JOURNAL OF VIROLOGY, June 1992, p. 3941–3945
0022-538X/92/063941-05$02.00/0

Vol. 66, No. 6

# Mechanism of Action of the Papillomavirus E2 Repressor: Repression in the Absence of DNA Binding

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Received 17 January 1992/Accepted 5 March 1992

**Repression of papillomavirus E2-dependent gene expression was studied by using transient transfections into mouse embryo fibroblast cells. Cotransfection of a gene corresponding to the naturally occurring repressor E2-TR along with the full-length E2 gene resulted in up to 98% repression of E2-dependent reporter gene expression. A series of E2 DNA-binding domain mutants were transferred into the E2-TR form and characterized for their ability to repress E2-dependent transactivation. All mutants which were defective for DNA binding but were dimerization competent repressed E2 transactivation as well or nearly as well as the wild-type repressor. E2 mutants which lacked dimerization activity repressed transactivation poorly or not at all. These results indicate that the E2 repressor can inhibit transcription, in the absence of DNA binding, by forming heterodimers with full-length E2.**

Papillomaviruses are a family of DNA viruses which cause benign and, in some cases, malignant epithelial tumors of the skin and mucosa. The complex transcriptional pattern of these viruses is orchestrated to a large extent by the products of the E2 open reading frame (9, 10, 13, 28, 32, 33). E2 protein binds as a preexisting homodimer to the sequence ACCN$_6$GGT (often ACCGN$_4$CGGT), which is present in multiple copies in all papillomavirus genomes (1, 11, 12, 19, 25). In bovine papillomavirus type 1 (BPV-1), the most extensively studied papillomavirus, the full-length 48-kDa E2 protein can increase the level of transcription initiation directed by BPV-1 promoters or heterologous promoters containing E2 binding sites by as much as 300-fold (11, 12, 21, 24, 32, 33). E2 is a multidomain protein with a large amino-terminal transactivation domain, a proline-rich "hinge" region, and a carboxy-terminal region required for sequence-specific DNA binding and dimer formation (6, 7, 20, 21, 23, 26). The carboxy-terminal 85 amino acids (residues 326 to 410) of BPV-1 E2 are sufficient for DNA binding (22, 23). This portion of E2 can form stable dimers in the absence of DNA (20, 27). The amino acids which mediate DNA recognition and dimerization have been resolved only recently (27).

In addition to the full-length transactivator, BPV-1 expresses two shorter forms of E2 which possess DNA-binding and dimerization activity but lack the amino-terminal transactivation domain (4, 14, 17, 18, 21). These are a 31-kDa protein known as E2-TR, whose RNA originates from a promoter within the open reading frame, and a 28-kDa E8-E2 fusion protein, generated through alternative splicing. Both contain a common carboxy-terminal 205 amino acids and, hence, the entire DNA-binding and dimerization domain. These shorter forms of E2 repress the transactivation activity of the full-length E2. It is likely that the regulation of papillomavirus gene expression, and possibly activation of latent virus, could depend on the balance between the E2 transactivator and repressors.

The mechanism by which the shorter forms of E2 repress E2-dependent transcription is not understood. The repressors may act by competitively binding to the ACCN$_6$GGT sequence and thus competing with the transactivator E2 for occupancy of these sites. Alternatively, the repressors could act by forming inactive heterodimers with full-length E2. In an attempt to resolve these two possible mechanisms, we used BPV-1 E2 mutants defective for DNA binding or dimerization (shown in Fig. 1). These mutants were generated by random mutagenesis and screening in *Saccharomyces cerevisiae* (27). In addition, some E2 mutants were generated by site-directed mutagenesis (27). The DNA-binding and dimerization activities of these mutants are summarized in the first four columns of Table 2 (see Prakash et al. [27] for experimental results). Although these mutants were generated in full-length E2, they were found to have identical DNA-binding and dimerization profiles in truncated forms (27) (data not shown).

With these mutant E2 proteins, it was determined previously that the DNA contact region includes amino acids 337 to 344. Dimerization is provided by the carboxy-terminal domain of the protein, with a tryptophan at position 360 mediating a critical hydrophobic contact between the two monomers (27). E2 mutants 316Y, 366Y/376L, 386W, 399I, 408*, and 411* were all capable of both DNA binding and dimerization. Mutants 337L, 339M, 340F, 340R, 340Y, and 344L did not bind DNA but could form dimers in the absence of DNA. Mutants 317STOP (truncated at position 317), 333V, 360N, 360S, and 374S/375L/391I could neither bind DNA nor dimerize efficiently. Since no DNA-binding-competent mutants were found which were dimerization defective, it is likely that dimer formation is required for DNA binding. This result is predictable for a protein which binds to a palindromic DNA sequence.

A transient-transfection repression assay was used to test the repression activity of these mutants. BALB/c 3T3 mouse embryo fibroblast cells were transfected by electroporation with an E2-dependent reporter plasmid in the presence and absence of an E2 expression vector. Repression activity was determined by cotransfection of an E2 repressor along with the E2 transactivator. In the E2-dependent reporter plasmid pC515-9 (12), expression of the chloramphenicol acetyltrans-

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| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PRO** | **VAL** | **ASP** | **LEU** | **ALA** | **SER** | **ARG** | **GLN** | **GLU** | **GLU** | **GLU** | **GLU** |
| 285 | | | | | | | | | | | |
| **GLN** | **SER** | **PRO** | **ASP** | **SER** | **THR** | **GLU** | **GLU** | **GLU** | **PRO** | **VAL** | **THR** |
| | | | | | | | **tyr** | **stop** | | | |
| **LEU** | **PRO** | **ARG** | **ARG** | **THR** | **THR** | **ASN** | **ASP** | **GLY** | **PHE** | **HIS** | **LEU** |
| | | | | | | | 316 | 317 | | | |
| **minimal DNA binding and dimerization region** | | | | | | | | | | | |
| **LEU** | **LYS** | **ALA** | **GLY** | **GLY** | **SER** | **CYS** | **PHE** | **ALA** | **LEU** | **ILE** | **SER** |
| **val** | | | | **leu** | | **met** | **arg,phe,tyr** | | | | **leu** |
| **GLY** | **THR** | **ALA** | **ASN** | **GLN** | **VAL** | **LYS** | **CYS** | **TYR** | **ARG** | **PHE** | **ARG** |
| 333 | | | | 337 | | 339 | 340 | | | | 344 |
| **VAL** | **LYS** | **LYS** | **ASN** | **HIS** | **ARG** | **HIS** | **ARG** | **TYR** | **GLU** | **ASN** | **CYS** |
| | | | **ser,asn** | | | | | | | **tyr** | |
| **THR** | **THR** | **THR** | **TRP** | **PHE** | **THR** | **VAL** | **ALA** | **ASP** | **ASN** | **GLY** | **ALA** |
| | | | 360 | | | | | | | 366 | |
| | | | | | **ser** | **leu** | **leu** | | | | |
| **GLU** | **ARG** | **GLN** | **GLY** | **GLN** | **ALA** | **GLN** | **ILE** | **LEU** | **ILE** | **THR** | **PHE** |
| | | | | | 374 | 375 | 376 | | | | |
| | | | | | **trp** | | | | | **ile** | |
| **GLY** | **SER** | **PRO** | **SER** | **GLN** | **ARG** | **GLN** | **ASP** | **PHE** | **LEU** | **LYS** | **HIS** |
| | | | | | 386 | | | | | 391 | |
| | | | | | | **ile** | | | | | |
| **VAL** | **PRO** | **LEU** | **PRO** | **PRO** | **GLY** | **MET** | **ASN** | **ILE** | **SER** | **GLY** | **PHE** |
| | | | | | | 399 | | | | | |
| | | | **11 new a.a.** | | | **leu + 22 a.a.** | | | | | |
| **THR** | **ALA** | **SER** | **LEU** | **ASP** | **PHE** | **END** | | | | | |
| | | | 408 | | 410 | 411 | | | | | |

FIG. 1. BPV-1 E2 DNA-binding domain mutants. The amino acid (a.a.) sequence of the carboxy-terminal 126 residues of BPV-1 E2 is shown in the three-letter code. Mutants generated by chemical mutagenesis and screening and by site-directed mutagenesis are indicated in boldface above the altered amino acids. The numbers below each mutant position connote their position in the full-length E2 protein.

ferase (CAT) gene was directed by a truncated simian virus 40 (SV40) early promoter having three upstream E2-binding sites. In pXB332hGH, the CAT gene of pC515-9 was replaced with the human growth hormone (hGH) gene, which was removed as an *Hin*dIII-*Bam*HI fragment from pOGH (Allegro hGH gene expression kit; Nichols Institute, San Juan Capistrano, Calif.). Expression of the BPV-1 E2 transactivator, repressor, and repressor mutants was directed by the chicken β-actin promoter with the vector pXB101. pXB101 was constructed by replacing the SV40 enhancer-adenovirus major late promoter of pBG312 (3) with the chicken β-actin promoter (16, 29) so that the actin promoter was followed by a polylinker for insertion of the gene to be expressed. SV40 splice and polyadenylation signals were downstream of the polylinker. pXB101 was used in some transfections as an control plasmid which lacked an insert, in order to control for transcriptional inhibition due to an excess of transfected promoter DNA. The native full-length BPV-1 E2 transactivator was inserted into pXB101 as a 1,866-bp *Bam*HI fragment isolated from pCO-E2 (12) to create pXB323. The wild-type sequence E2 repressor was isolated as a 1,378-bp *Bam*HI fragment from pYE2-R (24) and inserted into pXB101 to create pXB314. This fragment encoded the carboxy-terminal 249 amino acids of E2 (residues 162 to 410), which corresponds to the naturally occurring repressor E2-TR. DNA-binding domain mutants were transferred as 429-bp *Kpn*I-*Bst*XI fragments inserted into

TABLE 1. Repression of E2-dependent hGH expression by wild-type and mutant E2 repressors[a]

| Transfection | | hGH[b] (ng/ $10^6$ cells) | Fold induction | % Repression[c] |
|---|---|---|---|---|
| Transactivator | Repressor | | | |
| None | None | 0.8 | | |
| E2 | None | 49.7 | 62.1 | |
| E2 | Wild type | 2.1 | 2.6 | 97.4 |
| E2 | 337L | 4.3 | 5.4 | 92.8 |
| E2 | 340R | 2.2 | 2.8 | 97.0 |
| E2 | 344L | 8.4 | 10.5 | 84.4 |
| E2 | 360S | 39.6 | 49.5 | 20.6 |

[a] In each case, the E2-dependent reporter pXB332hGH was transfected along with the transactivators and repressors shown at a fourfold excess of repressor to transactivator DNA. Transfections in which the repressor is listed as "none" included the insert-lacking vector pXB101 at a fourfold DNA excess over transactivator DNA.

[b] The value (counts per minute) from a control standard without hGH was subtracted prior to computation of the hGH concentration.

[c] Computed as described in the text.

*Kpn*I- and *Bst*XI-cleaved vector pXB314 (replacing the wild-type sequence). These vectors were designated by the letters pEC and the residue number and type of amino acid in the single-letter code. This cloning strategy was also used to insert some mutant sequences into the full-length E2 form. These were named pE2, followed by the mutation designation.

3T3 cells were transfected by a transient-electroporation protocol similar to that of Chu et al. (5). E2 repressor DNA was present in electroporations at a fourfold excess over the E2 transactivator DNA. When no repressor was present, pXB101 vector DNA was present at a fourfold DNA excess over E2 DNA.

Assays were performed 48 to 72 h after electroporation, with culture medium changed 24 h before the assay. In experiments in which the reporter pC515-9 was used, cell extracts were analyzed for CAT activity by the procedure of Gorman et al. (8) with equal amounts of total protein in each point. When the reporter pXB332hGH was used, culture medium was assayed for the presence of hGH by the method of Seldon (30) with the Allegro hGH transient gene expression kit (Nichols Institute). In the case of hGH assays, 3T3 cell numbers were determined at the time of medium harvest, and hGH levels were computed on a per-cell basis. Induction levels were determined as the increase in reporter protein production in the presence of the E2 plasmid. Repression levels were determined as the decrease in E2-dependent reporter expression in the presence of the repressor relative to that in transfections lacking repressor DNA. The percent repression was determined by the formula below, in which background equals reporter expression in the absence of E2 and E2 repressor, $T$ is reporter expression in the presence of the E2 transactivator alone, and $R$ is reporter production in the presence of both E2 and the E2 repressor: percent repression = $[1 - (R - \text{background})/(T - \text{background})] \times 100$.

The level of transactivation by E2 in these experiments was 30- to 70-fold. Table 1 shows the results of one experiment in which four mutant E2 repressors were compared with the wild-type repressor. The level of inhibition by the wild-type repressor, 97% in this experiment, was surprisingly high. In all experiments, the transfection of a fourfold excess of this repressor DNA to transactivator DNA led to an 85 to 98% decrease in E2-dependent reporter expression. Control transfections in which a fourfold excess of insert-

TABLE 2. Summary of mutant E2 repressor activity[a]

| Mutant | Mutation | DNA binding | Dimerization[b] | Repression as % decrease in transactivation by E2 | Repression as % of wild type E2R repression[c] |
|---|---|---|---|---|---|
| 316Y | D316→Y | + | + | 8.0 | 9.5 |
| 317STOP | G317→stop | − | − | 7.1 | 8.1 |
| 333V | G333→V | − | − | 0[d] | |
| 337L | Q337→L | − | + | 90.4 | 93.7 |
| 339M | K339→M | − | + | 76.2 | 78.3 |
| 340F | C340→F | − | + | 86.1 | 95.1 |
| 340R | C340→R | − | + | 95.6 | 99.9 |
| 340Y | C340→Y | − | + | 93.5 | 105.1 |
| 344L | R344→L | − | + | 82.6 | 91.8 |
| 360N | W360→N | −[e] | − | 11.8 | 12.6 |
| 360S | W360→S | −[e] | − | 32.3 | 33.1 |
| 366Y/376L | N366→Y, I376→L | + | + | 31.8 | 35.8 |
| 374S/375L/391I | A374→S, Q375→L, K391→I | Reduced | Reduced | 39.0 | 44.4 |
| 386W | R386→W | + | + | 81.4 | 84.6 |
| 399I | M399→I | + | + | 86.9 | 90.8 |
| 408* | 11-amino-acid insert | + | + | 59.5 | 63.5 |
| 411* | 23-amino-acid addition | + | + | 37.2 | 42.9 |

[a] All values represent the average of three to five assays. Results obtained with CAT and hGH reporters were incorporated here.
[b] Dimerization as assayed by UV cross-linking reaction (27).
[c] Each mutant repressor was compared with the wild-type repressor in the same assay.
[d] 0 indicates that activation was slightly greater in the presence of this mutant than in the controls.
[e] This mutant can bind E2-binding-site DNA only in the presence of an E2 monoclonal antibody ("supershift").

lacking vector pXB101 was transfected resulted in no decrease or a minimal decrease (less than 25%) in reporter expression, indicating that repression by the E2 repressor was not due to titration of transcription factors by excess promoter DNA. The high degree of repression may be related to the phenomenon of cooperativity of E2 transactivation at multiple E2 binding sites (31, 32). Decreasing the number of E2 binding sites in our reporter from three to two resulted in a 68% drop in reporter expression, while a reduction to one E2 binding site virtually eliminated transactivation (data not shown). Therefore, a very significant decrease in reporter gene expression would be expected from a small decrease in the amount of transactivation-competent E2 protein bound to the promoter.

Mutant E2 repressors 337L, 340R, and 344L, all of which were completely defective for DNA binding but were capable of stable dimer formation, repressed E2-dependent transactivation in this assay (Table 1). These amino acids form part of the predicted DNA contact region (27). Repression by 340R was equivalent to that by the wild-type repressor. Mutant 337L repressed to a slightly lesser degree and 344L displayed still lower repression, although these two mutants produced repression levels equal to that of the wild type in other experiments (Table 2 and data not shown). Cotransfection of the dimerization-defective mutant 360S resulted in only a 20% decrease in reporter expression, indicating that it was considerably weakened for repression.

Table 2 provides a compilation of data from experiments measuring inhibition of E2-dependent CAT and hGH expression. Repression by the mutants was measured relative to inhibition by the wild-type repressor in the same experiment. All DNA-binding-defective and dimerization-competent mutants repressed E2-dependent expression. Mutants 337L, 340F, 340R, 340Y, and 344L all repressed expression to a level equal to or within 90% of that of the wild-type repressor. Mutant 339M also repressed expression but to a lesser extent than the above mutants. Repression experiments with 339M gave a wider variation in results than the above mutants. The reasons for this variability and lower repression level have not been determined. Collectively, these results indicate that the binding of the E2 repressor to promoter DNA is not required for repression. Therefore, direct competition by the E2 repressor with the E2 transactivator for occupancy of E2 binding sites in the promoter is not necessary for repressor activity. These results were not dependent upon the particular E2-responsive reporter used in Tables 1 and 2, since similar results were obtained with a reporter in which hGH was expressed from the BPV-1 upstream regulatory region (data not shown). This indicates that the sequence of the E2 binding site used in the repression assay is not critical, as the BPV-1 upstream regulatory region has a variety of different E2 binding site sequences.

The dimerization-defective mutants 317STOP, 333V, 360N, 360S, and 374S/375L/391I (weak dimerization activity) were poor repressors, indicating that dimerization may be required for repression. The relative degree of repression probably reflects the presence of weak dimerization activity in some mutants. For instance, mutant 360S retained some ability to dimerize, while 333V and 360N could not be detected in dimeric forms (27).

Mutants 316Y, 366Y/376L, 408*, and 411* were poor repressors despite being capable of dimer formation in vitro. The reasons for their failure to repress are not yet known. The possibility that these four mutants have shorter half-lives in vivo than the wild-type repressor will be investigated.

The natural BPV-1 E2 repressors have deletions of the transactivation domain but possess DNA-binding and dimerization activities. We tested whether a deletion of the transactivation domain was required for repression in the absence of DNA binding. A full-length E2 mutant having a complete transactivation domain but lacking DNA-binding activity due to a cysteine to arginine substitution at position 340 was tested for its ability to inhibit E2-dependent gene expression. Full-length E2 bearing the 340R mutation (E2.340R) completely lacked transactivation activity (Table 3). This result lends in vivo support to the in vitro finding that mutant 340R was completely defective for DNA bind-

TABLE 3. Transactivation and repression of reporter pXB332hGH by full-length E2 having the 340R mutation[a]

| Transactivator | Repressor[b] | hGH (ng/$10^6$ cells) | Fold induction | % Repression |
|---|---|---|---|---|
| None | None | 0.5 | | |
| E2 | None | 23.6 | 47.2 | |
| E2.340R | None | 0.4 | 0 | |
| E2 | E2-TR | 1.0 | 2.0 | 97.8 |
| E2 | E2-TR.340R | 1.1 | 2.2 | 97.4 |
| E2 | E2.340R | 0.9 | 1.8 | 98.3 |
| E2 | E2.360S | 15.3 | 30.6 | 35.9 |

[a] E2.340R and E2.360S are full-length E2s with the 340R and 360S mutations, respectively; E2-TR.340R is the E2 repressor with the 340R mutation. For computation of data, see Table 1, footnotes *b* and *c*.

[b] Repressor DNA was present at a fourfold excess over transactivator DNA. When no repressor was present, the insert-lacking vector pXB101 was used at the same DNA concentration as was used for the repressors.

ing, since DNA-binding activity of E2.340R would be expected to result in detectable transactivation. Full-length E2 having the 340R mutation failed to transactivate even when overexpressed by use of a strong promoter (data not shown).

As shown in Table 3, full-length DNA-binding-defective and dimerization-competent E2 (E2.340R) repressed E2 transactivation as efficiently as wild-type and 340R mutant E2-TR. Similar results were obtained with full-length E2 bearing the 344L mutation (data not shown). Full-length E2 with a dimerization defect, E2.360S, repressed poorly. Since the E2.340R and E2.344L homodimers are DNA binding defective, they cannot compete with wild-type E2 for the specific binding sites. This implies that repression occurs through formation of heterodimers with wild-type E2 which fail to bind DNA and therefore cannot transactivate. These mutants appear to mimic the mode of repression by naturally occurring repressors such as Id (2) and I-POU (34). These cellular factors are unable to bind DNA but are capable of forming heterodimers with DNA-binding-competent transactivators to inhibit their DNA-binding and transactivation activity.

It is theoretically possible that the DNA-binding-defective mutants repress through formation of heterodimers with wild-type E2 which are capable of binding DNA and which inhibit transcription by competing for occupancy of E2 binding sites with E2 homodimers. This is unlikely, since the few E2 mutants tested did not bind DNA as heterodimers with wild-type E2 (data not shown). The results in Table 3 also discredit this model. If heterodimers between wild-type E2 and E2.340R did bind DNA, they should transactivate rather than repress, since this heterodimer would have a complete transactivation domain.

It is unlikely that portions of E2-TR outside of the DNA-binding and dimerization region are required for repression because the carboxy-terminal 125 amino acids of BPV-1 E2 can repress E2-dependent gene expression as efficiently as E2-TR (data not shown). The carboxy-terminal 85 residues which constitute the minimal DNA-binding domain did not function as a repressor. However, Western immunoblot analysis indicated that, following transfection, this fragment of E2 was present in cells at a level far below that of E2-TR, indicating that the lack of repression could be due to a short half-life (data not shown).

In summary, our results indicate that the E2 repressor acts by formation of inactive heterodimers rather than by direct competition for occupancy of E2 DNA-binding sites. We cannot rule out a contribution to repression by competition for promoter occupancy, but this would seem unlikely to be as efficient as inhibition by heterodimer formation, since E2 would be expected to bind promoter DNA more efficiently than E2-TR owing to the cooperativity of DNA binding by E2 but not by E2-TR (15). The finding that many DNA-binding-defective mutants of E2-TR inhibit E2-dependent gene expression as efficiently as the wild-type DNA-binding-competent E2-TR indicates that if repression can occur by competition for promoter binding, it is not significant relative to inhibition by heterodimer formation.

The naturally occurring E2 repressors have DNA-binding activity. The E2–E2-TR heterodimers are capable of binding DNA, since E2-TR can bind DNA when present in a heterodimer with full-length E2 in vitro (data not shown). It is not known why these heterodimers are defective for transactivation. It may be that two transactivation domains are required in each dimer for interaction with other transcription factors. Alternatively, E2–E2-TR heterodimers could be defective because of a short half-life. E2-TR may reduce the amount of E2 present by forming heterodimers, which are then degraded. Work is in progress to determine the reason for the lack of transactivation activity by these heterodimers.

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