Papillomavirus Polypeptides E6 and E7 Are Zinc-Binding Proteins

MIGUEL S. BARBOSA,* DOUGLAS R. LOWY, AND JOHN T. SCHILLER

Laboratory of Cellular Oncology, Building 37, Room 1B-26, National Cancer Institute, Bethesda, Maryland 20892

Received 12 September 1988/Accepted 22 November 1988

Papillomavirus proteins E6 and E7 have Cys-X-X-Cys repeats which have been suggested to mediate zinc binding. We have developed a modification of an assay that detects zinc binding to proteins immobilized on filters. Using well-characterized metalloproteins, we show that, under reducing conditions, this assay distinguishes proteins that coordinate zinc through cysteine residues from those that bind the metal through other amino acids. Under these conditions, E6 and E7 polypeptides of human papillomavirus type 18 and bovine papillomavirus type 1 exhibited high-affinity zinc binding. Our results suggest that E6 and E7 are metalloproteins and may coordinate the metal ions through cysteine residues.

The papillomavirus E6 and E7 genes have been implicated in the induction and maintenance of neoplasias. In human genital tumors and tumor-derived cell lines, these genes are selectively retained and expressed (19, 20). In vitro, the E6 of bovine papillomavirus type 1 (BPV-1) (16) and the E7 of human papillomavirus types 16 (HPV-16) (11, 24) and 18 (HPV-18) (4) have been shown to induce anchorage independence in established rodent fibroblasts. The HPV-16 E7, which has an adenovirus E1a-like *trans*-activation function, can cooperate with *ras*-like oncogenes to transform primary rodent cells (12, 14). In addition, the E6 and E7 genes appear to be required for HPV-16- and HPV-18-induced immortalization of human keratinocytes (17; data not shown; R. Schlegel, personal communication).

All papillomaviruses have clearly homologous E6 and E7 open reading frames (ORFs). Cole and Danos (7) have suggested that both of these genes were formed by duplication of a 33-amino-acid unit containing a cysteine doublet (Cys-X-X-Cys); E6 has four repetitions, and E7 has two complete repeats plus a third degenerate one that lacks one of the cysteines. Cys-X-X-Cys motifs have been suggested to coordinate metal binding and are frequently found in nucleic acid-binding proteins (5), where they are thought to be part of a nucleic acid recognition complex designated a zinc finger. The cysteine motif sequence present in papillomaviruses has some but not all of the features proposed by Berg for the zinc finger domains (6). Therefore, it was of interest to determine whether E6 and E7 proteins of papillomaviruses do indeed bind zinc. Recently, Schiff et al. (15) described a technique which allows identification of zinc-binding proteins when they are immobilized on nitrocellulose by Western (immunoblot) transfer. We have used a modification of this technique, labeling under reducing conditions, to show that BPV-1 and HPV-18 E6 and E7 polypeptides are zinc-binding proteins.

The proteins used in this study were obtained by expressing the respective ORFs fused to bacterial genes. The HPV-18 ORFs were fused 3' to the bacterial *trpE* gene in the fusion protein expression system pATH (kindly provided by M. Crivellone) (22). The TrpE-18E6 protein was expressed by inserting the *Bam*HI (nucleotide [nt] 119)-*Taq*I (nt 838) fragment into the pATH2 vector at *Bam*HI-*Cl*AI. The resulting protein fused 336 TrpE amino acids to HPV-18 E6 residues 6 through 158, yielding a polypeptide of approxi-

mately 54 kilodaltons (Fig. 1, TrpE-18E6). The *Hae*III (nt 511)-*Eco*RI (nt 2440) HPV-18 fragment was first cloned into pUC18 at *Eco*RI-*Sma*I; then the E7 ORF was removed by using the *Bam*HI site of pUC18 (nt 417) and the *Dra*I HPV-18 site (nt 1167) and inserted into pATH1 at *Bam*HI-*Cl*AI (after filling in the *Cl*AI site). The resulting fusion protein contains TrpE fused to the E7 ORF 27 amino acids upstream of the first AUG (Fig. 1, TrpE-18E7). The BPV-1 E6 and E7 sequences were expressed as fusion proteins in the pCO-5 vector, which fuses 12 amino acids from the lambda phage cII protein to the amino terminus of the inserted ORF. The cII-B1E6 fusion protein, previously described (2), contains the full E6 protein sequence fused to the cII leader (Fig. 1). For expression of the BPV-1 E7 sequences, the *Hpa*II (nt 670 to 946) fragment was inserted at the *Cl*AI site of pCO-5. This construct expresses the cII-B1E7 fusion protein containing the carboxyl-end portion of the putative E7 protein (amino acids 65 to 127), which contains both of the Cys-X-X-Cys motifs common to papillomavirus E7 proteins. The similar spacing of those motifs, depicted as vertical lines in both E6 and E7 sequences, is evident in Fig. 1. Both E7 fusion proteins migrate slower (18E7 at 58 kilodaltons [kDa] and B1E7 at 14 kDa) than expected from their calculated molecular sizes (52 kDa for 18E7 and 8 kDa for B1E7); similar aberrant migration has been described for the HPV-16 E7 protein and thus seems characteristic of E7 proteins (20). Furthermore, since B1E7 containing only the carboxyl-terminal 62 amino acids migrates aberrantly, we suggest that this characteristic is encoded within the carboxyl-terminal half of the molecule.

We first attempted the zinc-binding assay using the conditions described by Schiff et al., in which the proteins are not reduced (15). No ⁶⁵Zn labeling of the papillomavirus proteins was seen under these conditions (data not shown). The absence of conserved histidines in the papillomavirus conserved motifs suggested that if E6 or E7 bound zinc, the cysteines themselves might be involved. If this were the case, the oxidation state of the proteins might be critical to their ability to bind metal. Therefore, we modified the protocol so that labeling was done after the blotted proteins were reduced as follows. Proteins were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to an Immobilon (Millipore) filter. The blots were incubated in renaturing buffer (100 mM Tris, 50 mM NaCl, 10 mM dithiothreitol; pH 6.8) for 1 h with three changes of buffer. All buffers were adjusted to pH 6.8, which has been shown to increase the level of stringency for zinc binding (13, 21).

* Corresponding author.

FIG. 1. Fusion protein constructs. Each of the constructs is labeled on the left with the name of the bacterial protein followed by the papillomavirus type and its ORF. The bacterial portion is depicted as a broken line for the TrpE peptide and a solid line for the cII peptide. The papillomavirus ORF regions expressed in each fusion are drawn as open boxes. Within each ORF, the locations of the homologous cysteines are indicated by vertical lines. The papillomavirus amino acids, numbered in relation to the first methionine of the respective ORF, are given below each construct. The numbers on the right are those of the last residue in the ORF.

Labeling buffer (100 mM Tris, 50 mM NaCl; pH 6.8) was flushed with N_2 to remove dissolved oxygen and thus avoid oxidation of blotted proteins. Filters were then rinsed in labeling buffer twice. $^{65}ZnCl_2$ in labeling buffer at a concentration of 10 to 15 μM was added to the filters for 15 min. The filters were then rinsed twice in wash buffer (100 mM Tris, 50 mM NaCl, 1 mM dithiothreitol; pH 6.8) and washed for 1 h with three changes of buffer. Under these conditions, the BPV-1 and HPV-18 E6 and E7 proteins bound ^{65}Zn (Fig. 2B, lanes B, C, E, and F, respectively), while many other bacterial proteins (Fig. 2A, lanes B, C, E, and F) present on the blot did not.

To ascertain the specificity of zinc binding to the papillomavirus portion of the fusion proteins, we tested unfused TrpE expressed from the pATH vector (Fig. 2A, lane G). This control was especially important for the TrpE fusions, since the TrpE sequence contains a Cys-X-X-X-Cys motif which potentially could bind zinc (25). Comparison of the amido black stains for 18E6, 18E7, and TrpE (Fig. 2A, lanes E through G, respectively) indicated that each band contained similar amounts of protein, and yet their labeling with ^{65}Zn was markedly different. 18E6 (Fig. 2A, lane E) and 18E7 (Fig. 2A, lane F) labeled strongly, while TrpE (Fig. 2A, lane G) showed only a very low level of label that became visible only after longer exposures of the filter. As a control for the BPV-1 fusion proteins, B1E2 (Fig. 2A, lane A), a cII fusion protein containing the DNA-binding domain of BPV-1 E2 described previously (1) was tested. It showed no zinc labeling, even after longer exposures of the filter (Fig. 2B, lane A), while B1E6 (Fig. 2B, lane B) and B1E7 (Fig. 2B, lane C) labeled strongly. The level of zinc binding (Fig. 2B) to each protein has to be considered in relation to the amount

of protein present on the filter (Fig. 2A). In the case of HPV-18 E6 and E7 proteins, when the amounts of protein were similar (as in Fig. 2A, lane E versus G), E6 reproducibly bound more zinc per molecule than E7 did (Fig. 2B, lanes E and F, respectively). This might be expected if the cysteine motifs were involved in zinc binding, since E6 contains four such motifs and E7 contains two. Making this comparison requires the assumption, as yet unproven, that both proteins have renatured equally well under the conditions used and that they are equally affected by the TrpE portion of the fusion protein.

Since these papillomavirus proteins bind zinc only in their reduced state, we evaluated whether the binding was due simply to the availability of free cysteines or to a more specific characteristic of metalloproteins which coordinate zinc binding through cysteine residues. Bovine serum albumin (Fig. 2A, lane J), a protein which has 34 cysteines (all normally involved in disulfide bonds), was used as a negative control and bound negligible amounts of zinc under the conditions described above (Fig. 2B, lane J) (13). Three well-characterized zinc-binding metalloproteins were also tested: carbonic anhydrase (CA) (Fig. 2A, lane D; 30-kDa molecular size standard), which binds one zinc atom through three histidines (23); carboxypeptidase A (CpA) (Fig. 2A, lane H), which coordinates a single zinc atom through two histidines and a glutamate (its only two cysteines being involved in a disulfide bond) (23); and liver alcohol dehydrogenase (ADH) (Fig. 2A, lane I), which binds two zinc atoms per molecule, one being coordinated by two cysteines and a histidine and the other coordinated by four cysteines (23). As shown previously (15), we found that under nonreducing conditions CA bound zinc strongly and CpA bound a small

FIG. 2. Identification of zinc-binding proteins. (A) Amido black stain of Western blot. The positions of the papillomavirus fusion proteins on the filter are indicated on the left. The positions of CpA, CA, ADH and bovine serum albumin (BSA) are indicated on the right. Lanes: A, B1E2 fusion protein; B, B1E6 fusion protein; C, B1E7 fusion protein; D, molecular size markers of (from the top) 94, 67, 43, 30, 20.1, and 14.4 kDa; E, 18E6 fusion protein; F, 18E7 fusion protein; G, TrpE unfused protein; H, CpA; I, ADH; J, bovine serum albumin. (B) Autoradiogram of the Western blot shown in panel A after renaturation and labeling with $^{65}\text{ZnCl}_2$, as described in the text. The positions of the proteins positive for ^{65}Zn binding are indicated on the left for papillomaviruses and on the right for ADH.

amount, while ADH did not bind at all (data not shown). However, under reducing conditions the results were reversed: CA (Fig. 2B, lane D; 30-kDa molecular size marker) and CpA (Fig. 2B, lane H) bound no zinc, while ADH (Fig. 2B, lane I) was efficiently labeled by $^{65}\text{ZnCl}_2$. We conclude that the conditions described above may specifically allow the detection of zinc-binding proteins that coordinate binding of the metal atom through cysteine residues. Taken together, these results suggest that HPV-18 and BPV-1 E6 and E7 are metalloproteins and that metal coordination may involve the cysteine motifs.

The specificity for zinc was addressed by blocking experiments in which the zinc binding and washing steps were

FIG. 3. Competition of ^{65}Zn binding. Identical amounts of protein were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon filters. Lanes: A, 18E6; B, 18E7; C, molecular size markers of (from the top) 94, 67, 43, 30, 20.1, and 14.4 kDa; D, B1E6; E, B1E7. After renaturation of blotted proteins as described for Fig. 2, labeling with $10 \mu\text{M}$ $^{65}\text{ZnCl}_2$ was carried out in the presence of competing metals (10 mM MgCl_2 , 10 mM CaCl_2 , 0.1 mM CdCl_2 , or 0.1 mM ZnCl_2). Positions of the fusion proteins are indicated on the left, and that of bovine α -lactalbumin (α lact.) is indicated on the right.

done in the presence of different amounts of competing divalent metal chlorides (Fig. 3). We found that 0.1 mM zinc (Fig. 3, 0.1 mM ZnCl_2) or cadmium (Fig. 3, 0.1 mM CdCl_2) was sufficient to completely block binding of $10 \mu\text{M}$ ^{65}Zn to both the E6 and E7 proteins of HPV-18 and BPV-1 (Fig. 3, lanes A, B, D, and E, respectively). It has been previously shown that Cd efficiently substitutes for Zn in zinc-binding metalloproteins (23), and in some cases it binds more strongly than Zn itself (10). However, when labeling was done in the presence of 10 mM calcium (Fig. 3, 10 mM CaCl_2) or magnesium (Fig. 3, 10 mM MgCl_2), ^{65}Zn still efficiently labeled the papillomavirus proteins. In contrast, the calcium-binding protein α -lactalbumin, which has also been shown to bind other metals, was efficiently blocked by the competing CaCl_2 (Fig. 3, lane C) (9).

Two functions that can be mediated by zinc in eucaryotic cells are nucleic acid binding (5) and dimerization (10). As is true of the sequence-specific DNA-binding proteins with zinc fingers (5), E6 and E7 contain Cys-X-X-Cys repeats and bind zinc. However, they lack the two histidines, a leucine, and two aromatic amino acids positioned in the conserved fashion described for zinc fingers. Therefore, papillomavirus polypeptides E6 and E7 should probably not be considered true zinc finger proteins. We have assessed the DNA-binding activities of E6 and E7 and found that although these proteins show avidity for DNA (as determined by the McKay DNA immunoprecipitation assay [1]), they do not show sequence-specific binding to their respective genomes (M. S. Barbosa and J. T. Schiller, unpublished observations). In the absence of specificity, the biological significance of this binding activity remains uncertain. A role for zinc in protein dimerization was recently described for the human immunodeficiency virus Tat protein (10). Frankel et al. found that Tat formed dimers in the presence of zinc or cadmium and proposed a model by which the metal atom linked the monomers together by coordinating two cysteines in each molecule (10). Although at present we do not know whether this occurs with either of the papillomavirus proteins, E7 of HPV-16 (21) and cottontail rabbit papillomavirus (3) have been found to sediment faster than expected in

nondenaturing gradients, suggesting that they may form complexes.

The E6 and E7 requirement for reducing conditions suggests that coordination of the metal atoms could be mediated by the Cys-X-X-Cys motifs. Recently, adenovirus E1a protein was shown to bind zinc through a peptide containing a Cys-X₂-Cys-X₁₃-Cys-X₂-Cys (8). Interestingly, both E1a and the HPV-16 E7 proteins have also been found to transactivate the adenovirus E2 promoter and share a region of limited homology upstream of the Cys-X-X-Cys motifs (14). Although these similarities between E7 and E1a may suggest a similar mechanisms of action, a potential difficulty with this hypothesis is data indicating that E7 is a cytoplasmic protein (21) while E1a is a nuclear protein (18). Therefore, more studies are required for a better understanding of the basis of their similar activities.

In this report we have demonstrated that a modification of a previously described assay for zinc-binding proteins allows the detection of a class of metalloproteins that bind zinc only under reducing conditions. Using this assay, we have shown that under stringent conditions the papillomavirus E6 and E7 proteins bind zinc. Given the reducing environment in eucaryotic cells and the presence of zinc, we would predict that papillomavirus E6 and E7 proteins bind zinc in vivo. Although information on the exact coordination and biological significance of zinc binding awaits future structural and genetic studies, our results indicate that the metal content and redox potential of solutions should be considered when the biochemical properties of these two proteins are being evaluated.

We thank Claude Klee for helpful discussions.

Miguel S. Barbosa is a National Institutes of Health fellow (IRTA grant 5T32CA09030).

LITERATURE CITED

1. Androphy, E. J., D. R. Lowy, and J. T. Schiller. 1987. Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. *Nature (London)* **325**:70-73.
2. Androphy, E. J., J. T. Schiller, and D. R. Lowy. 1985. Identification of the protein encoded by the E6 transforming gene of bovine papillomavirus. *Science* **230**:442-445.
3. Barbosa, M. S., and F. O. Wettstein. 1988. Identification and characterization of the CRPV E7 protein expressed in COS-7 cells. *Virology* **165**:134-140.
4. Bedell, M. A., K. H. Jones, and L. A. Laimins. 1987. The E6-E7 region of human papillomavirus type 18 is sufficient for transformation of NIH 3T3 and Rat-1 cells. *J. Virol.* **61**:3635-3640.
5. Berg, J. 1986. Potential metal-binding domains in nucleic acid binding proteins. *Science* **232**:485-487.
6. Berg, J. 1988. Proposed structure for the zinc-binding domains from transcription factor IIIA and related proteins. *Proc. Natl. Acad. Sci. USA* **85**:99-102.
7. Cole, S. T., and O. Danos. 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome: phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. *J. Mol. Biol.* **193**:599-608.
8. Culp, J. F., L. C. Webster, D. J. Friedman, C. L. Smith, W.-J. Huang, F. Y.-H. Wu, M. Rosenberg, and R. P. Ricciardi. 1988. The 289-amino acid E1a protein of adenovirus binds zinc in a region that is important for trans-activation. *Proc. Natl. Acad. Sci. USA* **85**:6450-6454.
9. Desmet, J., I. Hanssens, and F. van Cauwelaert. 1987. Comparison of the binding of Na⁺ and Ca²⁺ to bovine alpha-lactalbumin. *Biochim. Biophys. Acta* **912**:211-219.
10. Frankel, A. D., D. S. Bredt, and C. Pabo. 1988. Tat protein from human immunodeficiency virus forms a metal-linked dimer. *Science* **240**:70-83.
11. Kanda, T., A. Furuno, and K. Yoshike. 1988. Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. *J. Virol.* **62**:610-613.
12. Matlashewski, G., J. Schneider, L. Banks, N. Jones, A. Murray, and L. Crawford. 1987. Human papillomavirus type 16 DNA cooperates with activated *ras* in transforming primary cells. *EMBO J.* **6**:1741-1746.
13. Peters, T., Jr. 1977. Serum albumin: recent progress in understanding of its structure and biosynthesis. *Clin. Chem.* **23**:5-12.
14. Phelps, W. C., C. L. Lee, K. Munger, and P. Howley. 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1a. *Cell* **53**:539-547.
15. Schiff, L. A., M. L. Nibert, and B. N. Fields. 1988. Characterization of a zinc blotting technique: evidence that a retroviral gag protein binds zinc. *Proc. Natl. Acad. Sci. USA* **85**:4195-4199.
16. Schiller, J. T., W. C. Vass, and D. R. Lowy. 1984. Identification of a second transforming region in bovine papillomavirus DNA. *Proc. Natl. Acad. Sci. USA* **81**:7880-7884.
17. Schlegel, R., W. C. Phelps, Y. Zhang, and M. S. Barbosa. 1988. Quantitative keratinocyte assay identifies papillomavirus types associated with human cervical carcinoma and detects two distinct viral activities. *EMBO J.* **7**:3181-3187.
18. Schmitt, R. C., M. L. Fahnestock, and J. B. Lewis. 1987. Differential nuclear localization of the major adenovirus type 2 E1a proteins. *J. Virol.* **61**:247-255.
19. Schwarz, E., U. K. Freese, L. Gissman, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen. 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature (London)* **314**:111-114.
20. Smotkin, D., and F. O. Wettstein. 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. *Proc. Natl. Acad. Sci. USA* **83**:4680-4684.
21. Smotkin, D., and F. O. Wettstein. 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. *J. Virol.* **61**:1686-1689.
22. Spindler, K. R., D. S. E. Rosser, and A. J. Berk. 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. *J. Virol.* **49**:132-141.
23. Vallee, B. L., and A. Galdes. 1984. The metallobiochemistry of zinc enzymes. *Adv. Enzymol.* **56**:283-430.
24. Vousden, K. H., J. Doniger, J. A. DiPaolo, and D. R. Lowy. 1988. The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. *Oncogene Res.* **3**:1-9.
25. Yanofski, C., T. Platt, I. P. Crawford, B. P. Nichols, G. E. Christie, H. Horowitz, M. VanCleave, and A. M. Wu. 1981. The complete nucleotide sequence of the tryptophan operon of *Escherichia coli*. *Nucleic Acids Res.* **9**:6647-6668.