Anomalous Transforming Behavior of a Bovine Papillomavirus Type 1 Mutant with an Upstream Promoter Mutation

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We show that the previously described high-transformation phenotype of the bovine papillomavirus type 1 mutant BPV730 is manifested only in an E6-dependent cell transformation assay. The BPV730 mutation was associated with superinduction of the putative E6 promoter, P₈₉, after cycloheximide treatment, and with reduced activity of the P₃₀₈₀ promoter.

Bovine papillomavirus type 1 (BPV-1) is a DNA tumor virus which causes benign skin warts in cattle. It has long been studied as a prototype for other animal and human papillomaviruses which transform tissue culture cells less efficiently than BPV-1 does. Two separate BPV-1 transforming genes (E5 and E6) have been identified (3, 11, 12, 16), although the relative contributions of these two transforming genes to cell transformation in C127 focus assays have been reported to differ markedly in separate studies (4, 7, 8, 10).

In this study we have analyzed the transforming properties of mutant BPV730, which was isolated by Schiller and colleagues from a library of random linker insertion BPV-1 mutants on the basis of its elevated transforming activity relative to that of wild-type (wt) BPV-1 in a C127 cell focus assay (10). This mutant has a deletion of 28 bp of the viral genome between nucleotides (nt) 7889 and 7918 and an 8-bp *Xho*I linker insertion. This mutation is located in a complex regulatory region of the viral genome, containing an E1- and E2-dependent origin of viral DNA replication (14, 15) and the transcription initiation sites of two early promoters, P₇₉₄₀ and P₈₉. An E2 binding site (nt 7896 to 7907) was removed by this deletion.

Transforming properties of BPV730. We transfected subconfluent C127 cell cultures in 5-cm-diameter tissue culture dishes with 0.5 µg of the wt BPV-1 recombinant plasmid DNA p142-6 (9) or with 0.5 µg of BPV730 recombinant plasmid DNA mixed in each case with carrier DNA (sonicated salmon sperm DNA) to give a total amount of 10 µg of DNA per dish. The recombinant plasmid DNAs were digested with *Bam*HI prior to transfection to separate the viral DNA sequences from the bacterial plasmid DNA sequences. Control C127 cultures were transfected with carrier DNA alone. As reported by Chiang et al. (2), we observed no significant difference in the focus-forming activities of wt BPV-1 DNA and BPV730 DNA (Table 1).

To investigate the relative contributions of the E5 and E6 genes to the focus-forming activity of BPV730, we tested the transforming abilities of BPV730 E5-minus (BPV730 E5⁻) or BPV730 E6⁻ double mutants and compared these with the corresponding E5 or E6 mutants in a wt BPV-1 background. For additional controls, we prepared a BPV730 E5⁻ E6⁻ triple

mutant as well as the corresponding E5⁻ E6⁻ double mutant in a wt BPV-1 genomic context.

As observed previously in our laboratory (5), inactivation of the E5 gene in the wt genetic background resulted in a drastic reduction in focus-forming activity, indicating that E5 was the dominant transforming gene under our assay conditions. In support of this conclusion, the BPV E6⁻ mutant displayed a transforming activity similar to that of the wt BPV-1 genome (Table 1). Transformed foci induced by the BPV E5⁻ mutant were also fewer in number and smaller and expanded more slowly than foci induced by the BPV E6⁻ mutant or by wt BPV-1. Because of the much weaker transforming activity of the E6 gene, transformed foci were scored 3 to 4 weeks after transfection as opposed to 2 to 3 weeks in experiments involving viral genomes with an intact E5 gene. As expected, the BPV E5⁻ E6⁻ double mutant was inactive in this focus assay, confirming that the weak E5-independent viral transforming activity was dependent on expression of the E6 gene (Table 2).

When these mutations were tested in a BPV730 genomic context, we observed a significant difference in the transforming activity of the BPV730 E5⁻ mutant compared with that of BPV E5⁻ (Fig. 1 and Table 2) but observed essentially no difference in the transforming activities of the corresponding E6⁻ mutants (Table 1). The BPV730 E5⁻ mutant induced approximately 10-fold more transformed foci than the BPV E5⁻ mutant did. Moreover, transformed foci induced by the BPV730 E5⁻ mutant were often larger and expanded more rapidly than the control BPV E5⁻-induced foci, while still displaying the more compact morphology characteristic of E6 transformed foci (5).

Despite the higher E6-dependent transforming activity of the E5-minus BPV730 derivative, the relative contribution of E6 to the focus-forming activity of the BPV730 mutant (with intact E5 and E6 genes) was still relatively minor because of the dominant activity of E5 as the major transforming protein in our assays. Thus, although the number of foci induced by the BPV730 E5⁻ mutant was similar to the number of foci induced by wt BPV-1 in the same experiment (not shown), transformed foci induced by this mutant were still very small (average diameter, 1 to 2 mm) after 2 weeks, whereas wt BPV-1-induced foci were much larger (average diameter, 4 to 5 mm) after the same time period. This likely explains why we observed no significant difference in focus-forming activities between the wt viral genome and the BPV730 mutant genome with intact E5 and E6 genes, since under such conditions we were monitoring primarily the transforming activity of E5, and as shown above,

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TABLE 1. Focus-forming activities of BPV linker insertion mutants

Expt	No. of transformed foci/dish ^a					
	WT BPV-1	BPV730	BPV E6 ⁻	BPV730 E6 ⁻	BPV730 E1 ⁻	BPV783 (E1 ⁻)
1	53	18	48	58	18	ND
2	57	68	46	54	ND	5
3	40	30	ND	ND	5	4

^a Each value is the average for three dishes transfected with 0.5 µg of *Bam*HI-cleaved recombinant plasmid DNA and counted 2 to 3 weeks after transfection. Mock-infected cells all had no transformed foci per dish. ND, not done.

there was no evidence for an altered level of expression of E5 in the BPV730 mutant.

Schiller and colleagues suggested that BPV730 may be defective in the expression of a full-length E1 transcriptional repressor protein, since it displayed a high-transformation phenotype similar to that of replication-defective E1 linker insertion mutants (10). Our results appear to be inconsistent with this proposal, because as reported elsewhere, we found that BPV730 replicated as a multiple-copy-number extrachromosomal plasmid at approximately wt copy number in C127 cell lines transformed by this mutant (13). The BPV730 E5⁻ double mutant also replicated as a plasmid in each of four transformed cell lines tested (not shown), showing that the high E6-dependent transforming activity of this recombinant plasmid was not an indirect consequence of a replication defect. In fact, the high E6-dependent transforming activity of mutant BPV730 E5⁻ was significantly reduced by insertion of an E1 frameshift mutation, showing that E1 was required for the efficient E6-dependent focus-forming activity of this mutant (Table 2).

Analysis of viral early promoter activity in BPV730. We next performed primer extension analysis in order to investigate the effect of the BPV730 upstream promoter mutation on viral mRNA transcription from the P₇₉₄₀ promoter and from the nearby P₈₉ promoter, which is believed to regulate expression of the E6 transforming gene. Total cytoplasmic RNA was prepared from cell cultures with or without cycloheximide treatment (25 µg/ml, 4 h) to induce viral transcription (6).

The results of a primer extension analysis of the P₈₉ and P₇₉₄₀ promoters are shown in Fig. 2A. We observed in the absence of cycloheximide, only a slight increase in the level of transcripts initiated from P₈₉ in the mutant genome. No

TABLE 2. High E6-dependent transforming activity of mutant BPV730

Expt	No. of transformed foci/dish ^a					
	BPV E5 ⁻	BPV730 E5 ⁻	BPV E5 ⁻ E6 ^{-b}	BPV730 E5 ⁻ E6 ⁻	BPV730 E1 ⁻ E5 ⁻	BPV760 E5 ⁻
1	4	53	0	0	ND	16
2	5	63	0	0	12	21
3	2	38	ND	ND	9	7

^a Each value is the average for three dishes transfected with 0.5 µg of *Bam*HI-cleaved recombinant plasmid DNA and counted 3 to 4 weeks after transfection. Mock-infected cells all had no transformed foci per dish. ND, not done.

^b The E5 and E6 mutations used in this study have been described previously, both consisting of frameshifting *Xho*I linker insertion mutations positioned at nt 3881 within E5 (12) and at nt 240 within E6 (7). Each of these mutations has earlier been shown to inactivate the transforming potential of the respective viral gene.

transcripts initiated from P₇₉₄₀ were observed for either the mutant or wt genome in untreated cell cultures. This result was not unexpected, because previous studies in this laboratory have shown that transcription from P₇₉₄₀ is very weak or undetectable in cells grown in exponential-phase cultures (1). After performing cycloheximide treatment, we observed transcriptional induction of the P₈₉ promoter in both the wt and mutant genomes, with a substantially higher level of induction seen for the mutant genome.

Cycloheximide treatment also resulted in induction of the P₇₉₄₀ promoter in the BPV730 mutant, demonstrating that this promoter was not inactivated by the promoter mutation. As with P₈₉, there was a higher level of induction of P₇₉₄₀ in the mutant than in the wt, which exhibited very weak signals from P₇₉₄₀ even after cycloheximide treatment. The multiple bands observed between the P₈₉ and P₇₉₄₀ initiation sites for the mutant genome were most likely due to premature termination of reverse transcriptase during the primer extension reaction rather than to novel transcription initiation sites. Nevertheless, we did see in repeated analyses that there was some heterogeneity in the start sites in the immediate vicinity of nt 7940, suggesting that the P₇₉₄₀ promoter mutation caused some variability in the positioning of the initiation sites in this promoter.

We also examined transcription from the two other major early viral promoters, P₂₄₄₃ and P₃₀₈₀. We found essentially no difference in the level of transcription from the P₂₄₄₃ promoter

FIG. 1. E6-dependent transforming activity of BPV730. The effect of an E5 frameshift mutation or combined E5 and E6 mutations on the focus-forming activities of wt BPV-1 (BPV) and recombinant BPV730 plasmids are shown. The cultures were fixed and stained 4 weeks after transfection with the indicated mutant plasmid DNAs (0.5 µg per dish), which had been digested with *Bam*HI prior to transfection.

FIG. 2. Primer extension analysis of viral early promoters. (A) Analysis of relative levels of transcription from the upstream promoters P_{89} and P_{7940} in wt BPV-1-transformed cells (WT) and BPV730-transformed cells (730) either before or after cycloheximide (+CH) treatment. Equal amounts of total cytoplasmic RNA from transformed cell lines and from control nontransformed C127 cells were subjected to primer extension analysis by using a 32 P-labelled 20-nt minus-strand primer with a 5' end at BPV-1 nt 208, as described previously (1). A DNA sequence ladder (GATC) obtained with the same end-labelled primer was run as a marker. (B) Primer extension analysis of the P_{2443} promoter using an end-labelled minus-strand 20-mer primer with a 5' end at BPV-1 nt 2551. The long arrow indicates the position of the 2443 nucleotide, and the shorter arrows indicate the positions of alternative transcription initiation sites observed in this experiment. (C) Primer extension analysis of the P_{3080} promoter using an end-labelled 20-mer primer with a 5' end at BPV-1 nt 3178.

between the wt and mutant genomes in the absence of cycloheximide and a somewhat greater level of induction with cycloheximide (Fig. 2B).

In sharp contrast to the other viral promoters analyzed, we observed a reduction in the level of P_{3080} transcription in the mutant genome compared with that in the wt genome both before and after cycloheximide induction (Fig. 2C). Both the constitutive and induced levels of transcription from this promoter were reduced by approximately one-half relative to those of the wt genome. This promoter regulates expression of the short-lived transcriptional repressor E2TR.

In conclusion, our present results appear to confirm the suspicion that there are significant differences in the assay conditions employed in different laboratories to monitor BPV-1-induced focus formation in C127 cells. In our laboratory, as in a number of others, E5 is the dominant viral transforming activity assayed by transformation of C127 cells. Regulatory mutations which specifically activate expression of the E6 transforming gene are likely, therefore, to be overlooked with such an assay system. In future studies of the regulation of BPV-1 transforming gene expression, it may be useful to determine the effects of specific promoter or enhancer mutations on E5 and E6 gene expression in separate E5- and E6-dependent transformation assays, as we have described here. In this study, we have shown that mutant BPV730 exhibits high transforming activity in an E6-dependent transformation assay, and we have presented evidence to indicate that this phenotype may be linked to reduced activity of the P_{3080} E2TR promoter and up-regulation of the E6 promoter. This viral mutant may be useful in further studies of the coordinate regulation of BPV-1 early promoters, which is complex and, at present, poorly understood.

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