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Genetic Evidence that Acute Morphologic Transformation, Induction of Cellular DNA Synthesis, and Focus Formation Are Mediated by a Single Activity of the Bovine Papillomavirus E5 Protein

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The bovine papillomavirus (BPV) type 1 E5 gene encodes a 44-amino-acid protein that can stably transform cultured rodent cells when expressed in the absence of all other viral genes. We have previously constructed a BPV-simian virus 40 recombinant virus (Pava-1) which efficiently expresses the BPV type 1 E5 gene in infected cells (J. Settleman and D. DiMaio, Proc. Natl. Acad. Sci. USA 85:9007–9011, 1988). Within 48 h of Pava-1 infection, the vast majority of mouse C127 cells underwent a dramatic morphologic transformation which was accompanied by cell proliferation. Infection of C127 cells made quiescent by contact inhibition and serum starvation caused a great induction of cellular DNA synthesis. These morphologic and mitogenic responses were proportional to the virus multiplicity of infection. Mutational analysis indicated that the E5 gene is both necessary and sufficient for these activities. Analysis of a variety of E5 missense mutants revealed a strong correlation between their phenotypes in the acute transformation assays following infection and in the stable focus-forming assay following transfection. Most of the defective mutants expressed normal levels of E5 protein following infection, indicating that their defective phenotypes are not due to the synthesis of an unstable protein. The failure to genetically resolve these E5 activities suggests that the ability of the E5 protein to cause acute morphologic transformation and reentry into the cell cycle may be intimately related to its ability to cause stable cell transformation and that these functions are probably mediated by a single biochemical activity of the E5 protein.

Cellular and viral oncogenes have been detected usually because they are able to cause tumors in animals or stable transformation of cultured cells. Tumorigenesis and stable transformation, however, are the culmination of a complex series of events including, at a minimum, stable expression of the oncogene, action of its encoded protein product, and, quite possibly, rare cellular events that allow the clonal expansion of transformed cells. Considerable effort has been expended in trying to elucidate the signal transduction pathways that lead from an oncogene product to mitogenesis and transformation, but this analysis can be complicated by secondary events during the development of stably transformed cell lines. Ideally, this problem can be alleviated by studying the biochemical activities of purified oncogenic proteins and the acute biologic effects of these proteins on cells.

The DNA tumor viruses have been a rich source of proteins with transforming activity, but it has been difficult to determine the specific biochemical activities of these proteins that mediate transformation. This is partly because the compact genomes of these viruses frequently require that a single viral protein carry out several different functions. The simian virus 40 (SV40) large T, polyomavirus middle T, and adenovirus E1a transforming proteins each exhibit several biochemical and biologic activities, some of which appear to play a role in cell transformation. Mutational analysis of the genes which encode these proteins has revealed that the various activities frequently map to separate protein domains, and the transforming domain(s) of the protein can often be resolved from domains which mediate its other activities (for reviews, see references 13–15). Moreover, more than one biochemical activity or protein domain often seems to be involved in transformation. For example, although microinjection of SV40 large T antigen into quiescent cells induces cellular DNA synthesis, which is thought to play a role in transformation, mutational analysis has revealed that some T-antigen mutants which retain this ability are unable to induce stable cell transformation (17, 23). Furthermore, the immortalizing and transforming activities of large T antigen can be at least partially resolved, suggesting that more than one activity of this protein may be involved in mediating various aspects of the transformed phenotype (25).

In contrast to these complex multifunctional transforming proteins, the bovine papillomavirus type 1 (BPV-1) E5 gene encodes a transforming protein of only 44 amino acids that, in the absence of all other viral genes, can cause the stable transformation of cells (1, 2, 4, 8, 19, 21, 26). The small size of the E5 protein makes it unlikely that it contains multiple domains with independent activities, and our previous mutational analysis of the E5 protein suggests that most of the specific amino acids responsible for its focus-forming activity are clustered in a single functional domain (8, 9). We have previously described the development of a BPV-SV40 recombinant virus (called Pava-1) which efficiently expresses the E5 transforming protein in infected cells and

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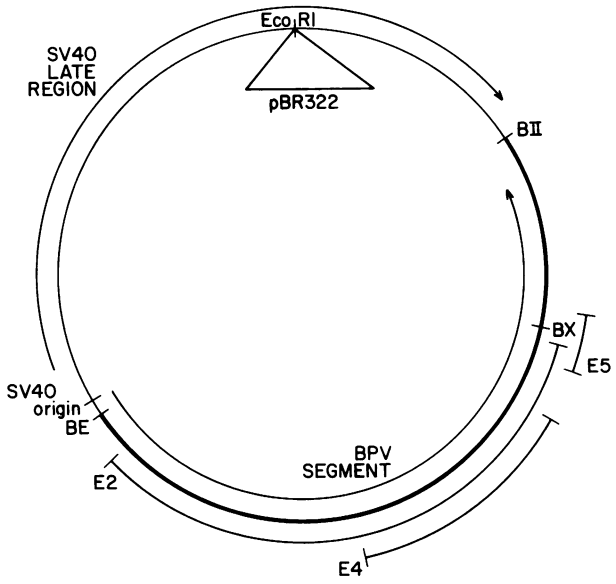


FIG. 1. Structure of pPava-2. pPava-2 contains the BPV-1 sequences encoding the E2, E4, and E5 ORFs (heavy line) linked in the correct orientation to be transcribed from the SV40 early promoter. Arrows indicate the direction of transcription. The plasmid also contains the intact SV40 late region and origin of replication. Viral DNA is inserted into pBR322 as indicated. Restriction sites for *Bgl*II (BII), *Bst*EII (BE), and *Bst*XI (BX) are also indicated (modified from reference 20).

causes rapid morphologic transformation of mouse C127 cells (22). Here, we further characterize this acute transformation and present evidence that a single E5 activity is probably responsible for causing acute morphologic changes, loss of contact inhibition, induction of cellular DNA synthesis, and stable cell transformation.

MATERIALS AND METHODS

Plasmids. Plasmid pPava-1 (see Fig. 1), which contains the SV40 late region and origin of replication and the 3'-transforming region (open reading frames [ORFs] E2, E4, and E5) of BPV-1, has been previously described (22). pPava-E2am1 and pPava-E5d29 are derivatives of pPava-1 containing a premature termination codon in ORF E2 (3) and a 29-base-pair deletion-frameshift in ORF E5 (22), respectively. pPava-E4am1 is a pPava-1 derivative which contains a premature amber termination codon in ORF E4 (16). pPava-BMV is a Pava-1 derivative in which a 101-base-pair *Bcl*I fragment at the extreme 3' end of ORF E2 is replaced with an *Eco*RV linker. This mutation inactivates both the E2 transactivation and repression functions (D. DiMaio, unpublished observations). To facilitate subcloning of the E5 mutants, pPava-1 plasmid DNA was modified by the insertion of a unique *Bgl*II site at the 3' end of the BPV-1 sequences to generate plasmid pPava-2 (Fig. 1). pPava-2 plasmid DNA was digested with *Bst*XI and *Bgl*II, and the large fragment was purified by gel electrophoresis. Plasmids containing E5 missense mutations in the full-length BPV-1 genome (8) were digested with *Bst*XI (nucleotide 3881) and *Bam*HI (nucleotide 4450), and the 569-base-pair fragment containing the E5 gene was purified by gel electrophoresis. E5 mutant fragments were then ligated to the pPava-2 fragment to generate recombinant viral plasmids containing the E5 missense mutations.

Preparing recombinant virus stocks. High-titer stocks of Pava-1 recombinant virus and its mutant derivatives were prepared as previously described (22). Briefly, pPava plasmid DNA was digested with *Eco*RI to release the pBR322 sequences, and the gel-purified viral sequences were circularized by ligation and transfected into CMT4 cells (5) which were induced with heavy metals to express high levels of SV40 large T antigen. After several days, virus particles containing the hybrid genome were harvested and then repassaged twice on fresh CMT4 cells to generate high-titer virus stocks to be used in experiments. To confirm the sequence of the E5 mutations in the various virus stocks, CMT4 cells were infected with each stock, and after 48 h, viral DNA was recovered from the cells and used as a template to directly sequence the E5 region.

Virus-mediated transactivation assay. To determine the relative titers of the various recombinant virus stocks, a virus-mediated quantitative transactivation assay which measures expression of the BPV-1 E2 gene was performed as previously described (22), with minor modifications. Briefly, NL-3D cells, which contain integrated copies of the BPV-1 long control region linked to the *Escherichia coli* β-galactosidase gene, were infected in quadruplicate with recombinant virus stocks at a dilution of 1:20,000 in 96-well plates, and after 48 h, β-galactosidase activity was determined in a colorimetric assay following the addition to each well of the β-galactosidase substrate chlorophenol red-β-D-galactopyranoside (1 mM) in a solution of 50 mM KPO_4–10 mM $MgCl_2$–0.2% Triton X-100. Enzyme activity, which is proportional to multiplicity of infection, was quantitated by spectrophotometry at 574 nm and compared with a standard curve constructed after infection with a wild-type stock of known titer.

Detection of the E5 protein. CMT4 cells in 10-cm-diameter dishes were infected at 80% confluence with 5 infectious units of mutant virus stocks per cell in the presence of 1 μM $CdSO_4$ and 100 μM $ZnCl_2$. After 48 h, the cells were metabolically labeled with 1 mCi of [^{35}S]methionine for 5 h, and total cellular protein was extracted and immunoprecipitated with an anti-E5 antiserum as previously described (21). The products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.

Cellular DNA synthesis assay. C127 cells in 24-well plates were infected in duplicate in serum-free medium immediately after reaching confluence and were maintained at 37°C in serum-free medium. At various times following infection, the cells were incubated for 2 h in medium containing 1.5 μCi of [^{3}H]thymidine per ml. The cells were then washed two times with cold phosphate-buffered saline followed by two washes in cold 10% trichloroacetic acid (TCA). During the second TCA wash, the cells were maintained at 4°C for 20 min. After the TCA was removed, the cells were incubated in 3% perchloric acid at 90°C for 25 min to hydrolyze the DNA. The perchloric acid was then added to scintillation fluor, and the radioactivity was counted. In general, values for duplicate samples differed by less than 15%.

Autoradiography. C127 cells at confluence on glass slides were infected with 250 infectious units per cell in serum-free medium and were maintained in serum-free medium. After 24 h, 5 μCi of [^{3}H]thymidine was added to the medium, and the cells were incubated at 37°C for 36 h. The cells were fixed after being washed in phosphate-buffered saline by the addition of methanol-acetic-acid (3:1). After 10 min at room temperature, the fixative was removed, the slide was dried in

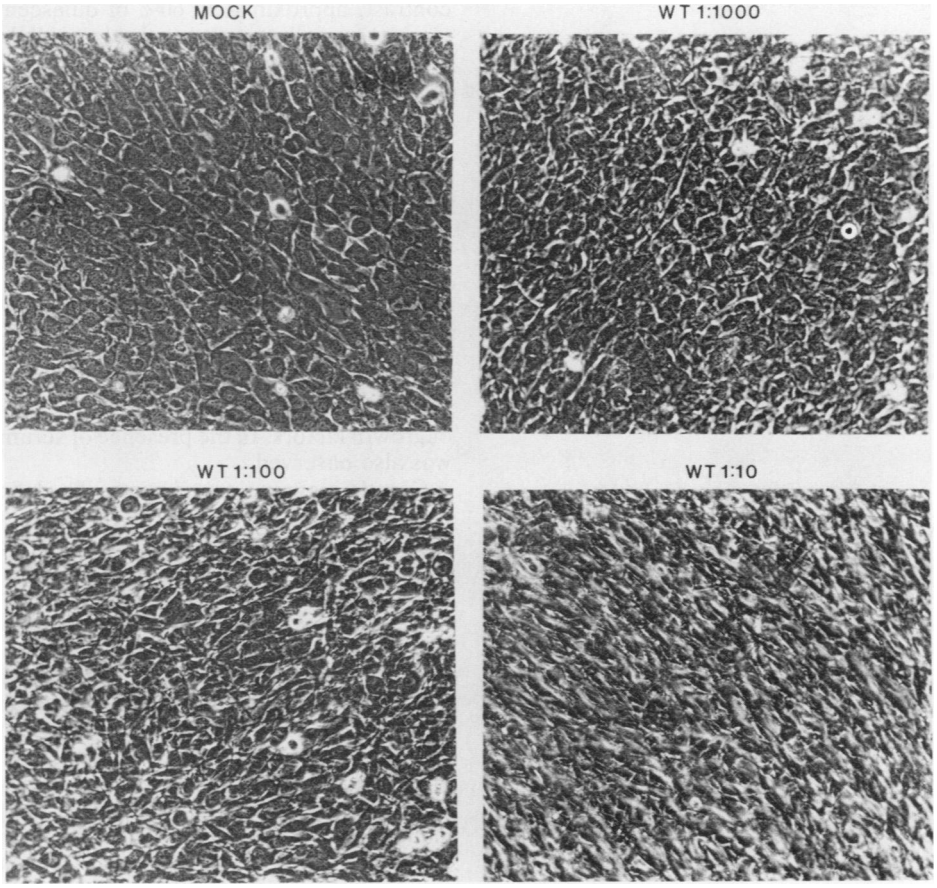


FIG. 2. Morphologic transformation of C127 cells after Pava-1 infection. C127 cells at 90% confluence were either mock infected or infected with various dilutions of wild-type (WT) Pava-1, as indicated. A 1:10 dilution of virus corresponds to about 1,000 infectious units per cell. Forty-eight hours later, the cells were photographed by using phase-contrast microscopy (magnification, ×150).

air, and the cells were treated with 5% TCA for 5 min at 4°C. The TCA was then aspirated, and the cells were washed three times with 70% ethanol and three times with 100% ethanol and allowed to dry in air. The slide was then coated with nuclear track emulsion (NTB 2; Eastman Kodak Co.) and allowed to expose for 10 days at 4°C. The slide was developed for 5 min with D19 developer, fixed for 10 min, rinsed with water, and mounted with 50% glycerol in phosphate-buffered saline. Labeled nuclei were photographed under phase-contrast microscopy.

RESULTS

Rapid morphologic transformation of Pava-1-infected C127 cells. To assess the rapid transforming ability of Pava-1 virions, C127 cells at 90% confluence were infected at a high virus multiplicity of infection, maintained in medium containing 10% fetal calf serum, and observed microscopically for the appearance of morphologic transformation. At 24 h after infection, the cells began to display morphologic alterations, which were most dramatic 72 to 96 h after infection. The cells became spindle shaped and refractile, overgrew the monolayer, and rapidly acidified the medium, appearing much like cells stably transformed by BPV-1 (Fig. 2). The degree of morphologic transformation was dependent on the dose of virus over a range of virus multiplicity between approximately 2 and 1,000 infectious units per cell. At a low virus multiplicity, the monolayer appeared disorganized and clearly distinct from the mock-infected monolayer. However, at this multiplicity, the cells did not significantly overgrow each other and were not as spindle shaped as stably transformed cells. As the multiplicity of virus was increased, the infected monolayer appeared increasingly transformed, until a plateau was reached, at which point increases in the virus dose had no apparent further effect on the cells. The morphologic changes detected after Pava-1 infection were accompanied by an increase in cell number which, at 4 days after infection, reached a maximum of about threefold over mock-infected cells (data not shown). This indicates that Pava-1-infected cells are able to escape contact inhibition and reach a higher saturation density than untreated C127 cells.

Pava-1 infection induces cellular DNA synthesis in quiescent C127 cells. Microinjected BPV-1 DNA or a chemically synthesized BPV-1 E5 peptide has been shown to cause an induction of cellular DNA synthesis in quiescent C127 cells (6, 11). To assess the ability of Pava-1 to induce cellular DNA synthesis in quiescent C127 cells and to quantitate the acute transforming activity of the virus, we tested the effect of Pava-1 infection on C127 cells made quiescent by contact inhibition and serum starvation. Cells at confluence were infected with Pava-1 and maintained in serum-free medium.

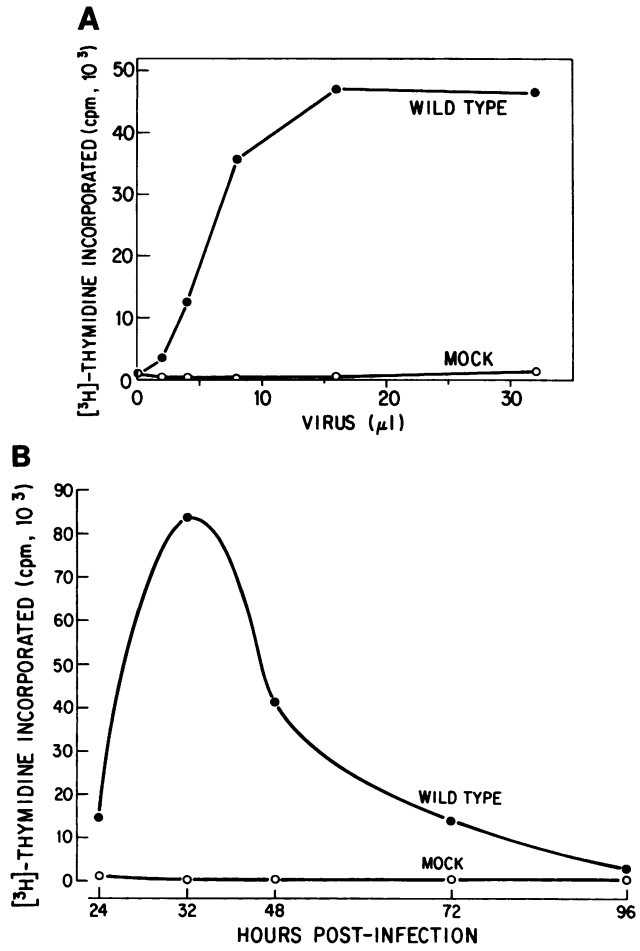


FIG. 3. Induction of cellular DNA synthesis in Pava-1-infected C127 cells. (A) C127 cells at confluence were infected in serum-free medium with various dilutions of virus and mock lysate. In this experiment, 1 μl of virus corresponds to about 15 infectious units per cell. After 40 h, cells were labeled for 2 h with 1.5 μCi of [^{3}H]thymidine per ml, and the incorporation of thymidine into cellular DNA was quantitated as TCA-precipitable counts. (B) The time course of the induction of cellular DNA synthesis following Pava-1 infection was determined by monitoring the incorporation of [^{3}H]thymidine into TCA-precipitable counts at several time points after infection at a multiplicity of infection of about 100 or after mock infection with an equivalent amount of CMT4 lysate.

After 40 h, DNA synthesis was measured as the incorporation of [^{3}H]thymidine into TCA-precipitable material during a 2-h labeling period. Viral infection caused an induction of cellular DNA synthesis which was proportional to the multiplicity of infection, and at a virus multiplicity of about 250 the extent of synthesis reached a maximum of about 200-fold over that seen in mock-infected cells (Fig. 3A). Maximal induction of cellular DNA synthesis occurred at about 30 to 40 h after infection (Fig. 3B). To determine the proportion of infected cells which were induced to undergo DNA synthesis, quiescent C127 cells were mock infected or infected with Pava-1 at a high multiplicity. At 24 h postinfection, the cells were incubated for 36 h with [^{3}H]-thymidine and then processed for autoradiography (Fig. 4). Only 2% of the mock-infected cells synthesized DNA, demonstrating that the cells were in fact made quiescent. In contrast, approximately 60% of quiescent cells exposed to virus were induced to synthesize cellular DNA. Furthermore, under the serum-free conditions, the majority of infected cells underwent a morphologic transformation. Unlike cells infected in the presence of serum, there was no substantial increase in cell number (data not shown). About 84% of the quiescent cells which were treated with medium containing 10% fetal bovine serum for 20 h prior to labeling underwent cellular DNA synthesis. Approximately 10 times less virus is required to detect morphologic transformation in serum-containing medium than is required to detect an induction of cellular DNA synthesis, suggesting that the former may be a more sensitive assay of Pava-1-induced transformation (data not shown). Thus, Pava-1 infection of contact-inhibited, serum-starved cells can induce morphologic transformation and the transit to S phase in the absence of growth factors. In the presence of serum, cell proliferation was also observed.

Genetic mapping experiments. To define the genetic requirements for the acute morphologic transformation of C127 cells following Pava-1 infection, several BPV mutations were separately subcloned into the recombinant viral genome. The mutant viruses were packaged, and the relative titers of mutant virus stocks were determined by using the virus-mediated quantitative transactivation assay, which measures expression of the E2 gene, which is also contained in Pava-1. These virus stocks were then tested for acute transforming ability after cells were infected with 150 infectious units per cell (Table 1). Infection of C127 cells with a virus containing a mutation which prevents expression of the E4 ORF (Pava-E4am1) caused rapid morphologic transformation of the monolayer and induction of cellular DNA synthesis to the same degree as seen in Pava-1-infected cells. In contrast, a deletion-frameshift mutation which prevents E5 expression (Pava-E5d29) completely abolished morphologic transforming activity, indicating that the E5 gene is required for the rapid transforming activity of the virus. The E5 mutant was also defective for stimulation of cellular DNA synthesis (Fig. 4). Thus, as is the case for stable transformation by transfected genomic BPV-1 DNA, ORF E5, but not ORF E4, is essential for efficient rapid transformation.

ORF E2 encodes both a transactivator and repressors of viral gene expression (12, 24), and we have recently shown that E2 products can regulate expression of the E5 protein in the context of the full-length BPV-1 genome (18). To examine the role of ORF E2 in this acute transformation system, the two ORF E2 mutations described in Materials and Methods were cloned into Pava-1. These Pava-1 derivatives were packaged, and their relative titers were estimated by quantitating the amount of replicated viral DNA obtained from CMT4 cells infected with each at a low multiplicity. Mutations that inactivate the E2 transactivator (Pava-E2am1) or simultaneously inactivate both transactivator and repressor (Pava-BMV) did not interfere with efficient induction of morphologic transformation and cellular DNA synthesis (Table 1). These results indicate that no recognized E2 product is required for acute transformation in this system (although, as noted previously, E2 mutations can affect the duration of the transformation response [22]).

Phenotypes of E5 missense mutations in the acute transformation assay. The results described above suggest that E5 is the major, and probably sole, transforming gene in the acute transformation of C127 cells after Pava-1 infection. We have previously described the phenotypes of a large number of E5 missense mutants in the standard C127 cell focus-forming

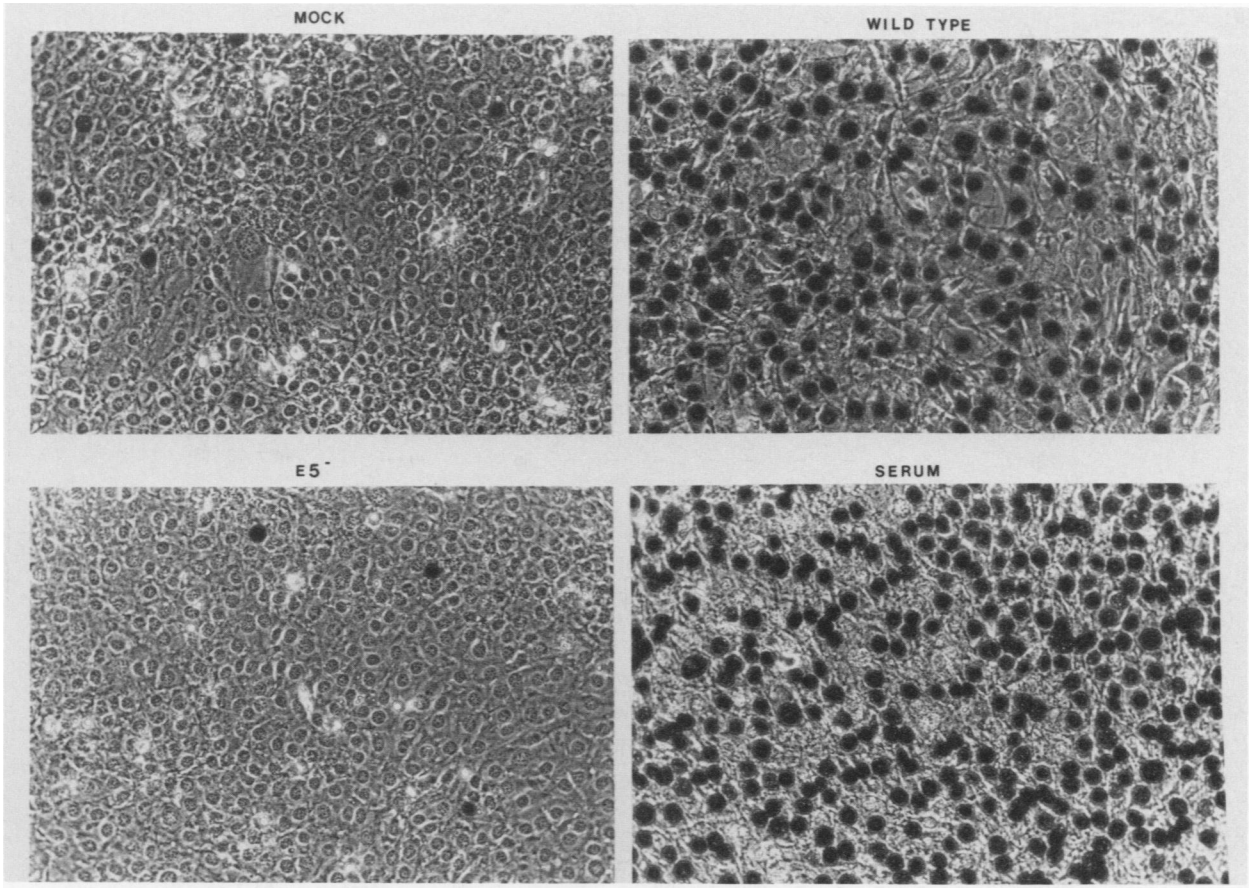


FIG. 4. Autoradiography of Pava-infected C127 cells. C127 cells at confluence in serum-free medium were either mock infected or infected with Pava-1 (wild type) or Pava-E5d29 ($E5^-$), as indicated, with 250 infectious units per cell. Cells exposed to medium containing 10% fetal bovine serum are also shown. After 24 h, the cells were incubated for 36 h with medium containing 5 μCi of [^{3}H]thymidine per ml and then processed for autoradiography. Labeled nuclei were visualized by phase-contrast microscopy.

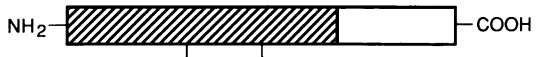
assay following transfection of full-length BPV-1 DNA (8). To determine the phenotypes of these various mutants in the acute transformation assay, several of the mutant E5 genes were subcloned into the plasmid pPava-2, and high-titer virus stocks of each were generated. The virus stocks were then individually tested, at a multiplicity of about 150 infectious units per cell, for the ability to stimulate cellular DNA synthesis in quiescent C127 cells. Each mutant was tested in duplicate in at least two infection experiments, using at least two different virus stocks of each, and the results are summarized in Fig. 5. Of the eight E5 mutants with mutations in the hydrophobic domain of the protein, three were very defective in the DNA synthesis assay (Fig. 5A). These defective mutants contained either a strongly basic amino acid or a replacement of the glutamine at position 17 with a nonhydrophilic amino acid. The requirement for a hydrophilic amino acid at position 17 has been noted by us previously (8, 9), but this requirement can be overcome by at least one random hydrophobic amino acid sequence (9). The other five mutants, all of which contain conservative substitutions, were competent to induce cellular DNA synthesis. Of the 10 mutants tested with substitutions in the carboxyl terminus of the protein, 3 were very competent at inducing cellular DNA synthesis, 2 were partially defective, and 5 were very defective (Fig. 5B). The mutations causing defects were located at the amino acid positions that are best conserved among related fibropapillomaviruses. Overall, a very strong correlation was found between the ability of each mutant to stimulate cellular DNA synthesis following Pava infection and to induce stable focus formation following transfection with full-length BPV-1 DNA (8) (Fig. 6). The minor discordancies between the two assays are within the range of experimental error of the focus-forming assay and are probably not significant.

TABLE 1. Biological activities of Pava mutants

Virus	Intact ORFs	Trans-activation	Acute morphologic transformation[a]	Stimulation of DNA synthesis (%)[b]
Pava-1	E2, E4, E5	+	+++++	100
Pava-E4am1	E2, E5	+	+++++	94
Pava-E5d29	E2, E4	+	−	0
Pava-E2am1	E4, E5	−	++++	80
Pava-BMV	E4, E5	−	+++++	110

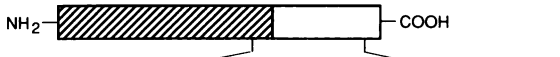
[a] Scored 48 h after infection by using a relative rating scale from − to +++++, with − indicating no morphologic change and +++++ indicating a morphologic change comparable to that seen in wild-type-infected cells.
[b] Percentage of wild-type activity.

A



Virus	15	16	17	18	19	20	21	22	23	Acute Morphologic Transformation	Cellular DNA Synthesis
Wild Type	ala	met	gln	leu	leu	leu	leu	leu	phe	+++++	100
16R	–	arg	–	–	–	–	–	–	–	–	-3 ± 0.3 (3)
17G	–	–	gly	–	–	–	–	–	–	++	1 ± 1.7 (5)
18R19K21F	–	–	–	arg	lys	–	phe	–	–	–	1 ± 3.4 (2)
15V21F22V	val	–	–	–	–	–	phe	val	–	++++	93 ± 11 (3)
15T17H	thr	–	his	–	–	–	–	–	–	+++++	43 ± 6 (2)
19V	–	–	–	–	val	–	–	–	–	+++++	75 ± 18 (4)
22M	–	–	–	–	–	–	–	met	–	+++++	107 ± 24 (3)
23I	–	–	–	–	–	–	–	–	ile	++++	44 ± 16 (3)

B



Virus	29	30	31	32	33	34	35	36	37	38	39	40	41	Acute Morphologic Transformation	Cellular DNA Synthesis
Wild Type	leu	val	tyr	trp	asp	his	phe	glu	cys	ser	cys	thr	gly	+++++	100
30L35S	–	leu	–	–	–	–	ser	–	–	–	–	–	–	+	3 ± 1.9 (3)
31F	–	–	phe	–	–	–	–	–	–	–	–	–	–	+++++	136 ± 25 (5)
31S	–	–	ser	–	–	–	–	–	–	–	–	–	–	++++	21 ± 7 (4)
32S	–	–	–	ser	–	–	–	–	–	–	–	–	–	+++	21 ± 7 (4)
33N	–	–	–	–	asn	–	–	–	–	–	–	–	–	++++	91 ± 16 (3)
33V	–	–	–	–	val	–	–	–	–	–	–	–	–	++	4 ± 2.2 (2)
34Q36D41A	–	–	–	–	–	gln	–	asp	-	–	–	–	ala	+++++	83 ± 24 (3)
37S	–	–	–	–	–	–	–	–	ser	–	–	–	–	+++	5 ± 2.4 (2)
39S	–	–	–	–	–	–	–	–	–	–	ser	–	–	+++	7 ± 3.4 (3)
37S39S	–	–	–	–	–	–	–	–	ser	–	ser	–	–	+	3 ± 2.5 (4)

FIG. 5. Primary structure of E5 missense mutants and their phenotypes in the acute transformation assays. A map of the E5 protein is shown at the top, with the hydrophobic portion indicated (▨). The score in the acute morphologic transformation assay (indicated by + or −) and the ability to stimulate cellular DNA synthesis (indicated as the percentage of wild-type activity ± standard deviation) are shown for mutants in the hydrophobic middle third (A) and the carboxyl-terminal third (B) of the E5 protein. Numbers in parentheses indicate the number of independent experiments performed with each mutant. Duplicate assays were done for each experiment. In these experiments, the values for mock-infected cells (generally less than 3% of the wild-type values) were subtracted from experimental values. Thus, the negative percentage obtained with mutant 16R indicates that cells infected with this mutant synthesize slightly less DNA than do mock-infected cells.

The ability of each of the E5 mutant virus stocks to induce rapid morphologic transformation of a C127 monolayer was also tested. The phenotype of each mutant was scored 48 h after infection by using a relative rating scale of − to +++++, with − indicating that the cells were indistinguishable from mock-infected cells and +++++ indicating that the cells were indistinguishable from cells infected with wild-type virus. Figure 7 shows the morphologic transformation of C127 cells 48 h after infection with some representative E5 mutants. The results for all of the mutants are summarized in Fig. 5. Again, a strong correlation was found between the acute and stable transforming abilities of the various mutants, although several mutants which showed a severe defect in the focus-forming assay and in the DNA synthesis assay induced a mild but significant morphologic transformation following infection. This is probably explained by our observation that acute morphologic transformation following infection appears to be the most sensitive assay of E5 activity. Thus, at the individual amino acid level, the same residues in the E5 protein are important for focus formation, rapid morphologic transformation, and the induction of cellular DNA synthesis in quiescent cells.

Stability of the mutant E5 proteins. To test the possibility that the various transformation-defective E5 mutants displayed such a phenotype because they produce an unstable E5 protein, the E5 mutant proteins in infected CMT4 cells were examined by immunoprecipitation with an E5-specific antiserum (21). This analysis was performed in CMT4 cells because we have had difficulty detecting the E5 protein in infected C127 cells, in which the recombinant virus does not replicate. We feel it is informative to examine the stability of the E5 protein in these cells because the half-life of the wild-type protein in infected CMT4 cells (about 1.5 h) is similar to the estimated half-life in stably transformed C127 cells (data not shown) (20). At 48 h after infection with E5 mutant virus stocks, CMT4 cells were metabolically labeled with [^{35}S]methionine for 5 h. The labeled proteins were immunoprecipitated with anti-E5 antiserum and analyzed by gel electrophoresis (Fig. 8). All five transformation-defective mutants with substitutions in the

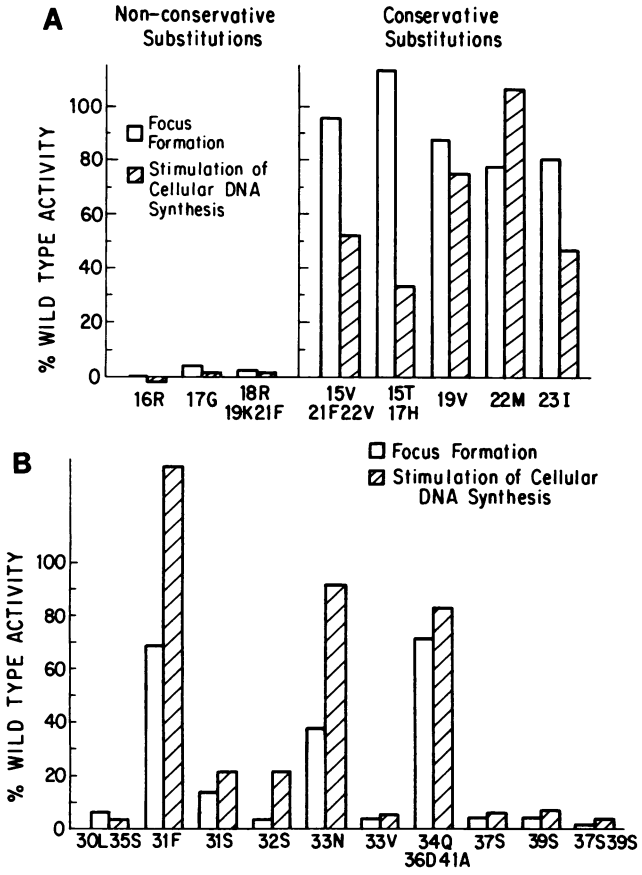


FIG. 6. Comparison of the E5 mutant phenotypes in the focus-forming and DNA synthesis induction assays. The activities of each mutant in the hydrophobic middle third (A) and the carboxyl-terminal third (B) of the E5 protein in these two transformation assays are presented as the percentage of wild-type activity. The focus-forming data for these mutants transfected as full-length BPV-1 DNA have been previously published (8), except for the value for 31S, which has been revised slightly to incorporate the results of additional experiments.

carboxyl terminus of the protein expressed levels of E5 protein comparable to that of the wild type, as did the defective mutant containing a glutamine-to-glycine substitution at position 17 in the hydrophobic portion. This result indicates that the synthesis of unstable E5 proteins is not responsible for the transformation defects displayed by these mutants. In contrast, there was little or no E5 protein present in cells infected with the two mutants which introduce a positively charged amino acid into the hydrophobic portion of the molecule.

DISCUSSION

We have presented several lines of evidence that indicate that the acute transforming activity of Pava-1 in C127 cells is closely related to the stable transforming activity of the BPV-1 E5 gene in these cells. Mutational analysis demonstrates that ORF E5 is required for both activities, and at the individual amino acid level, the sequence requirements within the E5 protein are closely similar or identical in both assays. Furthermore, it is possible to isolate stable transformants after infection with Pava-1 but not with a Pava E5 deletion mutant (J. Settleman, L. Nilson, and D. DiMaio, unpublished observations). Finally, the morphology and growth characteristics of cells infected with Pava-1 at a high multiplicity are similar to the properties of cells stably transformed by BPV-1 or by the isolated E5 gene. In contrast, C127 cells stably transformed by some other viral oncogene products, such as the polyomavirus middle T and SV40 large T antigens, are morphologically quite distinct from C127 cells acutely or stably transformed by Pava-1 or BPV-1 (D. DiMaio, unpublished observation). These similarities suggest that acute and stable cell transformation induced by the E5 gene is mediated by the same pathway(s).

This acute transformation system has been used to establish a number of basic facts about the transforming activity of the E5 gene. By varying the multiplicity of infection, we demonstrated that the extent of morphologic transformation is dependent on the dose of the viral E5 gene. A graded morphological response to different levels of expression of an inducible viral *src* gene has also been reported (10). Moreover, morphologic transformation appears to be a more sensitive indicator of E5 transforming activity than is the stimulation of cellular DNA synthesis, indicating that stimulation of quiescent cells to reenter the cell cycle requires more E5 protein than does induction of morphologic changes. The induction of DNA synthesis by the E5 protein is accompanied by morphologic transformation of the infected cells, whereas cells induced to synthesize DNA by treatment with serum maintain their normal morphology. This difference suggests that the pathway leading to DNA synthesis following serum treatment is different from that which is activated by the E5 gene or that the E5 gene activates multiple pathways.

The vast majority of C127 cells infected with Pava-1 underwent a rapid morphologic transformation, and a substantial fraction underwent DNA synthesis, demonstrating that most of the cells are at least transiently susceptible to the transforming activity of the BPV-1 E5 gene. In contrast, BPV-1 and Pava-1 virions or transfected viral DNA induced stable transformation at a low frequency. Our inability to genetically dissociate stable from acute transformation suggests that stable transformation does not require an additional E5 activity to which only a subset of C127 cells is susceptible. Rather, we believe that the low efficiency of stable transformation is due to a failure of the majority of the transfected cells to stably maintain and express the BPV-1 genes. In fact, over the course of 3 weeks, cells infected with Pava-1 gradually lose the extrachromosomal viral DNA (L. Nilson, J. Settleman, and D. DiMaio, unpublished results).

The genetic mapping experiments strongly suggest that acute transforming activity is due solely to ORF E5, the major viral gene required for focus formation by BPV-1 DNA. The efficient acute transforming ability of the Pava-E4am1 mutant is consistent with the failure to find a role for ORF E4 in stable cell transformation by the full-length BPV-1 genome (7, 16). Neither the full-length E2 transactivator nor the carboxyl-terminal E2 repressors are required for efficient transformation in this system. However, in testing a larger series of Pava E2 mutants, we found that there is a considerable range in the level of E5 protein expressed by these mutants and in their acute transforming activity when assessed 2 to 3 days after infection (J. Settleman and D. DiMaio, unpublished observations). The extent of transformation by these Pava E2 mutants parallels their

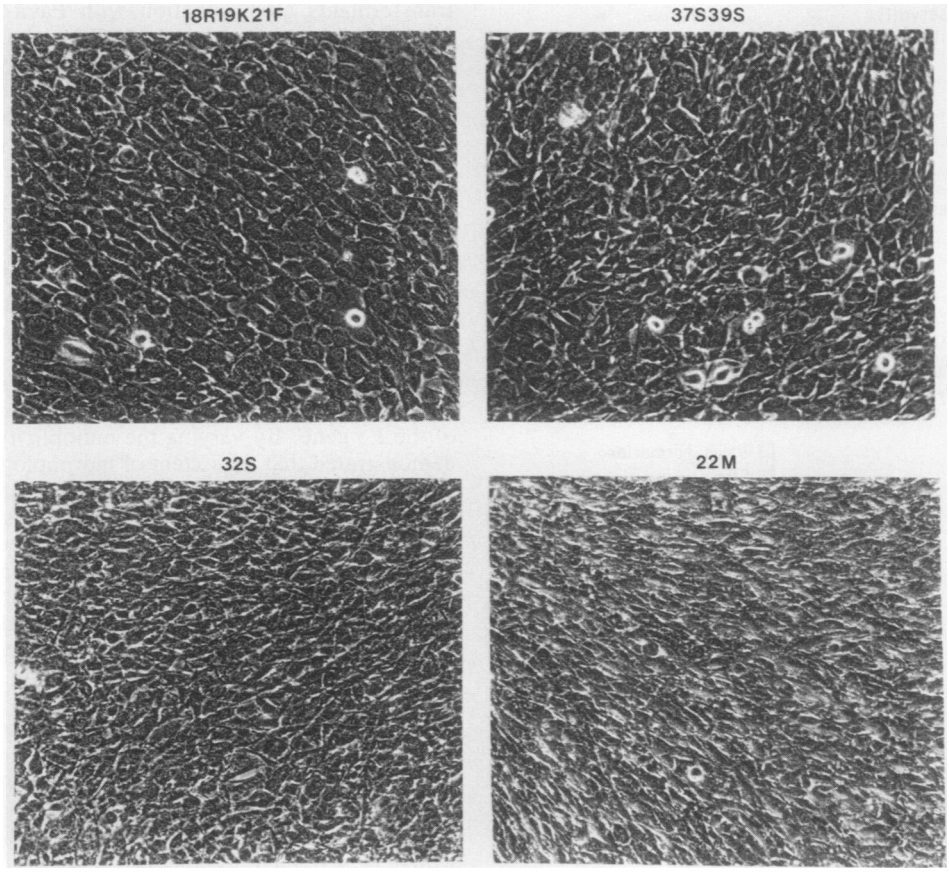


FIG. 7. Acute morphologic transformation of C127 cells infected with representative E5 mutants. Cells were infected at a multiplicity of infection of about 1,000 (as determined by transactivation titering on NL-3D cells) with the indicated virus stocks and photographed after 48 h by using phase-contrast microscopy.

ability to express the E5 protein following infection, with the most severe defects in transformation and E5 expression seen with mutants that overexpress E2 repressor relative to transactivator. Thus, although ORF E5 is not under the control of the BPV-1 control region in the Pava-1 genome, ORF E2 appears to play an indirect regulatory role in acute transformation by Pava-1.

The analysis of numerous E5 missense mutants in the acute transformation assay revealed requirements for a hydrophobic stretch of amino acids in the middle third of the protein and for specific amino acids in the hydrophilic carboxyl terminus of the protein. We have previously demonstrated similar requirements for stable transformation by ORF E5 in transfection assays (8, 9), and Green and Lowenstein have reported that the carboxyl-terminal segment of the E5 protein weakly stimulates cellular DNA synthesis (6). The failure to detect E5 protein in cells infected with the two mutants which contain a positively charged amino acid in the hydrophobic domain suggests that this region of the protein may be important for maintaining the stability of the protein. In contrast, there was a substantial amount of E5 protein present in cells infected with the glutamine 17-to-glycine mutant or with mutants containing substitutions in the carboxyl-terminal third of the protein. Therefore, the biochemical basis of the defect displayed by these mutants is likely to be different from that caused by the introduction of positive charges into the hydrophobic portion of the protein. Rather, the defects displayed by these mutants may be due to a failure of the mutant E5 proteins to interact with their putative cellular target(s). Except for the two mutants which did not express detectable E5 protein, all mutants tested were capable of inducing some morphologic transformation of infected cells. Evidently, the primary structure of the E5 protein can tolerate a variety of substitutions at many positions without a complete loss of transforming activity. We suggest that a level of activity sufficient to score in a sensitive transformation assay can be attained if enough of a partially defective E5 mutant protein is expressed.

The correlation between the phenotypes of the various E5 mutants in the acute and stable transformation assays is striking and suggests that the biochemical activity of the E5 protein responsible for acute transformation of Pava-infected cells is probably the same activity which mediates stable transformation. The identification of the biochemical activities of the E5 protein responsible for these biological activities remains a pressing question. Because Pava-1 infection rapidly generates a relatively homogeneous population of cells transformed by the BPV-1 E5 gene, this system should allow us to identify initial biochemical changes induced by this viral oncogene. The collection of virus stocks expressing various E5 mutants will be a valuable set of reagents for assessing the importance of such biochemical changes.

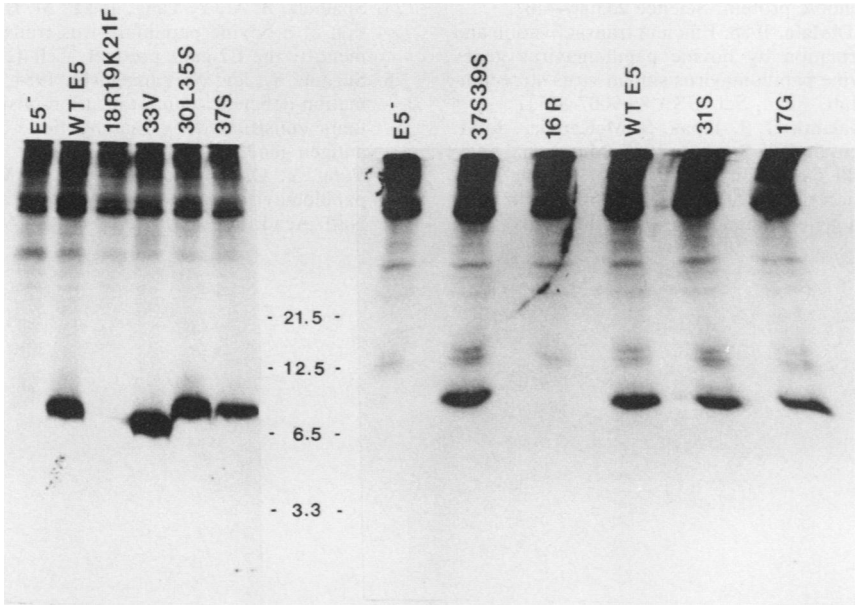


FIG. 8. Mutant E5 protein expression in Pava-infected cells. CMT4 cells were infected with wild-type Pava-1, the Pava-E5d29 mutant (designated E5⁻), or the transformation-defective E5 missense mutants, as indicated. Labeling with [^{35}S]methionine and immunoprecipitation were done 48 h after infection, as described in Materials and Methods. Autoradiograms of the samples after electrophoresis and fluorography are shown. Numbers indicate the sizes of molecular mass markers in kilodaltons. Similar results were obtained with cells infected with Pava-E516R and labeled with [^{35}S]cysteine (data not shown). Cells infected with Pava-E532S also expressed levels of E5 protein comparable to that of the wild type (data not shown).

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