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DNA-Binding Activity of Papillomavirus Proteins

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We demonstrate DNA binding by papillomavirus (PV) open reading frame (ORF) proteins that correspond to the early transforming and *trans*-activating (E6 and E2) and late structural regions (L2 and L1) from bovine PV type 1 and human PV types 6b and 16. All PV proteins were synthesized in *Escherichia coli* and had a common 13-amino-acid leader sequence from the expression vector pRA10. Antibodies have been generated in rabbits against these PV proteins. The PV ORF proteins bind double-stranded DNA, and this activity is demonstrated to be inherent to the PV proteins. DNA-binding activity by PV proteins is optimal at 50 mM NaCl and at pH 7.0. For some PV proteins (e.g., bovine PV type 1 E2), DNA binding is enhanced at a lower pH (pH 6.0) and NaCl concentration (50 to 100 mM). DNA binding is inhibited by the appropriate antibodies. The possible significance of these findings is discussed in relation to the genetic and structural evidence on the function of these ORFs.

Papillomaviruses (PVs) are members of the papovavirus family and consist of double-stranded circular DNAs of approximately 8,000 base pairs (bp) (24). Over 40 types of human PVs (HPVs) have been classified from various types of epithelial lesions, which can range in behavior from benign growths to invasive carcinomas (13). Bovine PV type 1 (BPV1) has served as a prototype for PV analysis, and a 69% fragment of BPV1 (69T) which contains the viral early region has been demonstrated to efficiently transform mouse cells in culture (9).

At least two (E6 and E5) BPV1 early open reading frames (ORFs) have been shown to encode transforming proteins (1, 23). Proteins corresponding to E6 and E5 have been immunologically identified in the mouse cell line C127, when transformed either by E6 or E5 under transcriptive control of a Moloney sarcoma virus long terminal repeat or simian virus 40 early promoter, respectively (1, 23). The E5 protein is very hydrophobic, and its function is unknown. The E6 protein is highly basic and possesses the repeat Cys-X-X-Cys five times in its sequence (attributes common to a number of nucleic acid-binding proteins [10, 29]). No biochemical characterization of the E6 protein has yet been accomplished.

The E7 protein of HPV16 has been identified in the tumor cell line Caski (27). Genetic studies of E7 in BPV1 indicate that this gene is required to maintain high virus copy numbers (17).

The E2 ORF of BPV1 has been implicated in transformation (22), viral replication, and plasmid maintenance (21). The transformation efficiency of a specifically mutated (termination codon inserted in the 5′ region of the E2 ORF) full-length BPV1 genome (8) is greatly reduced, but the role of the E2 ORF in transformation is ambiguous since other work maps the transforming and *trans*-activating functions of the BPV1 3′ early region to the E5 and E2 ORFs, respectively (33).

Despite the unclear role of E2 in transformation, its ability to *trans*-activate a BPV1 noncoding region (ncr) enhancer-dependent chloramphenicol acetyltransferase expression vector has been demonstrated (28). The mechanism for this induction of transcription is uncharacterized. There are at least two possible methods by which the BPV1 E2 protein could activate the BPV1 ncr regulatory element. The E2 protein could act indirectly, involving the promoter-specific protein factors from host cells, similar to the acquired immune deficiency syndrome (human T-cell lymphotropic virus type III) virus transcriptional control region (long terminal repeat) interaction with SP1 (a cellular transcription factor), and SP1 interaction with human T-cell lymphotropic virus type III gene product(s). This interaction apparently elicits a response of the long terminal repeat to virus-specific *trans*-activation (14). Alternatively, PV gene product(s) could interact directly with viral transcriptional regulatory elements similar to that which occurs in simian virus 40 with large T antigen (3).

Identification and characterization of early and late PV gene products is of importance both diagnostically and in understanding the role of early region ORFs in viral pathogenesis. To date, the biochemical characterization of PV proteins has been hampered by the inability to recover PV proteins from PV-transformed or -infected cells. To circumvent this problem, we have expressed the E2 ORFs of BPV1 and HPV6b (18), the E6 ORFs of HPV16, HPV6b, and BPV1, the L1 ORFs of BPV1 and HPV16, and the L2 ORF (BPV1) at high levels in *Escherichia coli*.

Our analysis of bacterially derived PV proteins is based on the fact that several oncogene proteins (*ras*, *abl*, *src*, *myc*, and *mos*) have been produced in *E. coli* successfully and have been shown to retain biochemical and biologic activities (4, 12, 26, 30–32).

The construction of the vectors for expression of the PV ORFs has been described previously (18) (Table 1). All 5′ junctions described in Table 1 enabled in-frame readthrough from the vector gene leader sequence into the PV ORF DNAs.

Expression plasmids for PV protein synthesis were analyzed by diagnostic restriction map analysis and by DNA sequence analysis of selected plasmids (data not shown).

All expression plasmids were found to have proper construction, and of note here is plasmid pH6E6*. This plasmid was formed by joining the *Eco*RI site of the pRA10 polylinker to the *Eco*RI* site at position 306 of HPV6b. This plasmid derives its designation pH6E6* from this construction and the resulting deletion of 207 bp (Table 1) from the

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TABLE 1. Construction of PV expression plasmids

Plasmid	Vector (bp)	Insert (bp)	Vector/insert at 5′ junction	Insert/vector at 3′ junction	Predicted fusion protein M_r (Da)	% ORF expressed
pH16E6	3,900	1,196	*Sma*I/*Nla*IV	*Nar*I/*Nar*I	19,250	93.2
pH16L1	3,900	1,810	*Bam*HI/*Bam*HI	*Hpa*II/*Nar*I	39,300	63.0
pH6E6*[a]	3,900	987	*Eco*Ri/*Eco*RI*	*Hpa*II/*Nar*I	12,320	54.0
pH6E6(FL)[b]	3,900	1,264	*Sph*I/*Ava*II	*Hpa*II/*Nar*I	20,900	100
pH6E2	3,900	1,969	*Hinc*II/*Sca*I	*Nar*I/*Nar*I	40,260	93.2
pB1E6	4,750	589	*Cla*I/*Hpa*II	*Hpa*II/*Cla*I	16,830	100
pB1E2	3,900	1,833	*Sph*I/*Sph*I	*Bam*HI/*Bam*HI	46,530	99.3
pB1L1	3,900	1,132	*Cla*I/*Nar*I	*Nar*I/*Nar*I	36,300	64.1
pB1L2	3,900	1,692	*Bam*HI/*Bam*HI	*Nar*I/*Nar*I	44,550	81.3
p*lac*Z	1,510	4,677	*Cla*I/*Cla*I	*Pst*I/*Pst*I	83,420	ND[c]

[a] HPV6b E6 ORF, 46% of 5′ sequence deleted.
[b] Full-length HPV6b E6 ORF.
[c] ND, Not determined.

HPV6b E6 ORF. Table 1 shows the percentage of PV ORF that was expressed by the expression plasmids.

PV fusion proteins synthesized in *E. coli* MZ1 were labeled with [^{35}S]methionine and were identified by immunoprecipitation with antiserum directed against the common *c*II 13-amino-acid (aa) amino terminal possessed by all *c*II-PV proteins.

Immunoprecipitated fusion proteins from induced MZ1 cells reacted against rabbit anti-*c*II antiserum are shown (Fig. 1); the M_rs of these bands corresponded well to those predicted (Table 1). No immunoprecipitate bands were seen with extracts of MZ1 cultures maintained at 32°C.

High-level expression of PV ORFs allowed a relatively simple and rapid purification procedure (26). This technique takes advantage of the relative insolubility of *E. coli*-synthesized PV proteins. Treatment with lysozyme, deoxycholate, 1.75 M guanidine hydrochloride, 1.0 M NaCl, and Triton X-100 (1%) removed most contaminating *E. coli* proteins, whereas only 8.0 M urea or 7.0 M guanidine hydrochloride efficiently solubilized PV fusion proteins. PV proteins solubilized in 8 M urea or 7 M guanidine hydrochloride were precipitated in ethanol and suspended in 6% sodium dodecyl sulfate (SDS), and samples were subjected to SDS-polyacrylamide gel electrophoresis for transfer to nitrocellulose and analysis of DNA-binding activity.

A partially purified HPV6b E2 protein is shown in Fig. 2 as an example. It has a predicted M_r of 40.2 kilodaltons (kDa). The removal of contaminating *E. coli* proteins (lanes 3 and 4) and the resulting purification of the HPV6b E2 protein (lane 4) is readily apparent. For proteins induced at high level, >90% homogeneity has been achieved, and this purification protocol was applied to all fusion proteins generated for this study.

Since direct interaction with DNA could be one possible mechanism for *trans*-activation by the PV E2 proteins (28) and the E6 proteins of BPV1, HPV6b, and HPV16 possess characteristics that are common to a number of nucleic acid-binding proteins (i.e., highly basic, Cys-X-X-Cys repeats), we tested for possible DNA-binding activity of renatured, nitrocellulose-bound E2 and E6 proteins (2). The DNA-binding potential of the E2 proteins of BPV1 and HPV6b was examined initially. Nitrocellulose-bound E2 proteins were incubated with ^{32}P-labeled (nick-translated) double-stranded DNAs corresponding to the ncr of BPV1 (*Hpa*I to *Hin*dIII fragment) and HPV6b (*Xho*II to *Nla*IV fragment) or to other viral DNA fragments that contain no transcriptional regulatory sequences.

Table 2 summarizes the results of experiments designed to test DNA-binding activity. It was found that DNA fragments that contained ncrs from HPV6b or BPV1 were bound by the HPV6b or BPV1 E2 proteins, respectively. Further analysis showed positive BPV1 ncr DNA fragment binding to the HPV6b E2 protein and positive HPV6b ncr DNA fragment binding to the BPV1 E2 protein. The E2 proteins also bound PV DNA fragments with no assigned transcriptional regulatory signals. Further characterization of the DNA-binding activity of these E2 proteins at 50 mM NaCl, pH 7.0, demonstrated that all [^{32}P]-labeled DNAs, including pBR322, were bound with equal efficiency by E2 proteins, with the HPV6b E2 protein binding DNA more efficiently than did the BPV1 E2 protein.

To evaluate possible sequence-specific DNA binding by the BPV1 and HPV6b E2 proteins, the binding of [^{32}P]-labeled DNAs was carried out in the presence of cold (unlabeled) competitor DNA fragments that consisted of ncrs or DNA fragments devoid of transcription regulatory signals. Cold competitor DNAs (regardless of sequence) caused a uniform reduction of E2 protein-DNA binding, whether to ncr or other DNA fragments (data not shown). Similar analysis of other PV fusion proteins (HPV6b E6, HPV16 E6, HPV16 L1, BPV1 E6, BPV1 L1, and BPV1 L2) also showed positive nonselective DNA binding for each of these proteins at 50 mM NaCl and pH 7.0 (Table 2).

To ensure that DNA binding was a property intrinsic to PV proteins, a truncated HPV6b E6 ORF expression vector (Tables 1 and 2) was constructed to evaluate the role of the vector-encoded (*c*II) aa sequence in DNA binding. The vector pH6E6* expressed the *c*II gene 13-aa leader sequence and an additional N-terminal 19-aa sequence encoded by the entire polylinker sequence of pRA10. Plasmid pH6E6* expressed a truncated (46%) HPV6b E6 ORF (Table 1). It was found that the *c*II-H6E6* protein did not bind DNA at pH 7.0, irrespective of NaCl concentration (Fig. 3; Table 2).

Further confirmation of inherent PV protein-DNA-binding activity came from analysis of the fusion protein expressed from plasmid p*lac*Z (Table 1; Fig. 1). The protein derived from this plasmid is a fusion of the vector-derived aa leader sequence and β-galactosidase. This protein exhibited no DNA binding at any pH or NaCl concentration (Table 2), thus indicating no leader sequence involvement in DNA binding.

Since optimal conditions for specific PV protein-DNA binding are unknown, we tested PV protein-DNA binding under more stringent conditions, which consisted of either an elevated NaCl concentration or an altered pH. As at 50 mM NaCl, the PV proteins in NaCl concentrations of up to

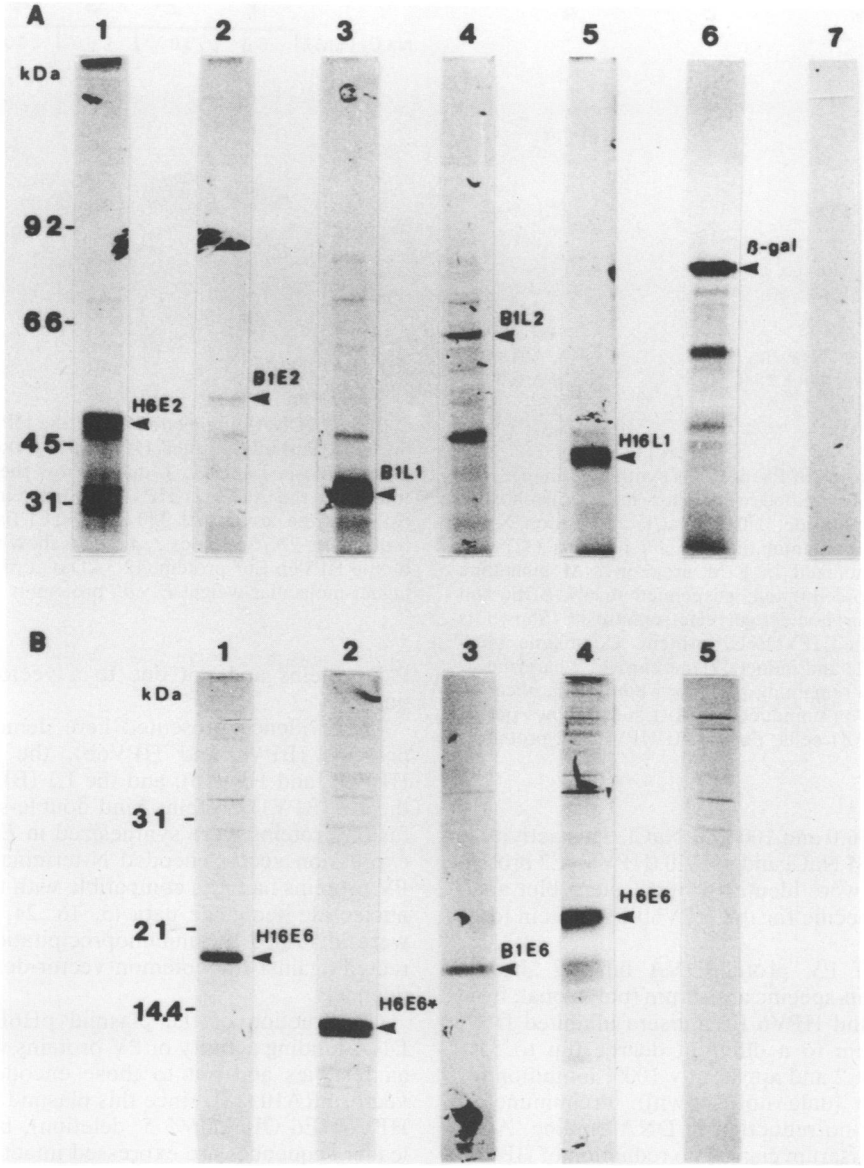

FIG. 1. Synthesis and identification of *c*II-PV ORF [^{35}S]methionine-labeled proteins with *c*II-specific antibody. Proteins from [^{35}S]methionine-labeled *E. coli* MZ1 cells were immunoprecipitated with *c*II-specific antibody, as previously described (23). (A) Lanes: 1 through 6, immunoprecipitates of, HPV6b E2, BPV1 E2, BPV1 L1, BPV1 L2, HPV16 L1, and β-galactosidase *c*II fusion proteins, respectively, on an SDS-8% polyacrylamide gel; 7, control (uninduced cells). BPV1 L2 protein (44.5 kDa) consistently displays an anomalous migration pattern (64 kDa). (B) Lanes: 1 through 4, immunoprecipitates of HPV16 E6, HPV6b E6*, BPV1 E6, and HPV6b E6 *c*II fusion proteins, respectively, on an SDS-12% polyacrylamide gel; 5, control.

350 mM exhibited no detectable selective DNA binding (Table 2). The strongest DNA binding occurred between 50 and 200 mM NaCl (pH 7.0) and decreased at higher (>200 mM) NaCl concentrations. An example of a typical DNA-binding blot is shown in Fig. 3 for intact HPV6b E6 and HPV6b E6* proteins. The HPV6b E6 (20.9-kDa) protein displayed peak DNA-binding activity between 50 and 200 mM NaCl (pH 7.0). At 200 mM NaCl, the background (DNA-binding activity of *E. coli* or degraded E6 proteins) is reduced, and at higher (>200 mM) NaCl concentrations, the DNA-binding activity of the HPV6b E6 protein is also reduced. At 350 mM NaCl, HPV6b E6 protein-DNA-binding activity is 27.3% of that occurring at 50 mM NaCl.

Further characterization of PV protein-DNA binding showed that certain PV proteins bound DNA with greater efficiency at pH 6.0. Figure 4A shows HPV6b E6* protein (12.3-kDa)-binding activity at pH 6.0 and 100 mM NaCl; for comparison, the DNA-binding activity of the BPV1 E6 (16.8-kDa) and HPV16 E6 (19.2-kDa) proteins is shown at the same pH and NaCl concentration. At pH 6.5 and above, the HPV6b E6* protein displayed no DNA-binding activity (data not shown). Figure 4B shows enhanced BPV1 E2 (46.5kDa) DNA-binding activity at pH 6.0 and 100 mM NaCl. This activity is detectable up to 150 mM NaCl; for comparison, DNA binding by the BPV1 E2 protein is shown at pH 7.0 and 100 mM NaCl. Included in Fig. 4B for reference is the HPV6b E2 protein (40.2 kDa). Of note is detection of DNA-binding activity by the HPV6b E2 degra-

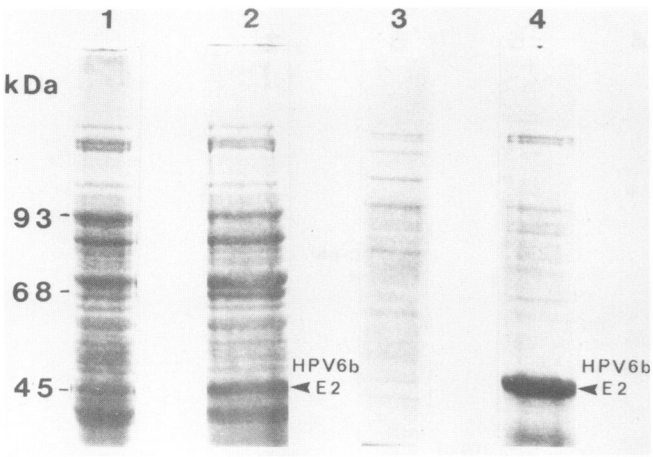

FIG. 2. Partial purification of PV proteins synthesized in *E. coli*. Induced-cell extracts were treated with lysozyme, deoxycholate, 1.75 M guanidine hydrochloride, 1.0 M NaCl, and Triton X-100 (1.0%) to remove most contaminating *E. coli* proteins (32). PV proteins were then solubilized in 8 M urea or 7 M guanidine hydrochloride, ethanol precipitated, suspended in 6% SDS, and analyzed by SDS-polyacrylamide gel electrophoresis. Shown is partial purification of the HPV6b E2 protein. Coomassie blue-stained uninduced (lane 1) and induced (lane 2) pH6E2 carrying *E. coli* MZ1. Lane 3 shows remaining proteins when the purification procedure is carried out on uninduced cells. Lane 4 shows partial purification of induced MZ1 cells. Fusion *c*II-HPV6b E2 protein is shown.

dation products at pH 6.0 and 100 mM NaCl. This activity is undetectable at 100 mM NaCl and pH 7.0 (HPV6b E2 protein degradation products were identified by Western blot analysis with antiserum specific for the HPV6b E2 protein [data not shown]).

Further analysis of PV protein-DNA binding showed inhibition by PV protein-specific antiserum (polyclonal, from rabbits). HPV6b E2 and HPV6 E6 antisera inhibited DNA binding of each protein to a different degree (up to 55% inhibition for HPV6b E2 and apparently 100% inhibition for HPV16 E6 antiserum [data not shown]). Preimmune or anti-*c*II serum caused no reduction in DNA binding. Also, HPV16 E6-specific antiserum caused no reduction of HPV6b E2 protein-DNA-binding activity, nor did HPV6b E2-specific antiserum affect HPV16 E6 DNA-binding activity. Blockage of DNA binding by PV protein-specific antiserum further confirmed that DNA binding is a genuine attribute of

TABLE 2. DNA-binding activity of PV proteins at pH 7.0

Viral protein	DNA binding at NaCl concn[a] (mM)					
	50	100	150	200	350	400
HPV16 E6	+	+	+	+	−	−
HPV16 L1	+	+	+	+	+	−
HPV6b E6	+	+	+	+	+	−
HPV6b E6*[b]	−	−	−	−	−	−
HPV6b E2	+	+	+	+/−	−	−
BPV1 E6	+	+	+	+	−	−
BPV1 E2	+/−	+/−	+/−	−	−	−
BPV1 L1	+	+	+	+	+	−
BPV1 L2	+	+	+	+	+	−
β-Galactosidase	−	−	−	−	−	−

[a] +, Positive DNA binding; +/−, weakly positive DNA binding; −, negative DNA binding.
[b] Asterisk indicates truncated protein.

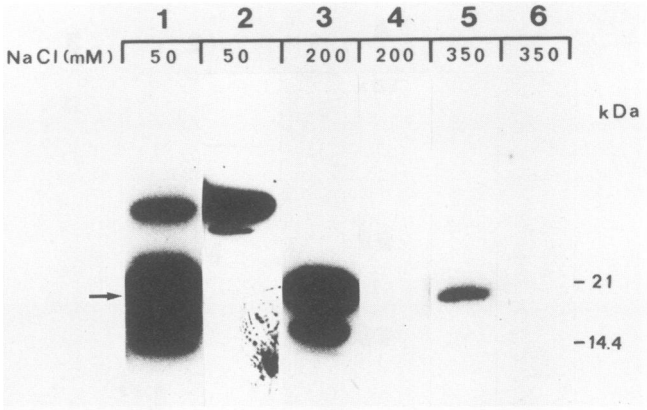

FIG. 3. DNA-binding activity of the HPV6b E6 and HPV6b E6* proteins. Partially purified HPV6b E6 proteins were transferred to nitrocellulose. Lanes 1, 3, and 5 show the effect of NaCl concentration on the 20.9-kDa HPV6b E6 protein-DNA binding, as indicated by the arrow. At 350 mM NaCl (lane 5), DNA binding is reduced by 72.7%. Lanes 2, 4, and 6 show the lack of DNA binding by the HPV6b E6* protein (12.3 kDa) at pH 7.0. DNA binding by a higher-molecular-weight *E. coli* protein(s) is shown in lane 2.

PV proteins and not due to a vector-encoded leader sequence.

The evidence presented here demonstrates that the E2 proteins (BPV1 and HPV6b), the E6 proteins (BPV1, HPV6b, and HPV16), and the L1 (BPV1 and HPV16), and the L2 (BPV1) proteins bind double-stranded DNA. These fusion proteins were synthesized in *E. coli* with a common expression vector-encoded N-terminal 13-aa sequence. All PV proteins had M_rs compatible with those predicted by the nucleotide sequence data (5, 16, 24, 25), and all proteins were identified by immunoprecipitation with antiserum directed against the common vector-derived 13-aa leader sequence.

Construction of the plasmid pH6E6* ensured that the DNA-binding activity of PV proteins was attributable to PV aa residues and not to those encoded by the expression vector pRA10 (18), since this plasmid expresses a truncated HPV6b E6 ORF (46% 5′ deletion), but the vector-derived leader sequences are expressed intact. DNA binding by the HPV6b E6* protein was not detected at pH 7.0 and 50 to 350 mM NaCl, but weak DNA binding by this protein occurred at pH 6.0 and 50 to 150 mM NaCl. Another plasmid, p*lac*Z, which expressed a fusion *c*II-β-galactosidase protein, exhibited no DNA binding at any pH or NaCl concentration. It was therefore concluded that weak DNA binding by the HPV6b E6* protein at pH 6.0 is due to a residual PV aa sequence. It is also evident that the amino-terminal portion of the HPV6b E6 protein is important for efficient DNA binding.

Analysis of the HPV6b E6 protein (11) suggests that the two predicted α helices preserved in the HPV6b E6* protein are responsible for weak DNA binding (pH 6.0 and 50 to 100 mM NaCl). It is believed that many DNA-binding proteins recognize DNA operator sequences by using helix-turn-helix units (32). The predicted structures for the BPV1 and HPV16 E6 proteins also reveal two prominent α helices, but distribution is dissimilar from HPV6b E6 and also from each other.

Other features common to the BPV1, HPV6b, and HPV16 E6 proteins are their Cys-rich composition (10.9, 8.7, and 8.8%, respectively) and the occurrence of the ordered repeat

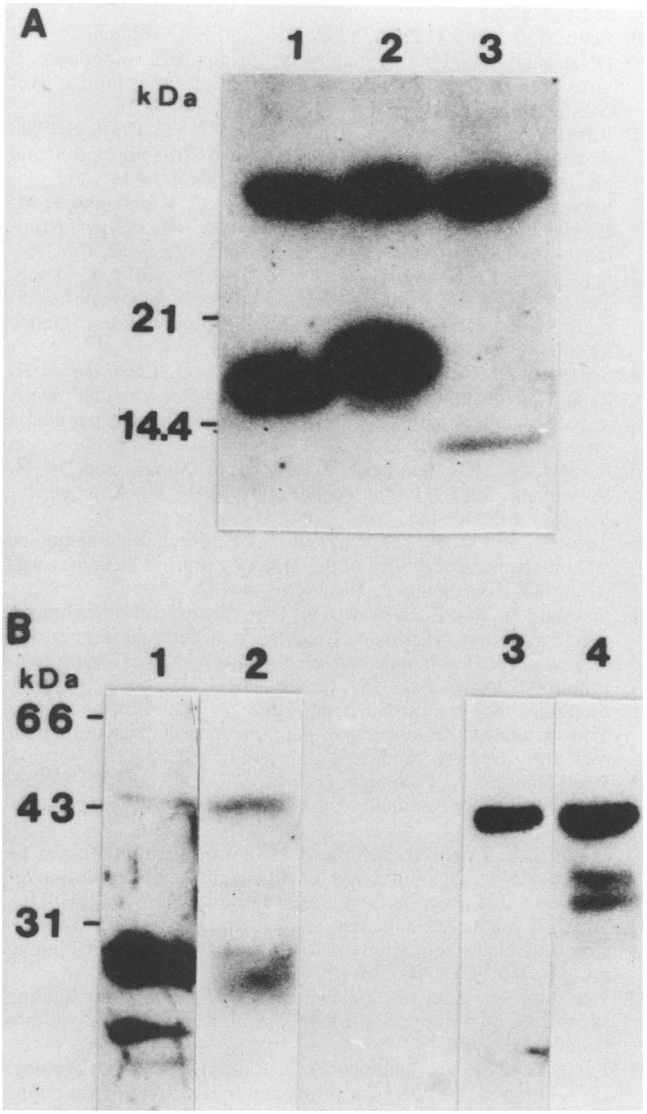

FIG. 4. Effect of lowered pH on PV protein-DNA-binding activity. (A) Lanes 1 and 2 show the partially purified protein of BPV1 E6 (16.8 kDa) and HPV16 E6 (19.2 kDa) binding DNA at 100 mM NaCl and pH 6.0. Lane 3 shows the 12.3-kDa HPV6b E6* protein-DNA-binding activity at 100 mM NaCl and pH 6.0. Weak HPV6b E6* DNA binding occurred up to 150 mM NaCl (results not shown), only at pH 6.0. (B) Lanes 1 and 2 show DNA binding by the BPV1 E2 protein (46.5 kDa) at pH 7.0 and 6.0 at 100 mM NaCl. Lanes 3 and 4 show the 40.2-kDa HPV6b E2 protein-DNA binding at pH 7.0 and at pH 6.0. Additional bands in lane 4 are the HPV6b E2 protein degradation products, as identified by Western blot analysis (data not shown).

Cys-X-X-Cys five times in BPV1 and four times in HPV6b and HPV16. The BPV1, HPV6b, and HPV16 E6 proteins are also highly basic (16.8, 11.3, and 18.2% Lys plus Arg, respectively). The E6 features described above are quite similar to those of other nucleic acid-binding proteins (e.g., polyomavirus t antigen and simian virus 40 T/t antigen [10]).

As indicated above, genetic evidence demonstrated that the E2 protein of BPV1 is a diffusible *trans*-acting factor (28). The finding that the E2 proteins of HPV6b and BPV1 bind double-stranded DNA suggests that *trans*-activation is mediated by direct protein-DNA interaction. In common with other DNA-binding proteins, E2 proteins are basic (10.9 and 11.4% Lys plus Arg, respectively, for HPV6 b and BPV1), and both E2 proteins are predicted (11) to possess prominent α helices (helix-turn-helix) near the N-terminal third of each protein. DNA binding by the E2 proteins is therefore expected, given the genetic evidence and protein analysis.

The DNA-binding activity displayed by the PV structural proteins L1 (BPV1 and HPV16) and L2 (BPV1) suggests the involvement of these proteins in virion assembly. Precedent exists for the interaction of viral capsid proteins with nucleic acids. Hepatitis B virus core (p22) proteins specifically bind hepatitis DNA, and the portion of the hepatitis p22 protein important in DNA binding is the carboxy-terminal region that is enriched for basic aa residues (19, 20). Similarly, the L1 proteins of BPV1 and HPV16 and the L2 protein of BPV1 possess clusters of basic aa residues at their carboxy terminal, suggesting that the carboxy-terminal region of the PV structural proteins is involved in DNA binding.

Nitrocellulose-bound PV protein did not exhibit sequence-specific binding of DNA in competitive or noncompetitive assays, possibly reflecting limitations of this assay. More refined analyses are currently under way to determine the sequence-specific PV protein-DNA interaction. Such factors as DNA structure (i.e., DNA template or strandedness) or PV protein modification (e.g., phosphorylation) may be important for the interaction of a specific PV protein and DNA. Also possible (and likely) is the requirement of ancillary host cellular (or PV) protein(s) for sequence-specific interactions.

PV early proteins, particularly E2, may directly associate with specific PV ncr regulatory sequences. In HPV11, two 12-bp inverted repeats (ACCGAAAACGGT) occur just upstream of the TATA box (7). This repeat also occurs in the corresponding position of HPV7, -13, -16, -18, -30, and -33 (6). Screening of HPV1, -6, -11, and -16, BPV1, and cottontail rabbit PV sequences reveals that the 12-bp element ACCG N_4 CGGT (N = A, C, T, or G) is present in differing amounts exclusively in the ncr of each virus (7). It seems plausible that such a common conserved PV ncr sequence, in close proximity to putative early promoters, may constitute a recognition element for the E2, E6, or other PV ORF gene products. PV proteins may also possess the ability to stimulate (*trans*-activate) cellular promoters and thereby play a role in cell immortalization or transformation. A number of viral oncogenes are able to stimulate expression from heterologous promoters (e.g., simian virus 40 large T antigen and adenovirus Ela protein) (15), indicating the plausibility of this suggestion.

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