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Biochemistry

Conserved cysteine residue in the DNA-binding domain of the bovine papillomavirus type 1 E2 protein confers redox regulation of the DNA-binding activity *in vitro*

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ABSTRACT **The bovine papillomavirus type 1 E2 open reading frame encodes three proteins involved in viral DNA replication and transcriptional regulation. These polypeptides share a carboxyl-terminal domain with a specific DNA-binding activity; through this domain the E2 polypeptides form dimers. In this study, we demonstrate the inhibition of E2 DNA binding *in vitro* by reagents that oxidize or otherwise chemically modify the free sulfydryl groups of reactive cysteine residues. However, these reagents had no effect on DNA-binding activity when the E2 polypeptide was first bound to DNA, suggesting that the free sulfydryl group(s) may be protected by DNA binding. Sensitivity to sulfydryl modification was mapped to a cysteine residue at position 340 in the E2 DNA-binding domain, an amino acid that is highly conserved among the E2 proteins of different papillomaviruses. Replacement of this residue with other amino acids abrogated the sensitivity to oxidation–reduction changes but did not affect the DNA-binding property of the E2 protein. These results suggest that papillomavirus DNA replication and transcriptional regulation could be modulated through the E2 proteins by changes in the intracellular redox environment. Furthermore, a motif consisting of a reactive cysteine residue carboxyl-terminal to a lysine residue in a basic region of the DNA-binding domain is a feature common to a number of transcriptional regulatory proteins that, like E2, are subject to redox regulation. Thus, posttranslational regulation of the activity of these proteins by the intracellular redox environment may be a general phenomenon.**

There are several ways in which the activity of regulatory proteins can be modulated posttranslationally. Recently, an unusual mechanism of regulation of the nucleic acid-binding properties of several proteins has been identified. Changes in the redox state of these polypeptides *in vitro* affects their ability to bind to RNA or DNA and leads to the hypothesis that oxidation–reduction could be an important regulatory mechanism *in vivo* (1–4). In this study we demonstrate that the DNA-binding activity of the bovine papillomavirus type 1 (BPV-1) E2 polypeptide is also regulated by oxidation–reduction *in vitro*.

The papillomaviruses are small DNA viruses that induce squamous epithelial and fibroepithelial lesions in their natural hosts. Proteins encoded by the *E2* open reading frame (ORF) of the viruses regulate viral gene expression and are required for viral DNA replication (reviewed in ref. 5). In BPV-1 the *E2* ORF encodes three proteins: a trans-activator that activates transcription from several viral promoters and two smaller repressor proteins that antagonize the function of the trans-activator (6–9). All three polypeptides share a C-terminal domain with a sequence-specific DNA-binding activity (10–13); through this domain the E2 polypeptides form dimers (14–16). The trans-activator contains an additional domain at the N terminus that is required for trans-activation (14, 17, 18). The trans-activator is also required in addition to the viral E1 polypeptide for viral DNA replication (19). In this study we demonstrate that the *in vitro* DNA-binding activity of the E2 polypeptides is regulated by oxidation–reduction of a cysteinyl residue located in the DNA-binding domain.

MATERIALS AND METHODS

Plasmid Constructions. Plasmids pTZE2$_{162-410}$, pTZE2$_{290-410}$, and pTZE2$_{310-410}$ have been described (12). Point mutations were generated in the background of pTZE2$_{290-410}$ by inserting synthetic oligonucleotides between the *Sty* I (nucleotide 3535) and *Pfl*MI (nucleotide 3683) sites to generate pTZE2$_{290-410}$S340, -G340, -A340, -S327, -S356, -S327/S356, and -S327/S340/S356 in which the substituted residue is shown in single-letter code—e.g., Ser-340, Gly-340, or Ala-340 is substituted for Cys-340.

***In Vitro* Transcription and Translation.** Capped RNA was synthesized from the pTZ plasmids by T7 RNA polymerase and translated in a rabbit reticulocyte lysate (Promega) as described (12).

Electrophoretic DNA Binding Assays. Gel retardation analysis was carried out as described (14). Two different double-stranded oligonucleotides, each containing a single E2 protein–DNA binding site (italic letters), were used as probes: am3–4 (5′-GGTCAA*ACCGTCTTCGGT*GCTCGA-3′) and am66–67 (5′-TCGA*ACCGAAAACGGT*GTCGA-3′).

Treatment of Lysates with Sulfydryl-Modifying Reagents. Diamide, *N*-ethylmaleimide (NEM), and copper *o*-phenanthroline were added directly to the DNA–protein binding reactions either before or after the addition of the DNA probe, as described in the text. Rabbit reticulocyte lysates containing *in vitro* translated E2 proteins were dialyzed against buffer D by using a Pierce system 100 microdialyzer.

Determination of DNA-Binding Affinity. DNA-binding assays were carried out as described above with equivalent amounts of wild-type or mutated E2 proteins. Various concentrations (4.6 pM to 10 nM) of the labeled oligonucleotide am66–67 were used as probes. The amount of bound DNA was determined with a radioanalytic imaging system (AMBIS Systems, San Diego).

RESULTS

E2–DNA Binding is Inactivated by *N*-Ethylmaleimide. To investigate whether the modification of free sulfydryl groups alters the interaction of the E2 protein with its DNA binding site, *in vitro* translated E2 proteins were treated with the reagent NEM, which alkylates free sulfydryl groups. Reticulocyte lysates containing a ^{35}S-labeled C-terminal E2 DNA-

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Abbreviations: BPV, bovine papillomavirus; ORF, open reading frame; NEM, *N*-ethylmaleimide.

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binding domain, E2-(290–410) polypeptide, were treated with various amounts of NEM for 10 min, and the reactions were stopped by the addition of 10 mM cysteine. The modified polypeptide was assayed for DNA binding by a gel electrophoresis mobility-shift assay as described (14). An unlabeled 24-base-pair (bp) oligonucleotide containing a single E2-binding site was used as a probe, and the DNA–protein complex was detected on nondenaturing polyacrylamide gels by virtue of the ^{35}S-labeled E2 protein. E2 DNA-binding activity was almost completely abolished after the addition of 1 mM NEM (Fig. 1 *Upper*, lanes 6–8). The addition of 10 mM cysteine to the lysate before NEM prevented inactivation of E2 DNA-binding activity at the lower concentrations of NEM (Fig. 1 *Upper*, lanes 3–5). When the E2 protein was bound to DNA before the addition of NEM, there was little effect on binding activity, and a small decrease in binding was only observed at high concentrations of NEM (Fig. 1 *Upper*, lanes 9–11). This could indicate that the free sulfydryl group(s) is in close proximity to the DNA and therefore protected from alkylation by NEM.

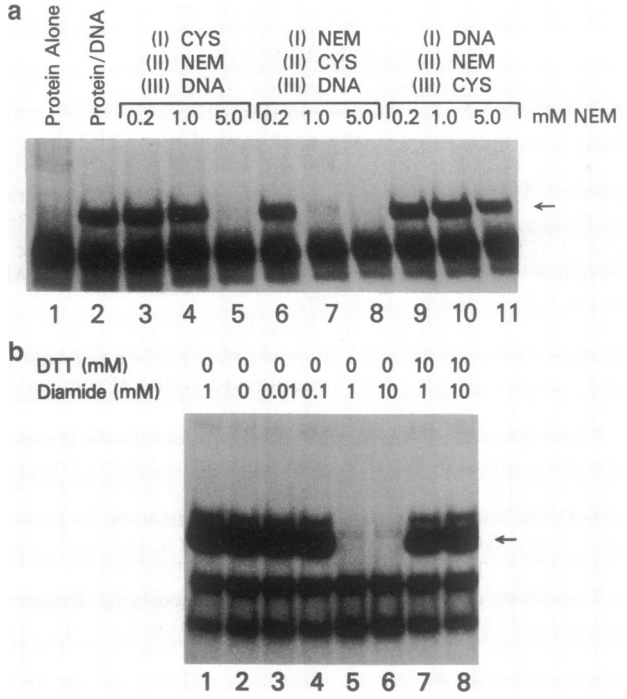

FIG. 1. (*Upper*) Inactivation of E2–DNA binding by NEM. *In vitro* translated ^{35}S-labeled E2-(290–410) protein was incubated with a double-stranded oligonucleotide (am3–4) containing a single E2 protein–DNA binding motif and was analyzed on a native gel (no NEM treatment) (lane 2). Other lanes: 1, protein without oligonucleotide incubation or NEM treatment; 6–8, modification of protein with 0.2 mM (lane 6), 1.0 mM (lane 7), or 5.0 mM (lane 8) NEM and termination of the reaction by the addition of 10 mM cysteine before incubation with oligonucleotide; 3–5, addition of 10 mM cysteine before treatment with NEM; 9–11, incubation of protein with the oligonucleotide before treatment with NEM and termination of the reaction with 10 mM cysteine. The roman numerals show the order of addition of reagents. The arrow indicates the position of the specific E2 protein–DNA complex. (*Lower*) Inactivation of E2–DNA binding by diamide. *In vitro* translated E2-(290–410) protein was incubated with a ^{32}P-labeled double-stranded oligonucleotide (am3–4) containing a single E2 protein–DNA binding motif and was analyzed on a native gel (lane 2). Other lanes: 3–8, incubation of protein with diamide before the DNA binding reaction; 7 and 8, reduction of proteins with dithiothreitol (DTT) after diamide treatment and before incubation with the oligonucleotide; 1, incubation of E2-(290–410) with the oligonucleotide before treatment with 1 mM diamide.

E2 DNA-Binding Activity is Reversibly Inactivated by Oxidizing Reagents. The E2 polypeptide was treated with reagents that oxidize free sulfydryl groups and reagents that reduce oxidized sulfydryl groups. Diamide [1,1′-azobis(*N,N*-dimethylformamide)] can chemically catalyze the oxidation of free sulfydryl groups (20). Treatment of the *in vitro* translated protein E2-(290–410) with diamide also inactivated the DNA-binding function. E2 DNA binding was inactivated by 1 mM diamide (Fig. 1 *Lower*, lane 5). After diamide treatment, binding activity could be fully recovered by the addition of dithiothreitol. Similar results were obtained by treating the E2 polypeptide-containing lysate with copper *o*-phenanthroline, which also can catalyze the oxidation of free sulfydryl groups, but by a different mechanism (21). E2 DNA-binding activity was abolished by pretreatment with 400 μM copper *o*-phenanthroline, and activity could be recovered by treatment with dithiothreitol (see Fig. 5). In both cases, when the E2 protein was bound to DNA prior to treatment with either diamide or copper *o*-phenanthroline, no inactivation of DNA binding was observed (Fig. 1 *Lower*, lane 1; also see Fig. 5 *Bottom*, lane 13). This confirmed the results obtained with NEM and suggested that a free sulfydryl group might be located in or near the DNA-binding site of the protein and demonstrated that modification of this residue abolished DNA-binding activity.

Identification of the Cysteine Residue That Is Sensitive to Oxidation or Modification. The BPV-1 E2 DNA-binding domain contains three cysteine residues (Cys-327, Cys-340, and Cys-356), one of which (Cys-340) is highly conserved among almost all of the papillomavirus E2 proteins sequenced so far [Fig. 2 (see also Fig. 6)]. To determine which of the three residues was responsible for the sensitivity of the DNA-binding property of the E2 protein to oxidizing agents, each of the cysteine residues was replaced with a serine residue (Fig. 2). *In vitro* translated E2-(290–410) polypeptides containing one or more of these amino acid changes were assayed for their ability to bind DNA and for their sensitivity to diamide. All mutated proteins were still able to bind to the E2 protein–DNA binding sites (Fig. 3). However, only the Cys-340 → Ser change gave rise to a protein, [Ser^{340}]E2-(290–410), whose DNA binding was no longer sensitive to inactivation by diamide. As noted above, Cys-340 is well-conserved among the papillomavirus E2 proteins; it is located in the conserved basic region of the polypeptide that is likely to interact with DNA (ref. 22; A.A.M., unpublished data).

Cys-340 was also replaced with glycine and alanine residues. Like [Ser^{340}]E2-(290–410) protein, [Gly^{340}]E2-(290–410) and [Ala^{340}]E2-(290–410) proteins were also able to bind DNA, and binding was no longer sensitive to sulfydryl modification. The relative binding affinity of each of the proteins mutated at position 340 was determined in comparison with wild-type E2-(290–410). All three mutated proteins bound DNA with an affinity less than that of the wild-type protein (data not shown). However, the affinity of [Gly^{340}]E2-

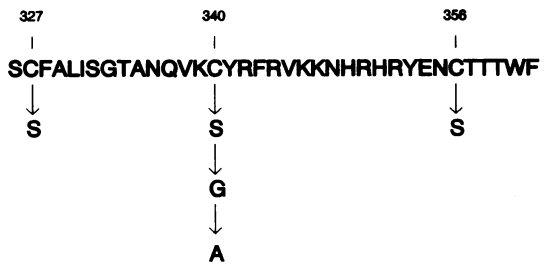

FIG. 2. The amino acid sequence of the region of the BPV-1 E2 DNA-binding domain that contains cysteine residues at positions 327, 340, and 356. Site-specific substitutions were generated at each position as shown.

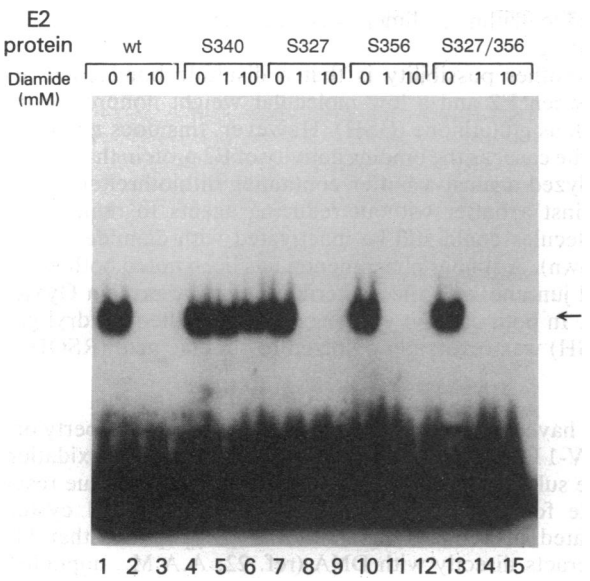

FIG. 3. Diamide treatment of mutated E2 proteins. *In vitro* translated E2-(290–410) proteins containing the indicated amino acid substitutions (single-letter code) were incubated with a ^{32}P-labeled double-stranded oligonucleotide (am66–67) containing a single E2 protein–DNA binding motif and was analyzed on a native gel (lanes 1, 4, 7, 10, and 13). Proteins in lanes 2, 5, 8, 11, and 14 were treated with 1 mM diamide and those in lanes 3, 6, 9, 12, and 15 were treated with 10 mM diamide before incubation with the oligonucleotide. wt, wild type.

(290–410) was decreased <50% as compared with wild type. [Ser^{340}]E2-(290–410) bound ≈50% less than wild type did, and the affinity of [Ala^{340}]E2-(290–410) was substantially decreased (at least 90%).

E2 dimers form during or shortly after protein synthesis and exist as stable dimers in solution (14). In the experiment shown in Fig. 4 *Upper*, the mutated E2-(290–410) proteins were cotranslated with a wild-type version of a smaller E2 protein, E2-(310–410). These protein lysates gave rise to three DNA–protein complexes on a gel-shift assay: the largest complex is a homodimer of E2-(290–410), the smallest is a homodimer of E2-(310–410), and the intermediate-size complex is a heterodimer of both E2 polypeptides (14). The DNA binding of the smaller wild-type protein served as an internal control and was inactivated by 5 mM diamide, while homodimers of proteins containing the alanine and glycine substitutions were resistant to this reagent. It also appeared that the heterodimers between the wild-type and mutated proteins were sensitive to diamide treatment. This was further examined by using heterodimers between wild-type E2 proteins and E2 proteins containing the serine substitutions for each of the three cysteine residues in the E2 DNA-binding domain. All of the heterodimers, including that containing the Ser-340-substituted polypeptide, appeared to be sensitive to oxidation (Fig. 4 *Lower*).

Does Diamide Treatment Result in the Formation of Intra- or Inter-molecular Disulfide Bonds? It seemed possible that diamide and copper *o*-phenanthroline were catalyzing the formation of a disulfide bond between two free sulfydryl groups in the E2 protein, thus inactivating the DNA-binding activity. These putative bonds could form intermolecularly between the Cys-340 residue in each subunit of the dimer or intramolecularly between Cys-340 and one of the other cysteine residues within the same subunit. The E2 protein dimer containing serine substitutions at positions 327 and 356 was sensitive to diamide, suggesting that a disulfide bond was not forming between these residues and Cys-340 (Figs. 3 and 4 *Lower*). However, the heterodimer between a wild-type E2

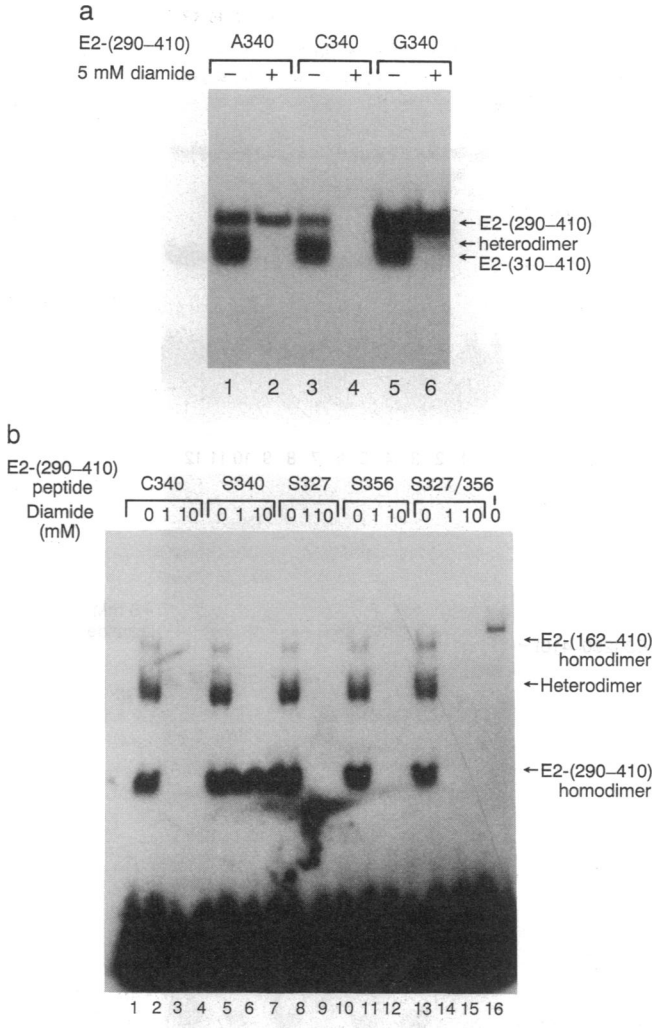

FIG. 4. Diamide treatment of mutated E2 proteins and heterodimers. (*Upper*) E2 heterodimers were generated by cotranslating polypeptides E2-(290–410) and E2-(310–410). In each case the smaller polypeptide was wild type and the larger was either wild type or contained amino acid substitutions, as indicated in single-letter code. Lanes: 1, 3, and 5, untreated protein lysates; 2, 4, and 6, proteins treated with 5 mM diamide. (*Lower*) E2 heterodimers were generated by cotranslating E2-(290–410) and E2-(162–410) polypeptides. In each case the larger polypeptide was wild type and the smaller was either wild type or contained amino acid substitutions as indicated in single-letter code. Lanes: 16, protein lysate without E2 proteins; 1, 4, 7, 10, and 13; untreated (lanes 0) protein lysates; 2, 5, 8, 11, 14, proteins treated with 1 mM diamide (lanes 1); 3, 6, 9, 12, 15, proteins treated with 10 mM diamide (lanes 10).

polypeptide and a polypeptide mutated at Cys-340 was also sensitive to oxidation, arguing against the formation of an intermolecular disulfide link between the Cys-340 residues in each subunit. To examine this further, heterodimers were generated between two differently sized E2 polypeptides, E2-(162–410) and E2-(290–410), containing serine substitutions for each of the cysteine residues. All E2 molecules containing a single cysteine residue, Cys-340, were sensitive to oxidation by either diamide or copper *o*-phenanthroline (Fig. 5). Even an E2 protein that contained three serine substitutions for Cys-327, -340, and -356 in one subunit and two serine substitutions for Cys-327 and -356 in the other subunit (Fig. 5, lane 11) was unable to bind DNA after treatment with these reagents. [The E2-(162–410) polypeptide contained an additional cysteine, Cys-242, but it seemed unlikely that it would be involved, as the shorter E2 proteins were sensitive to oxidation.] To further investigate the pres-

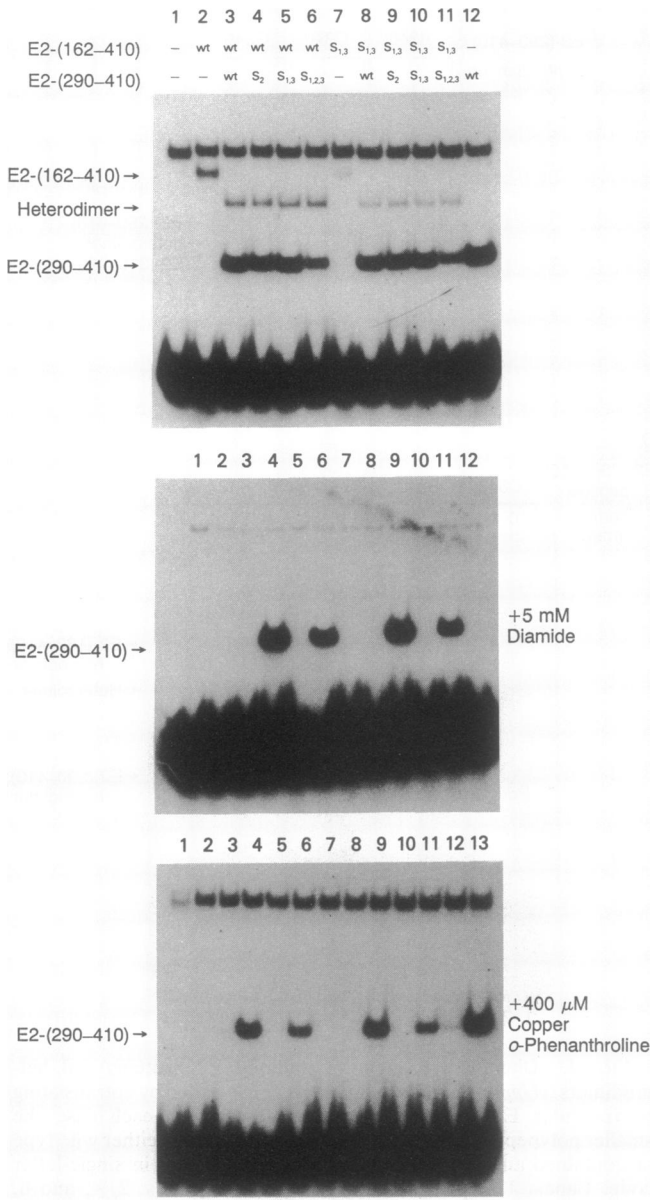

FIG. 5. Diamide and copper *o*-phenanthroline treatment of mutated E2 proteins and heterodimers. E2 heterodimers were generated by cotranslating polypeptides E2-(290–410) and E2-(162–410). In each case the larger E2-(162–410) polypeptide was wild type and E2-(290–410) was either wild type or contained amino acid substitutions as indicated. For ease of labeling, the serine substitutions at positions 327, 340, and 356 have been designated S_1, S_2, and S_3, respectively. As the E2-(290–410) polypeptide was in excess, most of the E2-(162–410) polypeptide has been sequestered into the heterodimer. (*Top*) Protein lysates were untreated. (*Middle*) Lysates were treated with 5 mM diamide. (*Bottom*) Lysates were treated with 400 μM copper *o*-phenanthroline. Assay for DNA binding used a ^{32}P-labeled am66–67 oligonucleotide. In lane 13 of *Bottom* the E2 proteins were incubated with DNA before treatment with copper *o*-phenanthroline.

ence of intermolecular disulfide bonds between the E2 subunits, *in vitro* translated E2-(290–410) was incubated with 50 mM diamide, immunoprecipitated, and analyzed by SDS/polyacrylamide gel electrophoresis under nonreducing conditions. A very small amount of covalently linked E2 dimer could be detected, and this was eliminated by the addition of reducing agents (data not shown). However, this small percentage of disulfide-linked protein was not sufficient to explain the complete inactivation of DNA binding by diamide. Thus, it appeared that oxidation of a single Cys-340 residue within a dimer was sufficient to inactivate DNA binding.

Another possibility is that a disulfide bond could form between E2 and a low molecular weight nonprotein thiol, such as glutathione (GSH). However, this does not seem to be the case, as the binding activity of E2 protein that had been dialyzed against a buffer containing dithiothreitol and then against a buffer without reducing agents to remove small molecules could still be inactivated with diamide (data not shown). A similar phenomenon has been noted both with fos and jun and with the bacterial regulatory protein OyxR (3, 23). In both cases it was suggested that the sulfydryl group (RSH) was reversibly oxidized to sulfenic acid (RSOH).

DISCUSSION

We have demonstrated that the DNA-binding property of the BPV-1 E2 proteins is sensitive to modification or oxidation of free sulfydryl groups. The sulfydryl-bearing residue responsible for this sensitivity is a highly conserved cysteine, located in a basic region of the polypeptide, that likely interacts directly with DNA (ref. 22; A.A.M., unpublished observations). The fact that E2 proteins already bound to DNA are no longer sensitive to oxidation further suggests that this polypeptide region contains the DNA-binding site.

The DNA-binding activity of the protooncogene products c-fos and c-jun and the related Epstein–Barr virus protein BZLF1 is also regulated by oxidation–reduction *in vitro*. The reactive cysteines have also been mapped to the basic regions of c-fos and c-jun, which interact directly with DNA (3, 4, 24) (Fig. 6). Notably, the amino acid sequences surrounding the reactive cysteines in c-fos and c-jun and the BPV-1 E2 protein are not dissimilar. The cysteines are situated in a region containing several positively charged amino acids, and in each case a lysine residue is located to the amino-terminal side of the cysteine. In addition, the cysteine residues and surrounding amino acid sequences are highly conserved

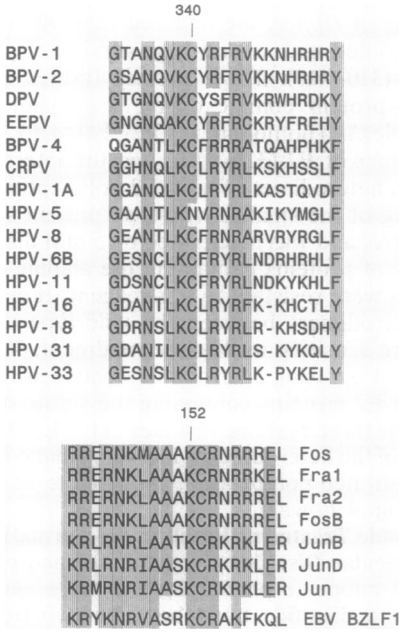

FIG. 6. (*Upper*) Sequence homology among several papillomavirus E2 proteins in the vicinity of BPV-1 Cys-340. Conserved residues and isofunctional substitutions are indicated by shading. The protein sequences in single-letter code were derived from papillomavirus DNA sequences as described in Baker and Cowsert (ref. 25 and references therein). (*Lower*) Cysteine residues responsible for sensitivity to oxidation in c-fos and c-jun (3) and sequence homology among fos- and jun-related cellular proteins and the fos-related Epstein–Barr virus protein BZLF1 (26).

among the papillomavirus E2 proteins and among the fos/jun family. It is likely that the local basic environment increases the reactivity of these cysteine residues, rendering them highly susceptible to oxidation and an ideal target for redox regulation (27).

It would be informative to identify the nature of the oxidation product that results in E2 inactivation. The fact that inactivation of DNA-binding activity can be reversed by reducing agents limits the possible candidates. By mutating all of the cysteine residues in the DNA-binding domain of the E2 protein, we have been unable to identify the formation of a disulfide bond within the E2 protein. Another possibility is that a disulfide bond could form between Cys-340 and a low molecular weight thiol molecule such as glutathione, but our data does not support this hypothesis. Alternatively, Cys-340 (RSH, where R is the residue) could be oxidized to the reversible oxidation product sulfenic (RSOH) acid. This possibility has been proposed for fos and jun and for the bacterial transcriptional regulatory protein OyxR which is activated by oxidation (3, 23). Cysteine sulfenic acid is very unstable in solution (28), but there is evidence for a stabilized cysteine sulfenic acid in the redox center of streptococcal NADH peroxidase (29). Presumably, such a modification in the E2 protein could alter its conformation or could directly interfere with the DNA–protein interaction. Notably, of the three amino acid substitutions for Cys-340 (glycine, alanine, and serine), the glycine-substituted protein had the highest affinity for DNA. It also has no side chain and therefore should offer the least steric hindrance. Therefore, it is feasible that oxidation of cysteine (RSH) to sulfenic acid (RSOH) would result in inactivation of DNA binding.

It is surprising that although Cys-340 is highly conserved, E2 proteins containing all three amino acid substitutions are still able to bind DNA, the glycine mutant having an affinity only slightly less than that of the wild-type protein. This further suggests that the conserved Cys-340 may have another role, perhaps as a target for regulation by a redox mechanism *in vivo*. To investigate this possibility we have studied the effect of the amino acid substitutions *in vivo* in the context of the full-length E2 trans-activator protein. The E2 proteins containing amino acid substitutions for Cys-340 are unable to activate expression from most enhancer/promoter configurations that are activated by the wild-type protein, and BPV-1 genomes encoding the mutated proteins are unable to transform cells (A.A.M., P.M.H., and T. Sarafi, unpublished observations). The E2 proteins containing Gly-340 can only activate transcription from a promoter with several adjacent E2 binding sites upstream and not from promoters dependent on BPV-1 enhancer elements. These results could mean that Cys-340 is important for some function in addition to DNA binding, such as the induction of a conformational change, interactions with other proteins, or synergy between E2 trans-activator molecules.

In support of a physiological role for redox regulation of c-fos and c-jun *in vivo*, it has been shown that a nuclear protein (Ref-1) copurifies with AP-1 proteins and is able to activate their DNA binding activity by reducing the reactive cysteine residues (3, 30). In addition, replacement of the reactive cysteines in c-fos and c-jun with serine residues enhances the transforming potential of these proteins (H. Iba, University of Tokyo and T. Curran, Roche Institute of Molecular Biology; P. Vogt and I. Morgan, University of Southern California School of Medicine, personal communications). This suggests that the oncogenic potential of fos and jun could be increased by deregulation of redox control.

For significant regulation to occur *in vivo*, the oxidized sulfydryl groups must be capable of existing in the highly reducing intracellular environment. This would depend on the oxidation equilibrium constant of the sulfydryl group, which is dependent on the local environment of the cysteine residue. It has been demonstrated for the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase that oxidized sulfydryl groups can exist in a buffer system in which the ratio of reduced glutathione to oxidized glutathione ([GSH]/[GSSG]) is very similar to that of the cytosol (31). It is also possible that the redox potential differs in localized subcellular areas and might change during the process of cellular differentiation. An attractive hypothesis based on the latter suggestion is that redox regulation could modulate the activity of the E2 polypeptides, which could in turn play a role in the activation of late viral functions within terminally differentiating keratinocytes.

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