the titration curve. For E2-103, 50% of the protein was denatured with 3.5 M urea, while for E2-127, 50% of the protein was denatured with 3.6 M urea. Interestingly, both E2-103 and E2-127 were stabilized to roughly the same extent, indicating that the 16 aa N terminal to E2-87 stabilize the structure of the DNA-binding domain of BPV-1 E2. Preliminary structural data for E2-103 obtained by nuclear magnetic resonance spectroscopy (NMR) support this notion (2a). Recently, the 80-aa form of the human papillomavirus type 16 (HPV-16) E2 DNA-binding region was tested for its susceptibility to denaturation by urea by a similar strategy (15). In this study, the changes in fluorescence resulting from denaturation were attributed to dimer dissociation. Since the DNA-binding domains of BPV-1 E2 and HPV-16 E2 are

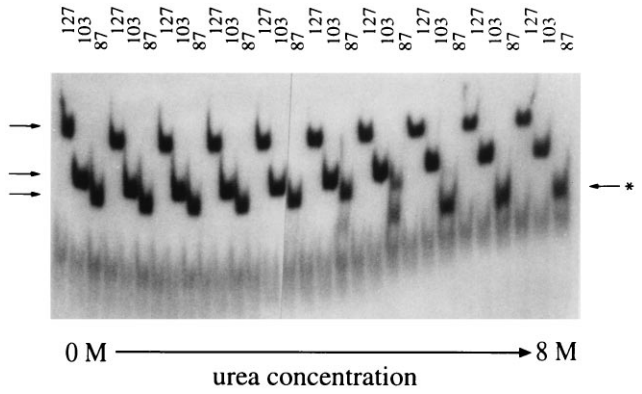

FIG. 2. Dissociation of E2-DNA complexes with urea. The samples were analyzed on a polyacrylamide gel containing a gradient of urea perpendicular to the direction of electrophoresis. Protein products were greater than 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Protein concentrations were estimated from A_{280}. E2-87 and E2-103 were loaded at 10 ng/lane, and E2-127 was loaded at 7.5 ng/lane. DNA containing a single E2 binding site was end labeled with [α-^{32}P]dGTP. Reactions were carried out in 20-μl reaction mixtures as previously described (6, 20). Arrows on the left of the figure indicate E2-DNA complexes for the three forms of E2. Under the conditions used for binding, all of the probe is shifted to a slower mobility. The asterisk on the right indicates free probe that was released as a result of denaturation of the E2-DNA complex.

30% identical in amino acid sequence and because of the similarity between these results and our data with BPV E2-87, we infer that the changes in fluorescence with BPV E2 are also due to dimer dissociation.

The spectral changes for BPV-1 E2 were more striking than those published for HPV-16 E2. While the shifts in emission wavelength for BPV-1 and HPV-16 E2 were identical, denaturation of BPV E2 resulted in an ~30% increase in signal intensity. There was no change in fluorescence intensity with HPV-16 E2. In BPV-1 E2, W-360 is the only amino acid contributing to its fluorescence spectrum, and therefore any fluorescence change reflects differences in its local environment. For HPV-16 E2, three tryptophans contribute to its spectrum. Even though W-360 is conserved in HPV-16 E2, the other

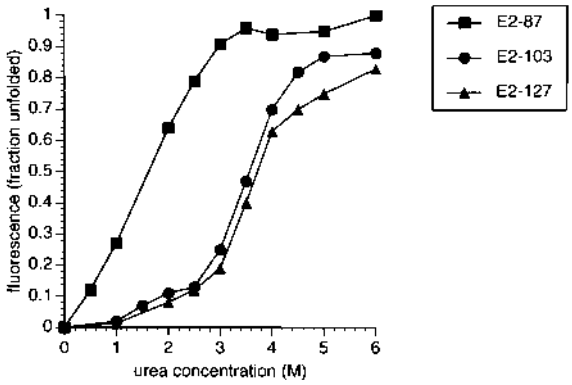

FIG. 3. Differences in E2 structure determined by fluorescence spectroscopy. The denaturation of E2 as a function of urea was measured by fluorescence spectroscopy in an Aminco-Bowman series 2 luminescence spectrometer. Samples containing 1 μM E2-87, -103, or -127 in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.0)–100 mM NaCl–1 mM dithiothreitol with various amounts of urea indicated were analyzed at 25°C with an excitation wavelength of 280 nm. Emission spectra from 300 to 400 nm were collected. Fluorescence data at 370 nm were plotted as a function of urea and standardized by using the change in fluorescence from 0 to 6 M urea for each of the E2 forms as a measure of the total fraction unfolded.

tryptophans presumably mask the effects on W-360 fluorescence. Since there are no tryptophan residues in the flanking sequences of BPV-1 E2, the spectra for E2-87, -103, and -127 all reflect changes in the W-360 environment and are directly comparable.

Functional differences were also apparent by monitoring repression of E2 transcriptional activation activity in mammalian cells (3, 19). In previous studies with BALB/c 3T3 cells and an E2 reporter system, activation (30- to 70-fold) by full-length E2 was reduced by 97% by both the wild-type E2 repressor (E2TR) or the C-terminal protein E2-127, while E2-87 showed no repression activity (3). When E2-103 was tested in this system, an intermediate level of 37% reduction in transcriptional activity was observed (data not shown), consistent with its intermediate phenotype exhibited in DNA-binding studies (Fig. 1). Studies with the HPV-16 E2 protein demonstrated trends in repression activity similar to those noted above for BPV-1 E2 (unpublished results). One explanation for these observations is that the differences in activity in cells are due to differences in the stability either of the E2 dimer or of the E2-DNA complex. Mok and coworkers speculated that, at least for HPV-16 E2-80, the core domain is predominantly monomeric and unfolded at concentrations at which DNA binding is often measured (10^{-11} M) (15). This notion is consistent with our data demonstrating that the BPV-1 E2-87 protein is unstable either alone or in a complex with DNA (Fig. 2 and 3) and suggests that the flanking regions of both proteins are necessary for stable complex formation with DNA.

Previously McBride and coworkers determined that E2 was a phosphoprotein and localized the major sites of phosphorylation to serine 298 and serine 301 (11). The functional significance of these sites is unknown. We generated phosphorylated E2-127 with casein kinase II and purified the phosphorylated protein. As expected, there was no effect of phosphorylation on DNA binding in gel shift mobility assays (unpublished results). Under the same conditions, E2-103 was not phosphorylated, supporting the specificity of the kinase reaction.

Specific residues in the flanking domain affect function. An E2 mutant containing an aspartic acid (D)-to-tyrosine (Y) substitution at residue 316 (D316Y) was isolated in a yeast (*Saccharomyces cerevisiae*)-based screen of random E2 mutants for transactivation-defective E2 proteins as previously described (20). This mutation was cloned into pET-8C, and 127-aa C-terminal peptides were purified as described previously (20, 22). Similar quantities, as assessed by immunoblotting of yeast or bacterial E2 proteins, were incubated with 1 ng of probe for 30 min at 4°C and resolved on polyacrylamide gels. Previously, we have used one-, two-, and four-E2-binding-site probes to evaluate the cooperative binding of E2 to DNA (16, 20). Bacterially expressed 127-aa proteins of E2 D316Y were incubated with a one-E2-binding-site probe (Fig. 4A) or a four-E2-binding-site probe (Fig. 4B), and full-length yeast-expressed E2 D316Y was incubated with a one-E2-binding-site probe (Fig. 4C). In all cases, DNA binding was substantially reduced or abolished with this flanking region mutant compared with that of the wild type. Furthermore, this reduction in DNA-binding affinity of E2 D316Y correlated with an absence of transcriptional activity. Transcriptional activation was tested by cotransfection into COS 7 cells of an E2-dependent chloramphenicol acetyl transferase reporter gene and full-length wild-type E2 or E2 containing the D316Y mutation, each expressed from the adenovirus major late promoter. Whereas wild-type E2 led to a 280-fold transactivation, E2 having the D316Y substitution caused only a 2.8-fold increase in transcriptional activity over the vector-alone control. Thus, specific residues adjacent to the core domain are required for function of the DNA-binding domain.

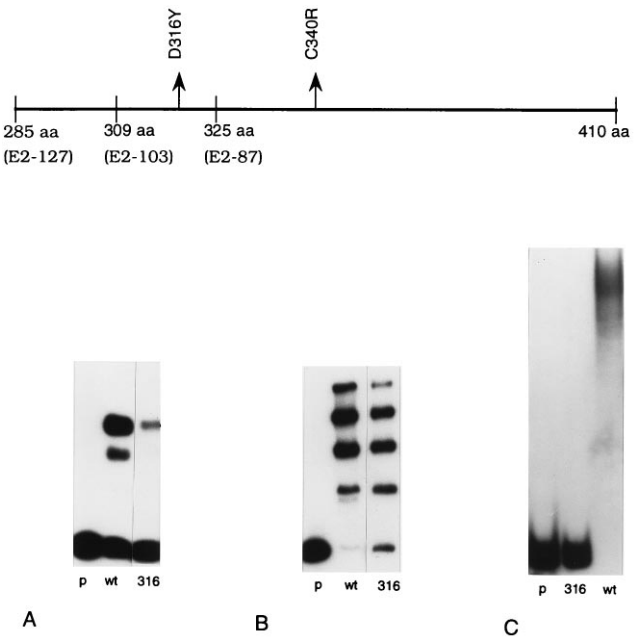

FIG. 4. DNA binding of E2-127 D316Y. Results of electrophoretic mobility shift assays of partially purified 127-aa C-terminal peptides and full-length yeast-expressed proteins of E2 mutant D316Y are shown. The binding reaction in panel A was done with a probe containing one E2 binding site, and that in panel B was done with a probe with four E2 binding sites (20). Probes were incubated with E2-127 D316Y (316) and E2-127 wild-type (wt) protein produced in *E. coli*. The binding reaction mixture in panel C contains a one-E2-binding-site probe incubated with full-length yeast-expressed protein. p indicates probe alone. The schematic at the top of the figure notes the positions of relevant amino acids in the E2 sequence.

Interactions between DNA-binding helix residues and flanking sequences impact DNA binding. In the center of the DNA-binding helix of papillomavirus E2 proteins is a highly conserved cysteine residue, present at aa 340 in BPV-1 E2 (13). In the crystal structure for the BPV E2-85–DNA complex (9), the C-340 sulfhydryl forms two bonds to DNA. Oxidation of this cysteine abrogates DNA binding in vitro (13). Through site-directed mutagenesis, C-340 was substituted with arginine (C340R) and the E2 C340R proteins in the full-length and 127-aa forms were shown to lack DNA-binding activity, but retained their ability to dimerize (20). Full-length E2 with this mutation was unable to activate transcription in yeast due to an inability to bind DNA (20). We have further characterized the C340R mutation in the context of the shorter E2 peptides discussed above. Proteins were purified from *E. coli*, and mobility shift experiments were performed as described for the wild-type proteins. Consistent with previously published results (20), E2-127 C340R was unable to bind the probe with one E2 binding site (Fig. 5, lane 1). However, when truncated to the C-terminal 103- or 87-residue forms, the C340R substitution mutants were DNA binding competent (Fig. 5, lanes 2 and 3), with the 87-residue form shifting all detectable probe to a slower mobility (Fig. 5A, lane 3). Immunoblots of the proteins confirmed that approximately equal amounts of protein were added to each lane and that the proteins migrated with mobilities expected for their predicted size (Fig. 5B). To investigate this result further, quantitative analysis was performed with purified 87-aa forms of the C340R and wild-type E2 peptides. The proportion of E2 protein bound to DNA was determined by densitometry and indicated that the E2-87 C340R protein displayed the same high-affinity binding for the E2 DNA motif as that of the wild-type E2-87 protein (data not shown). The

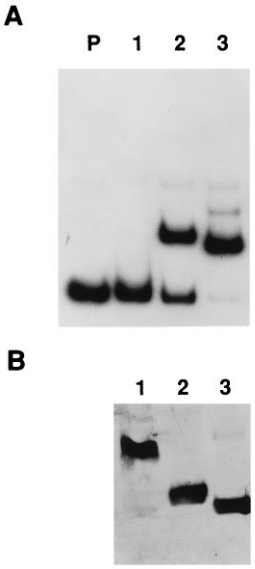

FIG. 5. DNA binding of C-terminal E2 C340R proteins. Results of electrophoretic mobility shift assays (A) and immunoblotting (17) (B) of C-terminal E2 C340R proteins, expressed and purified from *E. coli* (17), are shown. P indicates probe alone. Lanes: 1, E2-127 C340R peptide; 2, E2-103 C340R peptide; 3, E2-87 C340R peptide.

inability of E2-127 C340R to bind DNA under the same conditions in which E2-87 C340R binds with high affinity suggests that the 285 to 325 region of E2 folds back on the core domain near the DNA contact region, thereby influencing the structure and/or function of the binding helix. These predictions are supported by NMR studies of the 103-residue form that show contacts between the N-terminal extension and the 85-aa core of the protein (for example, F-318 to I-401, H-319 to I-331, H-319 to N-400, and H-319 to I-401 [2a]). The precise location and conformation of the N-terminal portion are being refined and will be reported elsewhere.

While the published high-resolution structure for BPV E2 lacks the flanking sequences, crystallographic data for the Epstein-Barr virus transactivating protein EBNA-1 provides precedent for our findings with E2. In EBNA-1, two domains contribute to DNA binding: the 100-aa core domain and a 33-aa flanking domain N terminal to the core (1, 5). EBNA-1 protein fragments that include the flanking region and the core domain have higher affinities for the EBNA-1 consensus site DNA than does the core domain alone (1). Unlike E2, the EBNA-1 core domain lacks high-affinity binding for its target DNA (1). The crystal structure of the core domain of EBNA-1 revealed a β-barrel structure remarkably similar to that of the E2 core protein (4), despite having no sequence similarity. A more recent crystal structure of the core plus flanking domain of EBNA-1 indicated that the EBNA-1 flanking domain makes direct DNA contacts distinct from those modeled with only the EBNA-1 core domain (5).

The results from the related EBNA-1 and HPV-16 E2 proteins provide further support for the involvement of the flanking region of BPV-1 E2 (aa 285 to 325) in DNA-binding function and dimerization. Specific residues in the 40 aa N terminal to the core 85-aa region of BPV-1 E2 contribute to E2 DNA-binding function and E2-directed transcriptional activation. This effect could be mediated through direct DNA contacts distinct from those of the core domain (as predicted for EBNA-1) or, as our results with E2 340R and NMR indicate, through intramolecular interactions between the flanking region and core domain. Together, these results show that the flanking residues 285 to 325 not included in the crystal structure of the E2 core domain are likely to play an integral role in the function of both full-length E2 and the repressor, E2TR.

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