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STIMULATION OF CELLULAR DNA SYNTHESIS BY WILD TYPE AND MUTANT BOVINE PAPILLOMAVIRUS DNA

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Abstract. Microinjection of recombinant plasmids containing bovine papillomavirus type 1 DNA into the nuclei of mouse C127 cells results in the stimulation of cellular DNA synthesis. Mutations in the viral E2 gene have no apparent effect on this activity even though the same mutations prevent efficient C127 cell focus formation and inhibit transactivation by this gene. © 1987 Academic Press, Inc.

The papillomaviruses are small, DNA-containing viruses that induce benign tumors in infected animals and humans (1,2), and some of these proliferative lesions progress to malignant neoplasms which contain the viral DNA. Although none of the papillomaviruses undergo productive infection in tissue culture, bovine papillomavirus type 1 (BPV1) and BPV1 DNA efficiently induce the appearance of transformed foci in mouse C127 cells, an established cell line derived from a mammary carcinoma of an RIII mouse (3,4). The BPV1 genome contains a number of long translational open reading frames (ORFs) that are thought to encode viral proteins responsible for the biological activities of the virus (5,6,7). Mutations in either ORF E2 or ORF E5 markedly inhibit the focus forming activity of the full-length viral DNA (8-13). The non-coding region of BPV1 DNA also contains an enhancer element that is active in the presence of a transactivation function expressed by ORF E2 which encodes a DNA binding protein (14-16).

Abbreviations: BPV1, bovine papillomavirus type 1; ORF, open reading frame; CAT, chloramphenicol acetyl transferase.

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We report here that molecularly cloned BPV1 DNA microinjected into the nuclei of mouse C127 cells stimulates synthesis of cellular DNA. By using this assay we have determined that stimulation of cellular DNA synthesis is not impaired by mutations in ORF E2 that block transactivation of the BPV1 enhancer and prevent efficient mouse cell transformation.

METHODS

Full-length BPV1 DNA cloned into the bacterial plasmid vector pML2d at its BamHI site was used for microinjection experiments. The structures of each mutant recombinant plasmid was identical to wild type (pBPV-142-6 (17)) except for the presence of a mutation that has been described previously (8,9,23). To make C127 cells quiescent by contact-inhibition, they were plated in DME medium plus 10% fetal calf serum six days prior to microinjection. At the time of microinjection, they had been confluent for three days. The manual microinjection technique of Graessmann and Graessmann (18) was used to introduce the plasmids (DNA concentration, 0.2 ug/ ml in 10mM Tris HCl, pH 7.6) into the nuclei of the quiescent cells. As controls, nuclei were injected with buffer alone or with pBR322. Immediately following microinjection, [^{3}H] thymidine (0.2 uCi/ml) was added to the medium for 40 hours and the fraction of cells in DNA synthesis was then assayed by autoradiography (19). To measure DNA content in individual cells, they were fixed 26 hours after microinjection, stained with acridine orange and analyzed with a Zonax microfluorimeter, as previously described (20).

For the transactivation assay, the wild type BPV1 DNA in c59 (14) was replaced with a mutant fragment derived from pE2am9 (to generate c59S4), pE2fs-1 (c59F2), or pE5XL-2 (c59X9). CV1 cells were cotransfected as described with the indicated BPV1 plasmid plus p407-1 which contains the *E. coli* CAT gene linked to the BPV1 inducible enhancer and the SV40 early promoter (14,21). 48 hours after transfection, cellular extracts were prepared and equal portions assayed for CAT activity in a 30 minute incubation at 37°C, followed by thin layer chromotography and autoradiography of the reaction products (22).

RESULTS AND DISCUSSION

The nuclei of quiescent C127 cells were microinjected with recombinant plasmids containing wild type or mutant BPV1 DNA. After incubation of the cells in radiolabelled thymidine, cellular DNA synthesis was assayed by autoradiography. Table 1 shows the results of several experiments expressed as the percentage of microinjected nuclei undergoing DNA synthesis. Injection of a plasmid containing wild type BPV1 DNA (pBPV-142-6) resulted in the induction of DNA synthesis in a high proportion of the microinjected cells. Few uninjected cells or cells injected with pBR322 or buffer incorporated [^{3}H] thymidine (data not shown). To quantitate DNA content in individual cells, microspectrofluorimetry was performed on uninjected cells and on cells that had been microinjected with a plasmid containing wild type BPV1 DNA (20). As shown in figure 1, a substantial proportion of cells receiving BPV1 DNA (but not uninjected cells) have a DNA content characteristic of cells that have undergone replication; thus the thymidine incorporation detected by autoradiography is attributable to cellular DNA

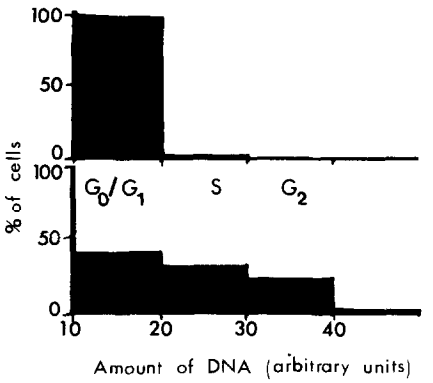

Figure 1. Amount of DNA in C127 cells. DNA content of individual uninjected C127 cells (top) or cells injected with pBPV-142-6 (bottom) on the same coverslip was determined by using a computer-operated microspectrofluorimeter to measure the green fluorescence of acridine orange-stained cells as previously described (20). DNA content of individual cells is proportional to fluorescence and is expressed as arbitrary units. The figure shows the percentage of cells with DNA content corresponding to specific stages in the cell cycle as indicated.

synthesis. These results demonstrate that introduction of cloned BPV1 DNA into growth-arrested cultured cells causes them to commence DNA synthesis.

To determine whether ORF E2 activity is required for stimulation, we have assayed the behavior of three constructed viral mutants with different mutations in the 5' half of ORF E2. pE2am9 contains a single base substitution mutation that generates an amber translation stop codon in this viral gene (8); pE2fs-1 and pE2-NIL contain a 16 base pair insertion and a 211 base pair deletion, respectively (23). Each of these mutants induces about 2 to 5% as many C127 cell foci as does an equivalent amount of wild type DNA (8,23). In several separate microinjection experiments, pE2am9 stimulates [^{3}H] thymidine incorporation to the same extent as does the wild type viral genome (table 1). Similar results were obtained with the other two ORF E2 mutants. Therefore, although a functional ORF E2 is required for efficient focus formation following transfection of mouse C127 cells, it is not required for stimulation of DNA synthesis following microinjection of DNA into these cells.

To determine the effects of two of these ORF E2 mutations on transactivation, each was inserted into an expression plasmid and tested for its effect on a co-transfected plasmid containing the chloramphenicol acetyl transferase (CAT) gene linked to the BPV1 upstream control region. In this assay, the CAT gene is expressed only in the presence of a functional BPV1 ORF E2. As expected, both ORF E2 mutations caused a marked defect in transactivation, as indicated by reduced CAT activity in transfected monkey CV1 cells (figure 2). Although different cells and methods of DNA delivery are used in these two assays, the results suggest that ORF E2-mediated

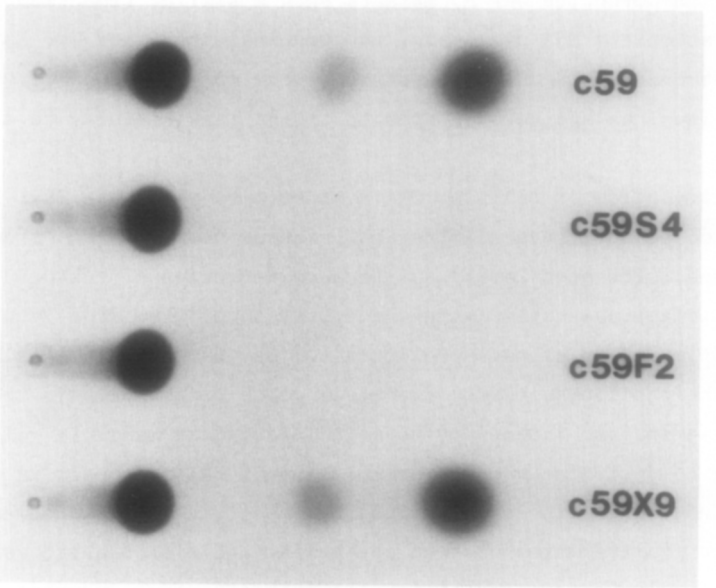

Figure 2. Extracts from CV1 cells that received the indicated BPV1 plasmid were assayed for CAT activity as described in the methods. The row of dark spots on the left is unreacted substrate, and the spots to the right are the products of the acetylation reaction. In the experiment shown, the percentage of initial substrate that was acetylated (as determined by scintillation counting) was 9.6% (wild type), 0.17% (c59S4), 0.09% (c59F2), and 13% (c59X9); background was <0.05%. Similar results were obtained in two additional transfection experiments.

transactivation is not directly required for stimulation of cellular DNA synthesis.

pE5XL-2 contains a frameshift mutation in ORF E5 that causes a severe focus forming defect but has no effect on transactivation (figure 2). This mutant reproducibly displays a substantial reduction in the ability to stimulate cellular DNA synthesis (table 1). Although many

Table 1
Stimulation of DNA synthesis by microinjected BPV DNA

Microinjected plasmid	% labelled nuclei[a]
pBPV-142-6	26.6, 20.2, 24.1[b]
pE2-am2	33.2, 20.3, 19.6
pE2-NIL	29.5
pE2-fs1	12.5
pE5-XL2	1.5, 1.1, 1.5

[a] Percentage of cells undergoing DNA synthesis after microinjection. The percentage of positive, uninjected cells on the same coverslip has been subtracted. This background ranged from 0.1% to 5% in different experiments.

[b] Multiple values are the results from separate microinjection experiments.

transformation-defective ORF E5 mutants show a similar reduction in the ability to stimulate DNA synthesis, the results with other ORF E5 mutants and with retroviral LTR/ORF E5 constructs were not reproducible (unpublished results). Thus, we are not yet able to assign this activity to a specific viral gene.

The stimulation of cellular DNA synthesis by wild type BPV1 DNA was not unanticipated because several other DNA viruses have similar effects (for examples, see references 24,25) and because infection of cultured cells with human papillomaviruses stimulates incorporation of thymidine into DNA (26,27). Stimulation of cell proliferation may be essential for productive growth of the papillomaviruses, a process which occurs only in the proliferative lesions induced by them in infected animals. It will be interesting to determine whether stimulation of DNA synthesis in microinjected C127 cells is restricted to papillomaviruses such as BPV1 which induce proliferation of both epithelial and fibroblastic cells or whether it is also a property of papillomaviruses which induce purely epithelial lesions.

Microinjection of SV40 DNA into the nuclei of mouse cells can result in expression of the late viral genes, an event that does not normally occur in these cells (28). By analogy to these results, it is possible that by microinjecting BPV1 DNA we have permitted the expression of a viral gene that is normally E2-dependent. Nevertheless, the analysis of the ORF E2 mutants suggests that activities encoded in the 5' portion of ORF E2 are not directly required for stimulation of C127 cell DNA synthesis. Further genetic and biochemical experiments are clearly required to identify the BPV1 gene(s) responsible for stimulation of cellular DNA synthesis and to establish the roles played by these genes in transformation.

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