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The DNA Binding Domain of a Papillomavirus E2 Protein Programs a Chimeric Nuclease To Cleave Integrated Human Papillomavirus DNA in HeLa Cervical Carcinoma Cells[▽]

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Viral DNA binding proteins that direct nucleases or other protein domains to viral DNA in lytically or latently infected cells may provide a novel approach to modulate viral gene expression or replication. Cervical carcinogenesis is initiated by high-risk human papillomavirus (HPV) infection, and viral DNA persists in the cancer cells. To test whether a DNA binding domain of a papillomavirus protein can direct a nuclease domain to cleave HPV DNA in cervical cancer cells, we fused the DNA binding domain of the bovine papillomavirus type 1 (BPV1) E2 protein to the catalytic domain of the FokI restriction endonuclease, generating a BPV1 E2-FokI chimeric nuclease (BEF). BEF introduced DNA double-strand breaks on both sides of an E2 binding site in vitro, whereas DNA binding or catalytic mutants of BEF did not. After expression of BEF in HeLa cervical carcinoma cells, we detected cleavage at E2 binding sites in the integrated HPV18 DNA in these cells and also at an E2 binding site in cellular DNA. BEF-expressing cells underwent senescence, which required the DNA binding activity of BEF, but not its nuclease activity. These results demonstrate that DNA binding domains of viral proteins can target effector molecules to cognate binding sites in virally infected cells.

Viral genomes present in lytically and latently infected cells provide unique DNA sequences that are absent from uninfected cells. Proteins that bind directly to viral or proviral DNA could act selectively in infected cells to modulate specific viral processes, such as gene expression or replication. Artificial DNA binding domains containing Cys_2-His_2 zinc finger motifs have been engineered to direct functional protein domains to DNA sequences of interest (61). These zinc finger proteins (ZFPs) have been used to construct designer transcription factors to activate or repress genes or to create site-specific endonucleases (reviewed in reference 36). ZFPs linked to the Krüppel-associated box repressor domain bind to and repress promoters of human immunodeficiency virus type 1 and herpes simplex virus type 1 (37, 43, 46), illustrating that engineered proteins can bind to specific sites in viral genomes. In addition, ZFPs designed to bind specific sites in human papillomavirus (HPV) DNA are able to inhibit the replication of HPV type 18 (HPV18) in transient replication assays (32).

The FokI restriction enzyme, a modular type IIS restriction enzyme, has a nonspecific nuclease domain that cleaves DNA adjacent to the recognition site of its DNA binding domain (27, 54). Engineered ZFPs and DNA binding modules of cellular proteins have been fused to the FokI nuclease domain to create site-specific endonucleases that can introduce DNA double-strand breaks at desired sequences (14, 21, 23–25, 45, 50, 51). The DNA binding domains of viral proteins have not been used to target chimeric nucleases to induce cleavage of viral DNA. ZFP-FokI-based nucleases have been used to stimulate homologous recombination in *Xenopus* oocytes and to enhance gene targeting in *Drosophila* and in human somatic cells (4–6, 41). The native FokI enzyme, as well as FokI-based chimeric nucleases, requires dimerization of the nuclease domain for efficient DNA cleavage (7, 57, 59).

Although ZFPs can be engineered to recognize many DNA sequences, the development and optimization of ZFPs that recognize particular sequences can be a lengthy and difficult process involving serial rounds of mutagenesis and selection. Furthermore, it has not been possible to generate highly sequence-specific ZFPs to some DNA sequences (35, 47). Therefore, we decided to test whether the DNA binding domain of a native viral protein could be used to direct a functional heterologous protein domain to viral DNA in infected cells.

Viruses contribute to the development of more than 10% of cancers worldwide (38). The high-risk HPVs, including types 16 and 18, play a central role in the development of cervical and other cancers, and HPV DNA is invariably present and often integrated into cellular DNA in cervical cancer cells (11). The HPV E6 and E7 oncogenes are expressed in cervical carcinomas and cell lines derived from them and encode proteins that inactivate cellular growth controls. The HPV E6 protein binds to the p53 tumor suppressor and targets it for ubiquitin-mediated degradation, and it induces expression of telomerase (29). The HPV E7 protein binds to hypophosphorylated members of the retinoblastoma (Rb) tumor suppressor family, resulting in their destabilization and loss of Rb/E2F complexes, thereby allowing the expression of cell cycle progression genes (33).

Continuous expression of the HPV oncogenes is required for the proliferation of cervical cancer cell lines. In cervical cancer, integration of HPV DNA into the cellular genome often disrupts expression of the HPV E2 gene (3, 48), which encodes a dimeric, site-specific DNA binding protein required

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for viral DNA replication, proper viral gene expression, and genome segregation (30). Loss of E2 results in the derepression of E6 and E7 expression and may play a key role leading toward cancer progression. When the E2 gene from bovine papillomavirus type 1 (BPV1) is introduced into HeLa cervical carcinoma cells, which contain integrated HPV18 DNA, the E2 protein binds to the E2 binding sites (BSs) in the HPV major early promoter, causing transcriptional repression of the E6 and E7 oncogenes. This results in restoration of p53 and Rb activity, inhibition of cellular growth, and entry into a state of cellular senescence (13, 15, 16a, 22, 55).

The E2 protein, the only papillomavirus protein that independently binds viral DNA with high affinity and site-specificity, has a modular structure. The full-length E2 protein contains an amino-terminal transcriptional regulatory domain and a carboxy-terminal DNA binding and dimerization domain sufficient for specific DNA recognition, separated by a phylogenetically unconserved hinge region (reviewed in references 18 and 31). An E2 dimer binds to the consensus sequence $ACCgN_4cGGT$ (lowercase letters are preferred but not required, N_4 denotes a spacer region) (28) present in multiple copies in papillomavirus genomes. The HPV16 and -18 genomes contain four conserved E2 BSs, all of which are in the viral long control region, including two (BS1 and BS2) immediately upstream of the promoter for the E6 and E7 oncogenes (28, 44, 55). The E2 protein from a variety of papillomavirus types binds to these E2 BSs, resulting in repression of the E6 and E7 genes. Thus, the well-characterized papillomavirus E2 protein can be used to test whether a viral DNA binding domain can target an effector protein to viral DNA sequences. Here, we report the generation of a chimeric nuclease that induces DNA double-strand breaks at E2 BSs in purified DNA substrates in vitro and in the integrated HPV18 genomes in HeLa cervical carcinoma cells, as well as at a nonviral E2 BS in cellular DNA.

MATERIALS AND METHODS

Construction and expression of BEF. BPV1 E2-FokI chimeric nuclease (BEF) was constructed by using recombinant PCR with overlapping primers to link the BPV1 E2 DNA binding domain (amino acids 310 to 410) to the glycine linker $(Gly_4Ser)_3$-FokI nuclease domain (amino acids 383 to 579) from pET15b:ZIFΔQNK (a generous gift from S. Chandrasegaran, Johns Hopkins) (50). For expression by in vitro transcription and translation, the PCR product encoding BEF was digested with XhoI and BamHI and subcloned into pET15b (Novagen), where expression is driven by a T7 promoter, to make pET15b-BEF, and the sequence was confirmed. BEF was transcribed and translated by using the TNT T7 quick coupled transcription/translation system (Promega) according to the manufacturer's instructions. Mock lysates were programmed with the pET15b empty vector.

To make adenovirus stocks, the BEF construct was subcloned into the shuttle vector pDualCCM (Vector Biolabs) to create pDualCCM-BEF. BEF was transferred from the shuttle vector into the viral genome by homologous recombination, stocks of recombinant replication-defective adenovirus with E1/E3 deleted were made and amplified, and titers were determined by Vector Biolabs (Philadelphia, PA).

pET15b-BEF was used as a template in standard site-directed mutagenesis reactions (QuikChange; Stratagene) to generate the following mutations: BEF-C340R (refers to E2 residue 340) and BEF-D467A (refers to FokI residue 467). PCR was used to add BamHI and XhoI restriction sites to BEF-C340R and BEF-D467A for subcloning into pDualCCM for the generation of adenovirus stocks, as described above.

DNA substrates. For in vitro digestion assays, plasmids containing an E2 BS were constructed by insertion of oligonucleotide duplexes containing HPV16 E2 BS1, BPV1 E2 BS10, or HPV18 E2 BS1 (see Table 1 for E2 BS sequences) into the PstI site in pACYC177 (New England Biolabs [NEB]). A total of 10 μg of the resulting plasmids was digested with BamHI and HindIII, and the resulting 3.1-kb bands, which lack endogenous E2 BSs, were gel purified. pSH99 was constructed by removing the E2 BSs in pUC19 by QuikChange site-directed mutagenesis of position 1388 from a C to a T and replacement of the NdeI-to-EcoRI fragment containing an E2 BS with the NdeI-to-EcoRI fragment of pET15b, which does not contain an E2 BS.

TABLE 1. E2 binding sites

E2 BS	Sequence (5′ to 3′)[a]
BPV1 BS10	ACCGTCTTCGGT
HPV16 BS1	ACCGAAACCGGT
HPV18 BS1	ACCGAAAACGGT[a]
HPV18 BS2	ACCGAAAACGGT[a]
HPV18 BS3	ACCGAAATAGGT
HPV18 BS4	ACCGATTTCGGT

[a] HPV18 BS1 and BS2 are identical.

In vitro digestion assays. Approximately 800 ng of gel-purified linear substrate DNA were incubated in 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol (i.e., restriction enzyme buffer 4 from NEB), 0.1 mg of bovine serum albumin/ml, and 3 μl of the TNT lysates at room temperature for 30 min. After digestion, the samples were treated with 10 μg of DNase-free RNase (catalog no. 11119915001; Roche Applied Science)/ml for 1.5 min, phenol-chloroform extracted, ethanol precipitated, resuspended in 20 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA buffer, and subjected to electrophoresis on 1% agarose gels. The products were run alongside a 1-kb DNA ladder (Gibco) and 0.9- and 2.2-kb size markers generated from PstI digestion of substrate DNA without an E2 BS. In some experiments, the protocol was modified as follows: 500 ng of substrate DNA and 4 μl of TNT lysate were used, and the incubation was done at 30°C. Variation of the incubation temperature (room temperature or at 30 or 37°C) did not significantly alter the cleavage properties or extent of digestion by the chimeric nuclease.

Cells. The HeLa/sen2 line was described previously (15). HeLa/sen2, Cos-1, and C33A cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, penicillin-streptomycin, and 20 mM HEPES (pH 7.3).

LM-PCR of DNA double-strand breaks. (i) From digestion with in vitro transcribed or translated BEF. Pairs of complementary oligonucleotides (07769 and 010067, left; 07769 and 014621, right; see Table 2 for the sequences of all primers) were annealed to generate double-stranded linkers with 5′ overhangs complementary to the mapped overhangs after BEF cleavage. BEF-cleaved substrate DNA containing the HPV18 E2 BS was ligated to 7 pmol of the appropriate linker in a total volume of 20 μl overnight at 18°C with T4 DNA ligase buffer and T4 DNA ligase (NEB). After heat inactivation of the ligase, 1 μl of a 1:10 dilution of the linker-ligated DNA was added to 50 μl (final volume) of PCR mixture with *Taq* DNA polymerase (NEB) and SPLKO (linker-specific outer primer, see Table 2) and either L-1 or R-1 substrate-specific primers. For PCR, the cycling conditions were as follows: initial denaturation at 95°C for 4 min and 10 cycles of heating (95°C, 45 s), annealing (60°C, 1 min), and elongation (72°C, 1 min). The PCR product was purified by using a QIAGEN PCR purification kit, and 1 μl of the sample was used as the template for a second round of PCR with the nested primers SPLKI (linker-specific nested primer, see Table 2) and either L-2 or R-2. The cycling conditions were as follows: initial denaturation at 95°C for 4 min, 25 cycles of heating (95°C, 45 s), annealing (62°C, 45 s), and elongation (72°C, 1 min). The reaction products were subjected to agarose gel electrophoresis and run alongside a 100-bp DNA ladder (Invitrogen).

(ii) From digestion with cell lysates expressing BEF. 4×10^5 HeLa/sen2 cells were seeded into 100-mm dishes and the next day mock-infected or infected at a multiplicity of infection (MOI) of 100 with Ad-BEF, Ad-BEF-C340R, Ad-BEF-D467A, or Ad-CMV (Vector Biolabs). Two days later, cell pellets were harvested and frozen. The cell pellets were lysed in FN-CD lysis buffer (10 mM Tris, 50 mM NaCl, 0.1 mM EDTA, 200 μg of bovine serum albumin/ml, 0.5% CHAPSO {3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate}, 0.25 mM deoxycholate, 50% glycerol [pH 7.4]) containing 5 μg of leupeptin and aprotinin/ml. DNA substrates containing no E2 BS or a BPV1 E2 BS were incubated with 10 μg of cleared lysate at 37°C for 30 min. After phenol-chloroform-extraction and ethanol precipitation, the samples were dissolved in 20 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA buffer. Ligation to the double-strand linker (07769 and 07770) and LM-PCR was done as described in the preceding paragraph using 62°C as the annealing temperature for the first

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TABLE 2. Oligonucleotides used for LM-PCR

Function and oligonucleotide	Sequence (5′ to 3′)	Function
PCR		
SPLKO	CGAATCGTAACCGTTCGTACGAGAA	Linker-specific outer primer
SPLKI	TCGTACGAGAATCGCTGTCCTCTCC	Linker-specific nested primer
L-1	TTGCCTTCCTGTTTTTGCT	Left outer primer; pACYC177
L-2	CCGAAGGAGCTAACCGC	Left nested primer; pACYC177
R-1	GCCAGTTACCTCGGTTCAA	Rt. outer primer; pACYC177
R-2	GATACGGGAGGGCTTACCAT	Rt. nested primer; pACYC177
1-1	ACCTTCTGGATCAGCCATTG	HPV18 E2BS1 outer primer
1-2	CTGGATTCAACGGTTTCTGG	HPV18 E2BS1 nested primer
4-1	CGTGTACGTGCCAGGAAGT	HPV18 E2BS4 outer primer
4-2	TGTGTTTGTATGTCCTGTGTTTGTG	HPV18 E2BS4 nested primer
13-1	TTTAAGAGGGAAAAGTCTAGGTTTCA	Chromosome 13 outer primer
13-2	TTCACAATCCAAAGGCAACA	Chromosome 13 nested primer
Linkers		
07769	CGAATCGTAACCGTTCGTACGAGAATTCGTACGA GAATCGCTGTCCTCTCCAACGAGCCAAGA	Top linker
09893	TCTTGGCTCGTTTTTTTTTGCAAAAA	Bottom linker: blunt ends
07770	CTGCTCTTGGCTCGTTTTTTTTTGCAAAAA	Bottom linker: BE2
010067	GTCGTCTTGGCTCGTTTTTTTTTGCAAAAA	Bottom linker: HPV18, left
014621	TATATCTTGGCTCGTTTTTTTTTGCAAAAA	Bottom linker: HPV18, right

round of the PCR and R-1 and R-2 as the nested primer set. The reaction products were subjected to agarose gel electrophoresis and run alongside a 100-bp DNA ladder.

(iii) From digestion of genomic DNA in HeLa cells. 5×10^5 HeLa/sen2 cells were seeded into 100-mm dishes and the next day infected at an MOI of 10 with Ad-CMV as a negative control or with Ad-BEF. Thirty hours later, cell pellets were harvested and frozen. Genomic DNA was prepared with a Genomic Tip (QIAGEN). Prior to linker ligation, T4 DNA polymerase and 0.2 mM concentrations of all four deoxynucleoside triphosphates were used to blunt staggered DNA lesions. After purification using a QIAGEN PCR purification kit, 200 ng of the blunted DNA was ligated with T4 DNA ligase to 7 pmol of a double-stranded blunt-ended linker (07769 and 09893) in a total volume of 60 μl overnight at 18°C. For PCR, 5 μl of ligated DNA was added to 50 μl of reaction mixture with primers specific for the linker and the relevant locus (see Table 2) and *Taq* DNA polymerase. The reaction mixture was incubated for 5 min at 72°C, followed by PCR as described above with the appropriate locus-specific primers. Half of the PCR product was resolved by electrophoresis on either a 2 or a 4% NuSieve agarose gel (Cambrex), transferred to a Nytran Supercharge membrane (Schleicher & Schuell) under neutral conditions, and cross-linked to the membrane by UV irradiation with a Stratalinker (Stratagene). The blots were hybridized with random-prime-labeled DNA fragments or a ^{32}P-labeled oligonucleotide locus-specific probe and analyzed with a PhosphorImager.

DNA sequence analysis of LM-PCR products. Amplified LM-PCR products were purified on 2% agarose gels. Gel extraction and purification was done with the QIAGEN MinElute gel extraction kit. The purified DNA was cloned into a TOPO cloning vector (Invitrogen), and individual clones were sequenced.

Immunoblotting. Protein from in vitro transcription and translation or from extracts of cells harvested in modified EBC buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 2 mM EDTA, 0.4% NP-40, 1 mM NaF, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 5 μg of leupeptin, and aprotinin/ml) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Millipore) in a 12.5 mM Tris–0.1 M glycine–20% methanol transfer buffer, and blocked in 5% milk-tris-buffered saline buffer. The membranes were probed with rabbit antiserum raised against the FokI endonuclease (a gift from S. Chandrasegaran, Johns Hopkins). The membranes were washed in TBS, incubated with rabbit horseradish peroxidase-conjugated donkey antibody (Jackson Immunoresearch), and washed again with TBS. The membranes were then incubated with ECL+ (Amersham), and the signals were detected with Hyperfilm (Amersham). Immunoblotting for E2 with a 1:10 dilution of the B202 tissue culture supernatant was done as described previously (16).

Autofluorescence. A total of 2×10^5 HeLa/sen2 cells were seeded into 100-mm dishes and the next day were infected with Ad-CMV, Ad-BEF, Ad-BEF-C340R, or Ad-BEF-D467A (MOI of 100). Flow cytometry assays were performed 5 days postinfection, as described previously (10).

SAβ-Gal assay. A total of 2×10^5 HeLa/sen2 or C33A cells were seeded into 100-mm dishes and the next day were mock infected or infected with either Ad-GFP (Vector Biolabs) as a control or Ad-BEF at an MOI of 100. After 5 days, 5×10^4 cells were replated into six-well dishes. Ten days after the initial infection, the cells were stained at pH 6.0 with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as described previously (12).

RESULTS

We used recombinant PCR to construct a gene that encodes a chimeric nuclease (BEF) comprised of the DNA binding domain and a portion of the hinge region of the BPV1 E2 protein (amino acids 310 to 410) fused in-frame N-terminal to the nuclease domain of the FokI endonuclease (amino acids 383 to 579) (Fig. 1A). The crystal structure of a dimer of the BPV1 E2 DNA binding domain bound to DNA revealed that the C terminus of the protein points away from the dimer interface and the DNA double helix, suggesting that adding protein sequences to the C terminus of the E2 protein would not interfere with its ability to dimerize or bind DNA (19). We used the C-terminal 101 amino acids of the E2 protein because this portion of the protein has increased stability and affinity for DNA compared to the minimal 85-amino-acid DNA binding domain (39). This region of E2 also contains a nuclear localization signal (1, 49). The nuclease domain of FokI used in these experiments has been well characterized and lacks site-specific DNA binding activity (27, 28, 58). A flexible 15-amino-acid glycine-serine linker [$(Gly_4Ser)_3$] separated the two domains.

Chimeric nuclease cleaves DNA substrates containing E2 BSs. The chimeric nuclease was expressed from a T7 promoter in a coupled in vitro transcription and translation system containing T7 RNA polymerase. Immunoblotting with a FokI antiserum or an E2 monoclonal antibody detected the chimeric nuclease at its predicted molecular weight (data not shown).

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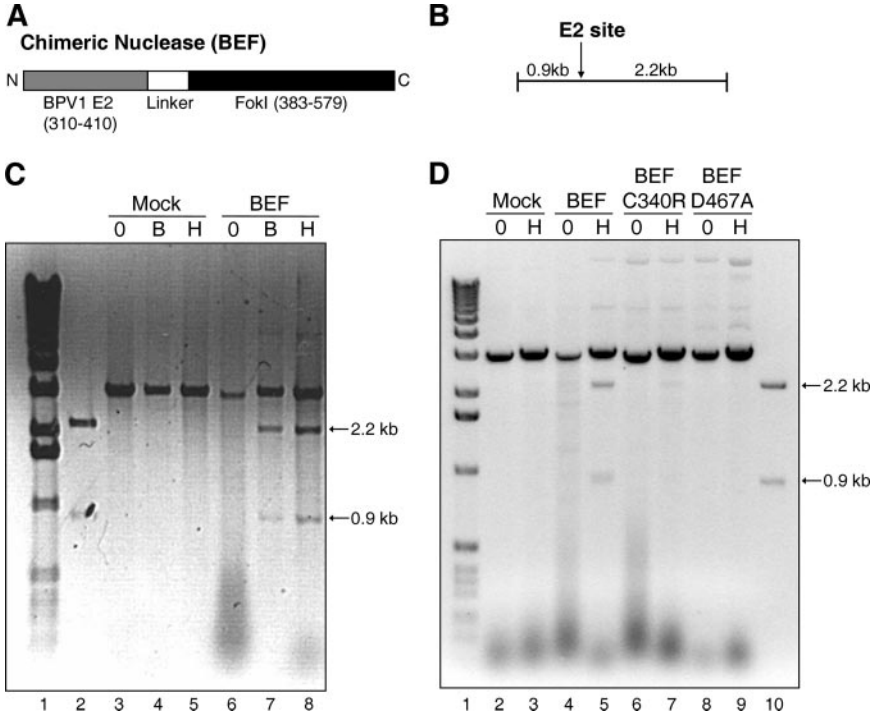

FIG. 1. Chimeric nuclease (BEF) containing the E2 DNA binding domain and FokI nuclease domain cleaves DNA substrates containing an E2 BS. (A) Schematic diagram of the BEF chimeric nuclease. The BPV1 E2 DNA binding domain (amino acids 310 to 410), shown in gray, was fused to the FokI nuclease domain (amino acids 383 to 579) in black by a glycine-serine linker, shown in white. (B) E2 BS substrate. Linear DNA substrates, with or without the E2 BS (at the location noted by the arrow) were used to test the activity of the chimeric nuclease. The approximate sizes of the cleavage products are noted. (C) The BEF chimeric nuclease cleaves DNA substrates containing E2 BSs. After digestion of substrate DNA containing no E2 BS (0), a BPV1 E2 BS (B), or an HPV16 E2 BS (H) with in vitro transcribed and translated BEF or a mock lysate, cleavage products were separated from uncut DNA by agarose gel electrophoresis and are noted by the arrows. Lane 1 shows a 1-kb DNA ladder, and lane 2 shows marker DNA fragments of the predicted sizes of the cleavage products. (D) The DNA binding and catalytic activity of BEF are required for DNA cleavage. BEF, BEF-C340R (the DNA binding mutant), BEF-D467A (the catalytic mutant), or a mock lysate were incubated with substrate DNA containing no E2 BS (0) or an HPV18 E2 BS (H). The cleavage products were separated by agarose gel electrophoresis and are noted by the arrows. The ladder and markers (lane 10) are the same as in panel C.

To determine whether BEF cleaved DNA containing E2 BSs, we incubated the in vitro transcribed and translated BEF with purified linear 3.1-kb DNA substrates lacking an E2 BS or containing a single E2 BS derived from BPV1 or HPV16 DNA (see Table 1 for the sequences of the E2 BSs). These substrates were identical other than the presence or absence of the E2 BS. BEF digestion generated specific cleavage products of the predicted size of 0.9 and 2.2 kb (Fig. 1B) with substrates containing an E2 BS from BPV1 or HPV16 (Fig. 1C, lanes 7 and 8). In contrast, no specific cleavage occurred in the substrates lacking an E2 BS (Fig. 1C, lane 6) or if a mock transcription-translation mix was used (Fig. 1C, lanes 3 to 5). These results indicated that BEF cleaves DNA at or near the E2 BS, and that cleavage requires only a single E2 BS. The presence of undigested DNA demonstrated that the cleavage reaction did not proceed to completion. The overall extent of cleavage was variable from experiment to experiment and not reproducibly increased by using substrates containing multiple E2 BSs (data not shown). BEF also specifically cleaved a DNA substrate containing E2 BS1 from HPV18 DNA (Fig. 1D, lane 5). In addition, a chimeric nuclease with a DNA binding domain from the HPV16 E2 protein generated specific cleavage products of 0.9 and 2.2 kb from substrates containing an HPV16 E2 BS, but was unable to cleave DNA substrates containing a BPV1 E2 BS with an A/T-poor spacer (data not shown), a finding consistent with the preference of the HPV16 E2 protein for E2 BSs with an A/T-rich spacer (20).

To test whether DNA binding was required for cleavage, we used site-directed mutagenesis to generate a cysteine-to-arginine mutation at position 340 in the E2 DNA binding domain of BEF, which impairs DNA binding (42). The DNA binding mutant displayed greatly reduced ability to cleave a DNA substrate containing an E2 BS (Fig. 1D, lane 7), demonstrating that BEF required DNA binding for efficient cleavage. To test whether the nuclease activity of BEF was required for cleavage, we also generated an aspartic acid-to-alanine mutation at position 467 in the FokI nuclease domain of BEF, which abolishes its catalytic activity (59). The catalytic mutant was unable to cleave a DNA substrate containing an E2 BS (Fig. 1D, lane 9), demonstrating that the catalytic activity of FokI was also required for BEF cleavage. Both mutants were expressed at levels similar to that of wild-type BEF (data not shown).

Mapping sites of cleavage by BEF. To determine the precise sites of cleavage relative to the E2 BS, we used DNA substrates containing the HPV18 E2 BS1 that were ^{32}P end labeled at either the 5′ or the 3′ end. After BEF digestion, the products of the cleavage reaction were analyzed by denaturing high

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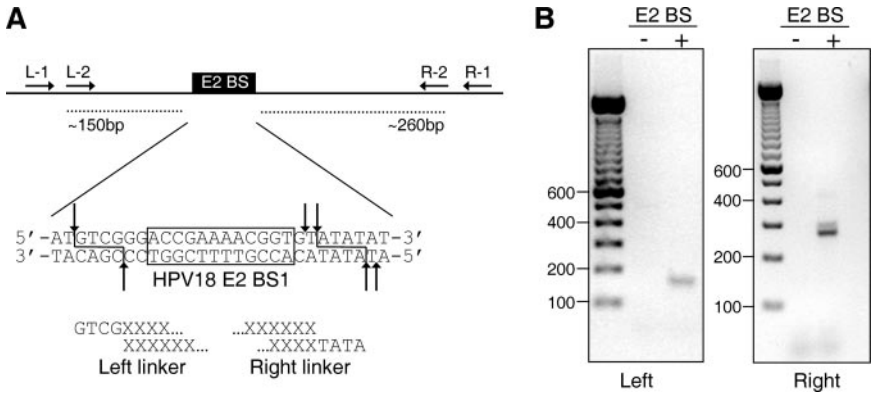

FIG. 2. Mapping the sites of cleavage by BEF. (A) Diagram of the substrate DNA used in digestion and mapping reactions indicating the position of the E2 BS and the left-side (L-1 and L-2) and right-side (R-1 and R-2) PCR primers. The dotted lines indicate the predicted sizes of the respective PCR products. The sequence of the E2 BS and flanking DNA, as well as the sequence of the linkers with single-strand overhangs, are shown at the bottom. The major sites of cleavage mapped on BEF-digested end-labeled DNA substrates are indicated by arrows, with the major four-base overhangs on each side of the BS shown. (B) After BEF digestion of DNA substrates containing an HPV18 E2 BS (+) or no E2 BS (−), a left-side or right-side linker was ligated to the DNA. The ligation products were amplified by PCR with linker-specific primers in combination with nested primers specific for the appropriate flanking substrate DNA and subjected to agarose gel electrophoresis along with a 100-bp DNA ladder shown in the far left lane of each gel.

percentage polyacrylamide gel electrophoresis, followed by autoradiography (data not shown). Figure 2A summarizes the mapping analysis of the cleavage of the end-labeled DNA substrates. These mapping data demonstrated that BEF cleaved substrate DNA on either side of the E2 BS to generate double-strand breaks. As was the case in previous studies of FokI-mediated cleavage, there was a slight heterogeneity of the sites of strand scission, with the predominant cleavage products having four-nucleotide 5′ overhangs (50, 51).

A variant of LM-PCR was used to confirm that BEF cleavage generated the four-nucleotide 5′ overhangs mapped with the end-labeled DNA. After digestion of substrates with or without HPV18 E2 BS1, a linker containing an overhang complementary to the predicted four-nucleotide overhang on the right or left of the E2 BS was ligated to the BEF-digested DNA. Nested PCR using primers specific to the linker and the appropriate flanking substrate DNA was used to amplify the ligation products (see Fig. 2A), which were subjected to agarose gel electrophoresis (Fig. 2B). No PCR product was detected when ligase or BEF was omitted from the reaction (data not shown) or when the substrate lacked an E2 BS. However, when linkers with overhangs complementary to the mapped overhangs near the E2 BS were ligated to DNA cleaved by BEF, discrete products of the expected size were amplified (Fig. 2B). Cloning and sequencing of the PCR products confirmed that they were all the result of linker ligation at the predicted site, including the faint upper band in the right-side PCR, which was generated by a duplication of the flanking substrate DNA primer in the PCR product (data not shown). This experiment confirmed that BEF cleaves at least some substrate DNA to generate double-strand breaks with 5′ overhangs of four nucleotides on both sides of the E2 BS.

Cell lysates expressing BEF cleave DNA substrates at E2 BSs. To test whether BEF expressed in cells was active, we generated replication-defective adenovirus vectors expressing the wild-type chimeric nuclease (Ad-BEF), the DNA binding mutant (Ad-BEF-C340R), and the catalytic mutant (Ad-BEF-D467A). HeLa cervical carcinoma cells were infected at an MOI of 100 with these viruses, a control adenovirus, or mock infected. Two days after infection, the cell lysates were harvested and incubated in vitro with DNA substrates containing no E2 BS or an E2 BS derived from BPV1 DNA. LM-PCR, as described above, was used to detect cleavage and generation of a four-nucleotide 5′ overhang 2 bp away from the E2 BS (Fig. 3). No specific cleavage was detected in substrates lacking an E2 BS (data not shown) or when substrates were incubated with a lysate from the cells that were mock infected or infected with the control adenovirus (Fig. 3, lanes 1 and 2). Cleavage adjacent to the E2 BS was detected by LM-PCR after incubation of the substrate with lysates from cells infected with Ad-BEF (Fig. 3, lane 3). BEF containing a mutation in either the

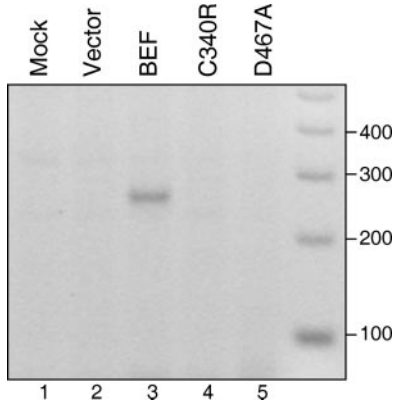

FIG. 3. Cell lysates expressing BEF cleave DNA substrates with a BPV1 E2 BS in vitro. HeLa cells were infected with adenoviruses (MOI of 100) expressing BEF, BEF-C340R (the DNA binding mutant), BEF-D467A (the catalytic mutant), a control adenovirus (vector), or mock infected. Two days after infection, cell lysates were harvested and incubated in vitro with DNA substrates containing a BPV1 E2 BS. LM-PCR was used to detect cleavage and generation of four-nucleotide 5′ overhangs on the right side of the E2 BS.

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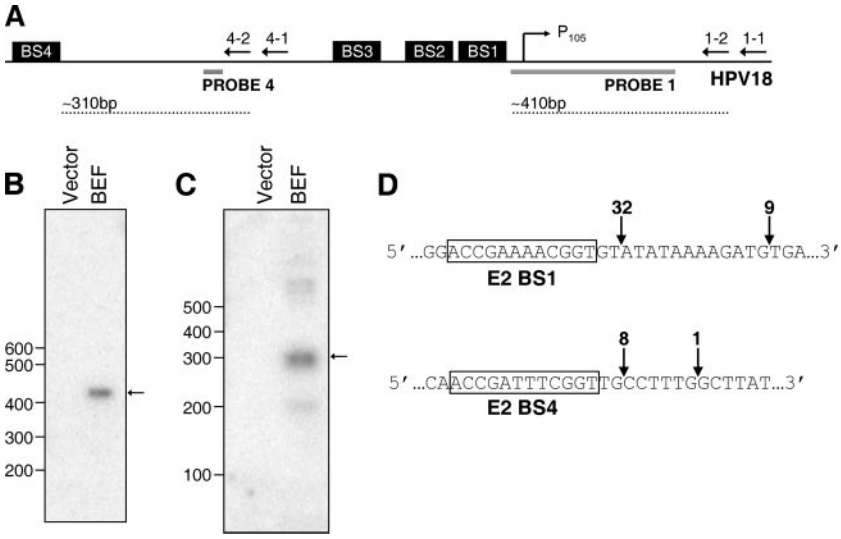

FIG. 4. BEF cleaves integrated HPV18 DNA in HeLa cells at E2 BS1 and BS4. (A) Diagram of the HPV18 long control region showing the four E2 BSs (BS1 to BS4), the E6/E7 transcription start site (P_{105}), and the primers used to detect cleavage at E2 BS1 (1-1 and 1-2) and at BS4 (4-1 and 4-2). The relative positions of the HPV-specific probes (probe 1 and probe 4) are also shown, as are the expected sizes of the LM-PCR products (dotted lines). Drawing is not to scale. (B) LM-PCR detects a DNA double-strand break at E2 BS1. HeLa cells were infected with an adenovirus expressing BEF or an empty vector. LM-PCR with nested primers (1-1 and 1-2) in combination with linker-specific primers, followed by Southern blotting with an HPV-specific probe (Probe 1), was done on genomic DNA ligated to blunt-ended linkers. (C) LM-PCR detects a DNA double-strand break at E2 BS4. The experiment in panel B was conducted with nested primers (4-1 and 4-2) and a probe (probe 4) specific to E2 BS4. (D) Location of DNA double-strand breaks in integrated HPV18 DNA in HeLa cells after expression of BEF. The DNA sequences of the top strand near E2 BS1 (top) and E2 BS4 (bottom) are shown, with arrows indicating the positions of the DNA double-strand breaks. Numbers above the arrows indicate the number of times the break was identified in individual cloned PCR products.

DNA binding or the catalytic domain was unable to cleave DNA substrates containing an E2 BS (Fig. 3, lanes 4 and 5). These results demonstrated that Ad-BEF expressed active enzyme in HeLa cells and that both DNA binding and catalytic activity of BEF were required for cleavage.

BEF cleaves integrated HPV18 DNA in HeLa cells at E2 BSs. To determine whether BEF could cleave at E2 BSs present in integrated HPV18 DNA in HeLa cells, we infected HeLa cells with Ad-BEF or a control adenovirus. Thirty hours later, total genomic DNA was harvested, treated with T4 DNA polymerase and all four deoxynucleoside triphosphates to fill in (or resect) any ends that were generated by BEF expression, and ligated to a blunt-ended linker. To detect cleavage at E2 BS1 (a high-affinity HPV18 BS for BPV1 E2 [53]), we amplified the ligated fragments by PCR with primers specific for the linker and the right side flanking HPV18 DNA (Fig. 4A). HeLa cells contain multiple copies of integrated HPV18 DNA, and the HPV18-specific primers used in the LM-PCRs were designed to detect all of these copies, including the active locus. The LM-PCR products were subjected to agarose gel electrophoresis and detected by Southern blotting with an HPV-specific probe. A DNA double-strand break at E2 BS1 is predicted to yield a PCR product of 410 bp (Fig. 4A). A specific band of approximately this size was seen after expression of BEF in HeLa cells (Fig. 4B), indicating that BEF generated a DNA double-strand break in HPV18 DNA near E2 BS1. No specific bands were generated by DNA from cells infected with a control adenovirus. The LM-PCR product generated from DNA from BEF-expressing cells was cloned and sequenced, revealing that this band did correspond to a DNA double-strand break on the right side of E2 BS1 in the HPV18 DNA. Of the 41 sequenced clones that contained both the linker and the HPV-specific primer, 32 corresponded to a DNA double-strand break two nucleotides from the end of the E2 BS, the site mapped in vitro, and 9 corresponded to a DNA double-strand break 14 nucleotides from the E2 BS (Fig. 4D, top). These data demonstrated that BEF cleaved integrated HPV18 DNA in HeLa cells at E2 BS1 at the precise position mapped in vitro with the end-labeled DNA.

We used a similar approach to detect a BEF-induced DNA double-strand break near E2 BS4, another high-affinity BS in integrated HPV18 DNA in HeLa cells. After expression of BEF in HeLa cells, LM-PCR with primers on the right side of E2 BS4 (see Fig. 4A) and Southern blotting with an HPV-specific probe detected a prominent PCR product of approximately 300 bp, indicating a DNA double-strand break in the HPV18 DNA near E2 BS4 (Fig. 4C). In addition to this prominent product, several additional minor PCR products were observed. No specific bands were generated by DNA from cells infected with a control adenovirus. Sequence analysis demonstrated that eight out of nine clones from the major LM-PCR product corresponded to a DNA double-strand break two nucleotides on the right side of E2 BS4 (Fig. 4D) and one to a DNA double-strand break eight nucleotides from E2 BS4. These data indicated that BEF cleaves integrated HPV18 DNA in HeLa cells at E2 BS1 and BS4. Cleavage was not detected at E2 BS2 or BS3 using LM-PCR with primers specific for these sites (data not shown). Because ends generated by cleavage may be rejoined by cellular DNA repair mechanisms, our results do not allow us to estimate the efficiency of cleavage. Nevertheless, we estimate that DNA breaks introduced by BEF at HPV18 E2 BS1 and BS4 persist in less than

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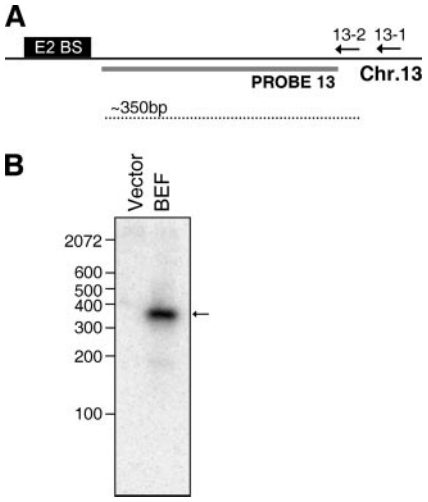

FIG. 5. BEF cleaves cellular DNA in HeLa cells at a nonviral E2 BS. (A) Diagram indicating the E2 BS in chromosome 13, the primers used to detect cleavage at this E2 BS (13-1 and 13-2), and the approximate position of the probe used for Southern blotting (probe 13). The drawing is not to scale. (B) Nested PCR with linker-specific and 13-1 and 13-2 primers amplified the linker-ligated genomic DNA isolated from HeLa cells infected with Ad-BEF or a control adenovirus, followed by Southern blotting.

1% of the cells because specific cleavage products were detected only when the DNA double-strand break was amplified by using LM-PCR and not directly using a Southern blot (data not shown).

BEF cleaves nonviral cellular E2 BSs in HeLa cells. BLAST searching of the human genome revealed two exact sequence matches to HPV18 E2 BS1: one in chromosome 13 and one in chromosome 18. To determine whether BEF cleaved these non-HPV E2 BSs, we used LM-PCR and flanking primers from these two chromosomes to amplify the cleaved genomic DNA isolated from HeLa cells infected with Ad-BEF or a control adenovirus (Fig. 5A). After expression of BEF in HeLa cells, LM-PCR and Southern blotting with a probe specific for chromosome 13 detected an amplification product of approximately 300 to 400 bp, indicating a DNA double-strand break near the E2 BS in chromosome 13 (Fig. 5B). No specific bands were generated by DNA from cells infected with a control adenovirus. These data indicate that BEF cleaved cellular DNA in HeLa cells at this E2 BS in chromosome 13. Cleavage by BEF was not detected at the E2 BS in chromosome 18 (data not shown).

Phenotype of HeLa cells after BEF expression. Repression of the HPV18 E6 and E7 genes in HeLa cells by the full-length BPV1 E2 protein causes virtually all of the cells to undergo senescence (15, 60), as assessed by growth arrest, altered cellular morphology, increased autofluorescence, and senescence-associated β-galactosidase (SAβ-Gal) activity. To determine whether BEF induced cellular senescence, we measured SAβ-Gal activity in HeLa cells. Ten days after mock infection or infection with a control adenovirus or Ad-BEF, HeLa cells were incubated with the chromogenic substrate X-Gal at pH 6.0 and examined by bright-field microscopy (Fig. 6). The mock- and control adenovirus-infected cells formed colonies that did not display SAβ-Gal activity. After BEF expression, although some of the HeLa cells formed colonies that did not display SAβ-Gal activity, a significant fraction of cells ceased proliferation, were larger and flatter than control cells, and displayed SAβ-Gal activity (Fig. 6, top panels). In contrast, infection with Ad-BEF did not induce SAβ-Gal activity in HPV-negative C33A cells (Fig. 6, bottom panels), even though BEF was expressed at similar levels in HeLa and C33A cells (data not shown).

We used flow cytometry to measure another marker of senescence, increased autofluorescence, in HeLa cells 5 days after infection with Ad-BEF or a control adenovirus (Fig. 7). BEF expression in HeLa cells induced a uniform increase in autofluorescence in a majority of the cells compared to cells infected with the control adenovirus. Taken together, these results demonstrated that BEF induces senescence in HeLa

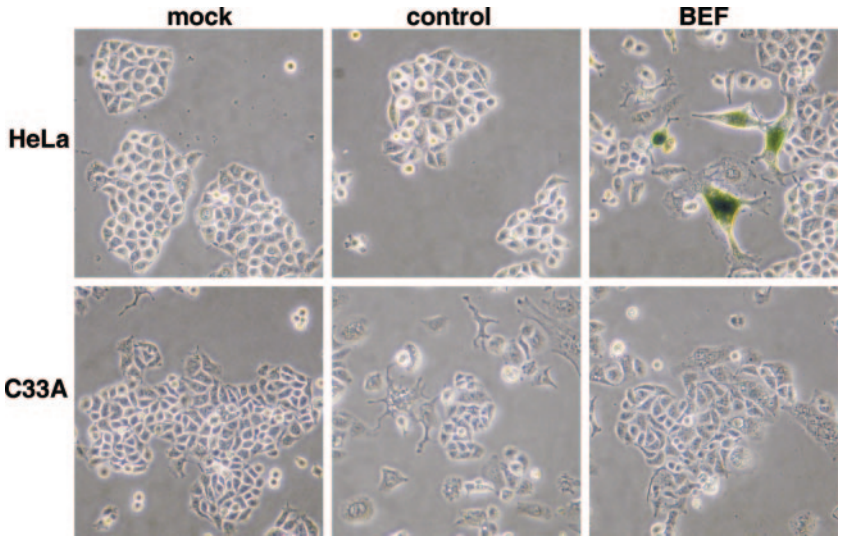

FIG. 6. Effect of BEF expression on SAβ-Gal activity. HeLa and C33A cells were stained for SAβ-Gal activity 10 days after mock infection or infection with a control adenovirus or an adenovirus expressing BEF. Cells were photographed with bright-field optics.

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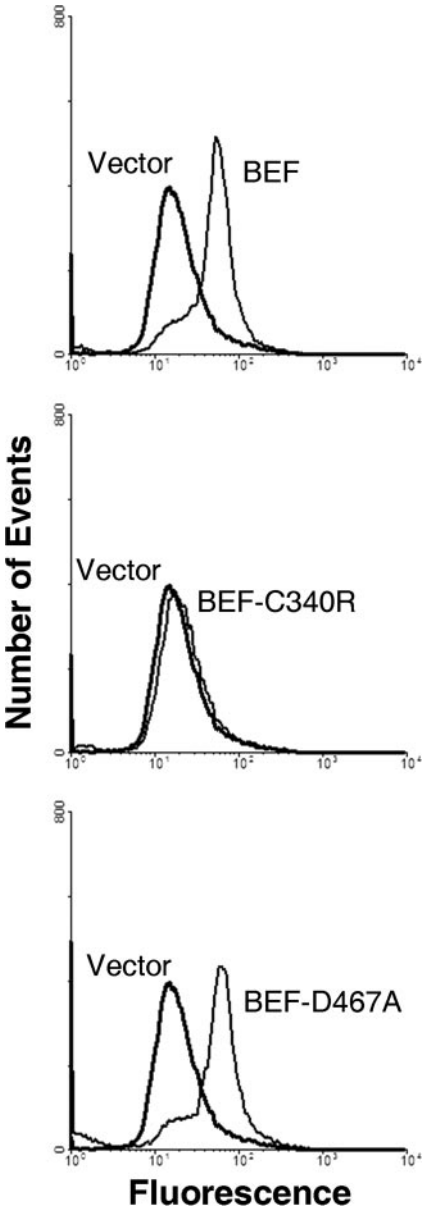

FIG. 7. Effect of BEF expression on autofluorescence. HeLa cells were assayed for autofluorescence by flow cytometry at 5 days after infection with adenoviruses expressing BEF, BEF-C340R (the DNA binding mutant), BEF-D467A (the catalytic mutant), or a control adenovirus (vector).

cells. BEF did not induce senescence as efficiently as the full-length E2 protein, as measured by the fraction of cells displaying increased autofluorescence or SAβ-Gal staining (data not shown). In addition, we used an annexin V binding assay to measure apoptosis at various times after expression of BEF in HeLa cells but did not detect a measurable increase in the extent of apoptosis compared to control adenovirus-infected cells (data not shown).

The induction of senescence in a substantial fraction of HeLa cells by BEF was surprising because the overall cleavage efficiency of BEF in HeLa cells appeared to be low. Quantitative real-time PCR analysis demonstrated that HPV18 E6 and E7 transcript levels decreased approximately two- to threefold in HeLa cells after expression of BEF compared to mock-infected cells (data not shown), suggesting that BEF induced senescence in HeLa cells by repressing the endogenous HPV18 oncogenes. To determine whether this phenotype required DNA cleavage or DNA binding by BEF, we measured autofluorescence in HeLa cells after expression of the DNA binding mutant or the catalytic mutant of BEF. Immunoblotting with FokI antisera demonstrated that the mutants were expressed at levels equal to or greater than wild-type BEF in HeLa cells (data not shown). Expression of the BEF DNA binding mutant did not induce an increase in autofluorescence (Fig. 7, middle panel) or SAβ-Gal staining (data not shown), indicating that the DNA binding domain of BEF is required for the efficient induction of senescence in HeLa cells. Strikingly, however, expression of the BEF catalytic mutant induced a uniform increase in autofluorescence (Fig. 7) and SAβ-Gal staining (data not shown), comparable to that seen with wild-type BEF. Thus, the catalytic activity of BEF was not required for the induction of senescence in HeLa cells. These data suggested that the BEF-mediated induction of senescence is due to DNA binding by BEF and not DNA cleavage, which is consistent with the apparent low cleavage efficiency of BEF in HeLa cells.

DISCUSSION

Integrated HPV DNA in cervical cancer cells provides a model system to test whether a viral DNA binding domain can direct a nuclease domain to cleave viral genomes present in infected cells. To determine whether we could harness the ability of the BPV1 E2 protein to selectively recognize integrated HPV DNA in cervical cancer cells, we fused its DNA binding domain to the nuclease domain of FokI and tested whether this chimeric nuclease, BEF, induced DNA double-strand breaks at papillomavirus E2 BSs.

BEF specifically cleaved DNA substrates containing an HPV or a BPV E2 BS and not DNA substrates lacking a consensus E2 BS, demonstrating that fusion of the FokI nuclease domain did not significantly alter the DNA binding activity of E2. In substrates without E2 BSs, BEF induced a low level of nonspecific cleavage, which was inhibited in the presence of an E2 BS. This finding suggests that BEF can be sequestered to the E2 BS, thereby preventing nonspecific cleavage at other locations in DNA. As is the case with other published studies using FokI-based chimeric nucleases, our mapping experiments revealed that in vitro cleavage by BEF generated DNA double-strand breaks with primarily four-nucleotide 5′ overhangs and that there is minor heterogeneity of the site of strand scission (21, 23–26, 51, 54). The cleavage efficiency of BEF in vitro was relatively low, since the DNA was not typically digested to completion in the cleavage reactions, which could be due to instability of the protein in the reaction, suboptimal cleavage conditions, or low intrinsic catalytic activity of this constructed enzyme.

Although previous studies indicated that FokI dimerization is required for efficient DNA cleavage (7, 57, 59), BEF required only a single E2 BS to induce cleavage in vitro. Similarly, a chimeric nuclease consisting of the FokI nuclease and the yeast Gal4 DNA binding domain, which also binds to its

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recognition site as a dimer, required only a single recognition site for cleavage (23). Because the DNA binding domain of the E2 protein is also a dimerization domain and binds to its recognition site as a dimer, dimerization of the E2 DNA binding domains in the BEF protein most likely brings two monomers of the FokI cleavage domain in close proximity to induce cleavage. The symmetry of the sequence of the E2 BS and of the dimeric E2 DNA binding domain presumably accounts for the ability of BEF to cleave symmetrically on either side of the BS.

BEF cleaved integrated HPV18 DNA in HeLa cells at E2 BS1 and BS4. Thus, BEF can locate and act at its recognition site in the context of genomic DNA in living cells. We detected a very low level of cleavage products from the integrated HPV18 DNA in HeLa cells after expression of BEF, a finding consistent with its relatively inefficient cleavage of DNA in vitro. However, we measured cleavage 30 h after infection, during which time the cellular DNA double-strand break repair machinery may have been activated to repair some of the induced DNA double-strand breaks. BEF cleavage occurred immediately adjacent to the E2 BSs at positions consistent with the results of the in vitro mapping experiments. At both of these E2 BSs, a small fraction of the cloned DNA displayed ends a few base pairs downstream from the primary sites of cleavage in vitro, possibly due to limited melting of the cleaved DNA in these A/T-rich regions and subsequent degradation by cellular exonucleases. The LM-PCRs for E2 BS4 detected several minor bands in addition to the prominent band, which could correspond to cleavage at degenerate E2 BSs or to nonspecific cleavage by BEF in the HPV18 DNA. Although we failed to detect cleavage at the other E2 BSs in the HPV LCR (S. M. Horner and D. DiMaio, unpublished data), it is possible that different reaction conditions or combinations of primers would reveal cleavage at these sites as well.

Expression of BEF, which lacks the transcriptional regulatory domain from BPV1 E2, induced senescence in HeLa cells. Quantitative real-time PCR analysis suggested that the induction of senescence was at least in part due to direct repression of E6 and E7 transcription by BEF, as is the case with the wild-type E2 protein. However, BEF-mediated induction of senescence did not require the nuclease activity of the protein, although it did depend on its DNA binding activity. Therefore, BEF-mediated induction of senescence was probably due to the ability of BEF to bind to the E2 BSs in the HPV promoter and repress expression of E6 and E7, perhaps by occluding transcription factor binding. Because BEF can cleave at a cellular E2 BS, it is also possible that the action of BEF at cellular E2 BSs may contribute to the induction of the senescent phenotype.

Previous studies showed that mutants of native BPV1 E2 lacking a functional transcriptional regulatory domain or chimeric proteins of the BPV1 E2 DNA binding domain and the herpes simplex virus VP16 transactivation domain were unable to repress E6 and E7 transcription or induce cellular growth arrest (13, 16, 34), suggesting that a function inherent to the E2 transcriptional regulatory domain is required for the repression of E6 and E7 and induction of growth arrest. Although BEF lacks the transcriptional regulatory domain of the native BPV1 E2 protein, the presence of the FokI nuclease domain may contribute to the repressive properties of BEF. In addition, in our experiments we used a recombinant adenovirus to express BEF, and the relatively high levels of expression of BEF in this system may obviate the requirement of the E2 transcriptional regulatory domain for HPV repression and induction of senescence.

Our work demonstrates that the papillomavirus E2 protein can be used to direct nuclease domains to induce site-specific DNA double-strand breaks at E2 BSs in cervical cancer cells. It may be possible to extend this approach to target, and perhaps eliminate, HPV-infected cells or the HPV genomes or gene expression in premalignant and malignant cells, either by induction of apoptosis or by inaccurate repair of DNA double-strand breaks. However, before this strategy can be used for this purpose, the design of the chimeric nuclease would have to be optimized to induce more efficient cleavage. Altering the length of the flexible linker between the two functional domains of the protein or varying the exact sites of fusion may increase the overall cleavage efficiency. In addition, the specificity of the chimeric nuclease for the viral E2 BSs compared to cellular E2 BSs or DNA lacking E2 BSs would need to be increased. The close proximity of the two E2 BSs (BS1 and BS2) immediately upstream of the transcriptional start site for the E6 and E7 genes provides an opportunity to increase specificity. These sites are separated by only four nucleotides in HPV18 DNA (see Fig. 4A). By engineering the chimeric nuclease to take advantage of this arrangement of the viral E2 BSs, for example, by appending protein-protein interaction domains to favor cooperative binding at these sites, it might be possible to effectively increase the recognition site to 16 bp, so that DNA could be cleaved preferentially at viral E2 BSs, rather than at any solitary E2 BS in cellular DNA. A 16-bp recognition site is predicted statistically to be unique in the human genome. If such sequence specificity is obtained, the chimeric nuclease may be able to cleave viral DNA in infected cells while sparing the DNA of uninfected cells.

Other approaches to target papillomaviruses at the DNA level have been described. Carson et al. exploited homologous recombination in papillomavirus-infected cells to induce expression of a suicide gene for selective killing of infected cells (8). Mino et al. constructed ZFPs that bind to HPV18 DNA, block the HPV18 E2 protein from binding to DNA, and inhibit HPV18 replication in transient assays (32). While this approach could prevent establishment of HPV infection, such a method that primarily interferes with viral DNA replication would not be effective against the integrated HPV DNA in cervical cancer cells, which replicates passively with the cellular DNA. In contrast, the induction of DNA double-strand breaks by a chimeric nuclease would target both episomal DNA present during an initial HPV infection and integrated HPV DNA in cervical cancer cells. These targeted DNA double-strand breaks could result in irreversible changes in the viral DNA, leading to either cell death or permanent growth arrest. Expression of BEF in virally infected cells could also be used to exploit cellular homology-directed repair mechanisms to introduce sequences to inactivate the viral genomes in these cells, as has been done for the targeted manipulation of cellular recognition sites with zinc finger nucleases in combination with repair templates (2, 40, 56).

We have demonstrated that a viral DNA binding protein can direct a nuclease domain to cleave at specific DNA sequences

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in virally infected cells. Unlike zinc finger nucleases which require extensive engineering and/or selection to achieve their requisite specificity, this chimeric nuclease did not require any modification of the viral DNA binding domain to act with its predicted specificity in cells. Many viruses encode DNA binding proteins with greater specificity than the papillomavirus E2 protein and may serve as the source of viral DNA targeting moieties. For example, Epstein-Barr virus (EBV) encodes a site-specific DNA binding protein Rta that binds to an 18-bp recognition site in EBV DNA and is involved in lytic reactivation of the virus (9, 17, 52). Therefore, it is possible that chimeric nucleases containing this DNA binding domain would induce site-specific cleavage in EBV but not cellular DNA, possibly inducing EBV-specific cell death or preventing viral DNA replication. Furthermore, although the native E2 protein, as well as BEF, repressed viral gene expression, most viral DNA binding proteins do not block virus replication or inhibit cell growth. Thus, chimeric nucleases using DNA binding domains of proteins from other viruses may well induce novel phenotypes not caused by their native counterparts. Indeed, increasing the cleavage efficiency and specificity of BEF may reveal phenotypes due to BEF-mediated cleavage of integrated papillomavirus genomes in cervical cancer cells. It may also be possible to use related strategies with viral DNA binding domains to direct other effector domains to viral DNA in infected cells.

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