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Genetics

# Successive steps in the process of immortalization identified by transfer of separate bovine papillomavirus genes into rat fibroblasts

**(transformation/oncogene cooperation/human papillomavirus type 16)**

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ABSTRACT **Transfer of *neo*$^r$ and bovine papillomavirus type 1 (BPV1) DNA into rat embryo fibroblasts led to colony formation in G418-containing medium, with no detectable background in controls with *neo*$^r$ DNA alone. More than 50% of the drug-resistant clones could be further propagated in culture. The genetic functions of BPV1 involved in colony formation and in long-term immortalization were investigated by both translation termination mutations in the full-length genome, which inactivate individual open reading frames, and constructs in which these open reading frames were separately expressed under control of long terminal repeat promoter enhancers. Expression of either open reading frame E2 or E5 was sufficient for formation of a drug-resistant colony, but long-term growth in culture required that of E6. No significant cooperative effect was observed upon cotransfection of BPV1 and *ras* oncogene DNAs. Expression of the early region of the human papillomavirus type 16 also led to immortalization of rat embryo fibroblast cells in the same assay, and, unlike what was previously reported in baby rat kidney cells, it required neither activation by a heterologous promoter, nor a cooperating *ras* oncogene.**

In spite of their obviously artificial nature, the established pseudonormal rodent cell lines (1) provide useful systems for the assay of viral and cellular oncogenes (see ref. 2 for review). One limitation of studies performed on cell lines is that one is always dealing with complex cooperative effects involving the oncogenes under study, the unknown cellular genes whose alteration originally resulted in the establishment of the cell line and the (equally unknown) oncogenes possibly activated during passages in cell culture (3, 4). Performing transformation assays on the more normal primary culture embryonal cells is therefore of interest. It has led to the identification of a first stage, designated immortalization, in the transformation of rat embryo fibroblast (REF) cells; immortalization can be induced by genes of DNA tumor viruses [polyoma virus large tumor antigen (T antigen), adenovirus E1A] and by a class of cellular oncogenes, all of them encoding transcriptional regulatory proteins (see refs. 2 and 5 for reviews). Much attention has been devoted to the process of immortalization. Conceptually, it provides a model for a very early stage of tumor progression that may correspond to changes in the expression of a set of critical cellular genes. It may also provide a way to establish differentiated cell lines of various types. Very little progress has been made, however, in the analysis of immortalization and a number of questions remain unanswered. For example, when the oncogenes that fully immortalize rodent cells, such as the T antigens of polyoma virus and simian virus 40, have been assayed on human primary fibroblast cultures, their expression has resulted in an increase in the number of generations that the cells can be propagated in culture, but not in permanent immortalization (ref. 6; unpublished observations), suggesting that in this case only part of a complex multistep pathway was induced by the oncogene.

Several systems of cooperative interactions have been provided by oncogenes of DNA tumor viruses. Extending this analysis to another group of viruses that are clearly distinct from the previously studied polyoma- and adenoviruses could therefore be informative. Papillomaviruses are of interest for the analysis of oncogenic transformation because, in several respects, they present original features as compared with polyoma, simian virus 40, or retroviruses. They are the causative agents of a variety of benign proliferative lesions of the skin and other epithelia, some of them with a high risk of progression to malignant stages. A subgroup of the family, including the bovine type 1 papillomavirus (BPV1), induce fibroepitheliomas *in vivo* and readily transform established fibroblast lines *in vitro*. Genetic analysis of BPV1 (see ref. 7 for review) determined that all the genes necessary for cell transformation were located within a subgenomic fragment that includes a noncoding upstream regulatory region [1 kilobase (kb)] and a coding region of ≈4.5 kb with 7 open reading frames (ORFs) designated E1–E7 (Fig. 1). ORFs E5 and E6 were shown to be important for transformation of established rodent lines. When isolated under control of a retroviral promoter, they could separately transform mouse C127 cells. Only E5, however, was efficient in inducing morphological transformation and tumorigenic properties in mouse NIH 3T3 and in rat FR 3T3 cells (refs. 8–11; unpublished data). Mutations in ORFs E1 and E2 also led to changes in transformation efficiency. However, since the isolated E1 and E2 genes have not been reported to transform any cell line, these effects have been thought to be indirect and possibly related with the known functions of E1 and E2 in the autonomous replication and transcription controls of the viral genome.

In the present work, we have addressed the question of a possible distinction of immortalizing and transforming functions among the early genes of BPV1. Immortalization of REF cells was assayed by first selecting for colony formation in medium containing the drug G418 after cotransfer of both the gene to be tested and a drug-resistance allele (*neo*$^r$) followed by subsequent testing of the cells from a repre-

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Abbreviations: REF, rat embryo fibroblast; BPV1, bovine papillomavirus type 1; ORF, open reading frame; HPV16, human papillomavirus type 16; LTR, long terminal repeat; T antigen, large tumor antigen.

sentative number of colonies for their capacity to further multiply in culture. The results suggest that *in vitro* immortalization of REF cells by papillomaviruses requires the sequential expression of distinct viral oncogenes, thus distinguishing two stages in the immortalization process.

## MATERIALS AND METHODS

**Recombinant DNAs.** Plasmid pPY1 (12) carries a complete polyoma genome inserted at the *Bam*HI site of pBR322, and pSVc-myc-1, the second and third exons of the mouse c-*myc* gene under control of the simian virus 40 promoter (13). pVV2 is a derivative of pBR322 containing the *neo*$^r$ gene expressed under control of the herpes simplex virus thymidine kinase promoter (14). Plasmid pVEJB was constructed by inserting the *Bam*HI fragment of plasmid pEJ6.6 carrying the Ha-c-*ras* oncogene (13) at the unique *Bam*HI site of pVV2. Plasmid pVH16 contains the complete genome of human papilloma virus type 16 (HPV16) virus (15), cloned at the *Bam*HI site of pVV2, with the early region expressed under control of the viral promoter–enhancer region in a counterclockwise direction with respect to *neo* transcription.

**BPV1 Mutants and Subgenomic Constructs.** A summary of the constructs and the relevant references are listed in Table 1 and genetic maps are shown in Fig. 1. Plasmids p142-6 (wild type) and mutants p760, p775, p797, p709, p793, and pE5XL-2 carry a BPV1 genomic DNA molecule cloned at the *Bam*HI site of vector pML2d. These mutations are frameshifts created by insertion of *Xho* I linkers. pE2am7 carries a nonsense mutation in ORF E2 created by oligonucleotide-directed mutagenesis. Plasmid pXH800 contains the E6–E7 region (nucleotides 1–1019) linked to sequences from 4040 to 4450, including the polyadenylylation signal, transcribed from the Moloney murine sarcoma virus long terminal repeat (LTR) promoter. Its derivatives pXH875 and pXH997 were obtained by *Xho* I linker insertion (same as in p775, E6$^-$) and by deletion of nucleotides 681–1019 (E7$^-$), respectively. pHLB1 contains the *Eco*RI/*Bam*HI fragment of BPV1 activated by the Harvey sarcoma virus LTR. Its derivatives pHLB1-500, -709, and -793 carry the same mutations as mutants pE5XL-2, p709, and p793, respectively.

**Preparation of REF Cultures, Transfection, and Drug Selection.** Primary cultures were prepared from 13- to 15-day-old Fischer rat embryos. Embryos were rinsed in Tris-buffered saline, minced, and digested at 40°C for 10 min in 0.2% trypsin. Cells were centrifuged at 220 × *g* for 5 min, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (GIBCO), and plated at a density of $2 \times 10^5$ cells per cm$^2$. Medium and floating cells were removed the next day, fresh medium was added, and 4 hr later, cells were transfected (21) with a total of 5 μg of DNA per 60-mm Petri plate. Medium with DNA was removed after 1 day, and cells were transferred in medium containing 10% fetal calf serum, at a density of $3 \times 10^5$ cells per 60-mm Petri plate. Six to eight plates were seeded for each condition. Three to 4 days later, G418 was added to a final concentration of 0.2 mg/ml. The selective medium was changed twice a week. After 2–3 weeks, cells were either fixed and stained with Giemsa or cloned and tested for long-term growth.

**Assay for Long-Term Growth in Culture.** Cells were picked from individual G418-resistant colonies and seeded in DMEM supplemented with 10% fetal calf serum. The first transfer was performed by seeding cells from one colony into a 2-cm$^2$ microwell; at transfer 2, cells were transferred in two microwells; at transfer 3, cells were transferred in a 60-mm plate. For subsequent transfers, they were diluted 1:8 in 60-mm plates.

## RESULTS

**Immortalization of REF Cells by Wild-Type BPV1 DNA.** Primary REF cultures prepared from 14- to 15-day-old Fischer rat embryos (see *Materials and Methods*) were transfected with a 1:1 mixture of plasmids p142-6 and pVV2, carrying a complete BPV1 genome and the *neo*$^r$ gene, respectively (Table 1). The BPV1 insert in p142-6 was excised from vector sequences by *Bam*HI cleavage before transfection. In the first stage of the experiment, the number of G418-resistant colonies was compared to that in controls transfected either with pVV2 DNA alone, or with a mixture of plasmids pPY1 (polyomavirus early region) and pVV2, or with a mixture of pSVc-myc-1 and pVV2 DNAs. pSVc-myc-1 (13) carries a rearranged form of the mouse c-*myc* gene capable of immortalizing REF cells. Representative results are shown in Table 2 and illustrated in Fig. 2. Cotransfection of pVV2 and BPV1 DNA produced drug-resistant colonies as efficiently as the two positive controls pSVc-myc-1 and pPY1, whereas cells that had received pVV2 DNA alone failed to form colonies.

Formation of a colony in G418 medium requires a limited number of generations, which may be comparable to the

Table 1. BPV1 mutants and subgenomic constructs

| | Genomic BPV1 DNAs | | Subgenomic regions under LTR control | | | | |
|---|---|---|---|---|---|---|---|
| | Mutation | | | Mutation | | | |
| Plasmid | Position | ORF | Early ORFs present | Position | ORF | Coding capacity | Ref. |
| p142-6 | — | — | | | | | 8 |
| p760 | 1019 | E1 | | | | | 8 |
| p709 | 3353 | E2–E4 | | | | | 11 |
| pE2am7 | 3081 | E2 | | | | | 16 |
| p793 | 2693 | E2 | | | | | 11 |
| pE5-XL2 | 3884 | E5 | | | | | 9 |
| p775 | 445 | E6 | | | | | 8 |
| p797 | 680 | E7 | | | | | 8 |
| pHLB1 | | | E2–E5 | — | — | E2–E5 | 11 |
| pHLB1-500 | | | E2–E5 | 3884* | E5 | E2–E4 | 11 |
| pHLB1-709 | | | E2–E5 | 3353 | E2–E4 | E5 | 11 |
| pHLB1-793 | | | E2–E5 | 2693 | E2 | E3–E5 | 11 |
| pXH800 | | | E6,E7 | — | — | E6,E7 | 8 |
| pXH875 | | | E6,E7 | 445 | E6 | E7 | 8 |
| pXH997 | | | E6 | — | — | E6 | 8 |

*Same mutation as in pE5-XL2.

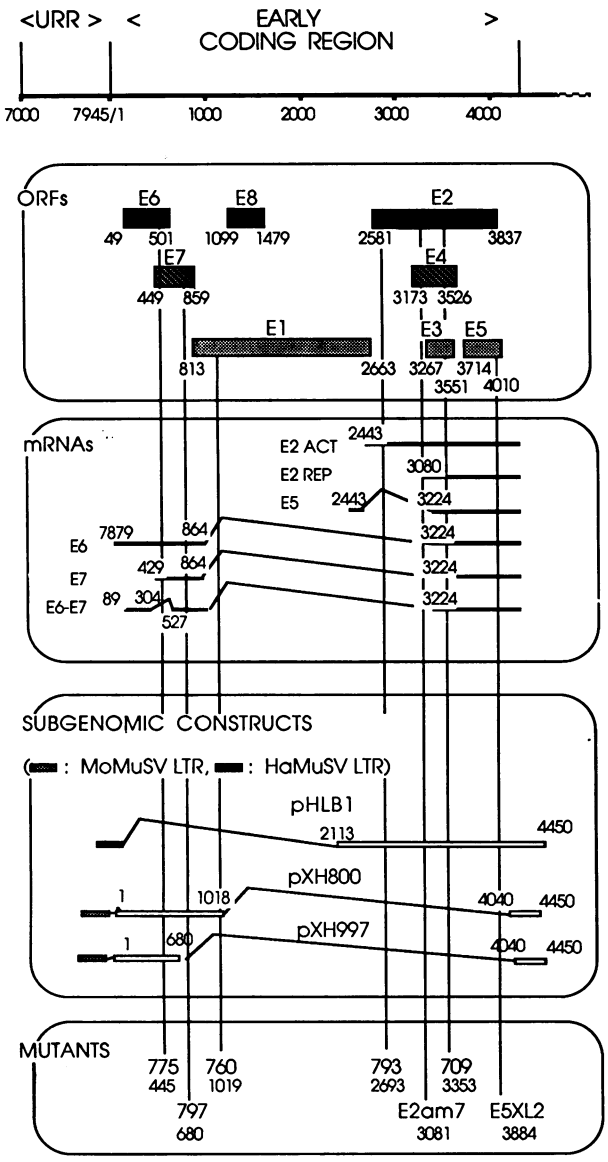


FIG. 1. Genetic maps of BPV1 early region. From top to bottom: upstream regulatory region (URR) and coding region with nucleotide numbers according to ref. 17; ORFS in the three reading frames (17); cytoplasmic mRNAs for ORFs E2, E5, E6, and E7 (18–20); subgenomic constructs and mutants (see Table 1). MoMuSV, Moloney murine sarcoma virus; HaMuSV, Harvey murine sarcoma virus.

growth potential of nonestablished cells before they reach crisis, especially if one takes into account the fact that, in these experiments, REF cells were transfected immediately after explantation. The long-term growth potential of the drug-resistant cells was measured by picking representative series of colonies, growing cultures in microwells, and serially transferring them whenever confluency was reached.

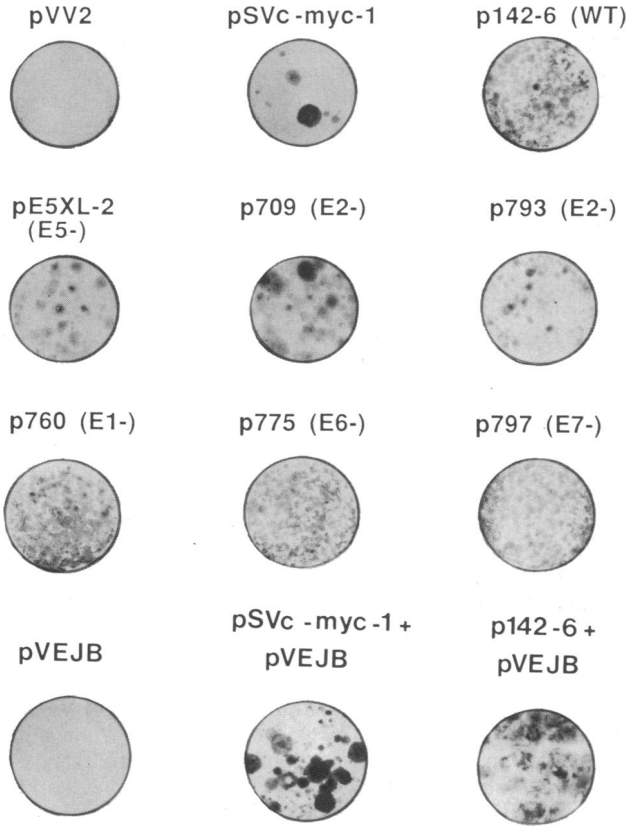


FIG. 2. Colony formation in G418 medium after transfer into REF cells of wild-type and mutant BPV1 genomes. Cultures were transfected with the indicated plasmid DNA as described in the text and stained with Giemsa 2 weeks later.

Table 2 shows the fraction of the clones tested in this way that could be propagated for at least three transfers (≈10 generations). Growth for longer periods was also assayed, with the general result that cultures that could be successfully maintained for the first three transfers could be maintained thereafter for much longer periods (30 generations and more), without any indication of the occurrence of a crisis. As expected, the frequency of establishment was high (although <100%) among cells that received pPY1 DNA. pSVc-myc-1 was somewhat less efficient, in agreement with our previous observations (E. Mougneau and F.C., unpublished results). BPV1 DNA was remarkably efficient, producing established lines with the same frequency as polyoma DNA.

The growth control and state of the viral genome in cell lines established after transfection of BPV1 DNA into REF cells were similar to what we had previously observed in cell lines derived from FR 3T3 rat fibroblasts (22): the transfected plasmid DNA was maintained in an autonomous form and the growth remained initially that of a normal fibroblast (low saturation density and poor plating efficiency in suspension) (data not shown). Whether these lines will progress toward

Table 2. Colony formation in G418 medium and subsequent growth in culture

| Plasmid | Coding capacity | Colonies in G418 medium* | Fraction giving rise to established cultures |
|---|---|---|---|
| pVV2 | *neo*$^r$ | 0 | — |
| pPY1 + pVV2 | Polyoma early + *neo*$^r$ | 48 | 18/27 |
| pSVc-myc-1 + pVV2 | *myc* + *neo*$^r$ | 30 | 5/47 |
| p142-6 + pVV2 | BPV1 wt + *neo*$^r$ | 18 | 6/12 |

wt, Wild type.
*Colonies per $10^6$ cells; average of six to eight plates.

transformed and tumorigenic states when passaged in culture, as did BPV1-transformed FR 3T3 lines, is not known.

**Neither BPV1 Nor HPV16 Requires the Cooperation of a Second Oncogene for Immortalizing REF Cells.** The efficient immortalization of REF cells by BPV1 DNA alone appears at variance with recent observations on the effects on rat cells of HPV16. In this case, immortalization required activation of the viral genes by a heterologous promoter and the simultaneous expression of the *ras* oncogene (23). These differences may mean either that the response of REF cells is different from that of baby rat kidney cells used in HPV16 studies, or that BPV1 and HPV16 immortalize primary cells by significantly distinct mechanisms. We therefore tested BPV1 and HPV16 DNA in the same series of assays, both with and without cotransfection of a *ras* oncogene. The results suggested that both explanations may be correct (Table 3). On the one hand, transfecting the early region of HPV16, expressed under control of its own promoter and upstream regulatory region, efficiently induced the appearance of G418-resistant colonies that could be further propagated in culture. On the other hand, cotransfection of *ras* did not increase to a significant extent the number of drug-resistant colonies after transfer of either HPV16 or BPV1 DNAs.

**Genetic Functions of BPV1 Involved in REF Immortalization.** Two series of constructs were used to delineate the individual BPV1 gene product(s) required for establishment of permanent cell lines: mutant genomes with one of their ORFs inactivated by *in vitro* mutagenesis, and subgenomic fragments corresponding to individual ORFs expressed under control of a retroviral LTR promoter (Table 1 and Fig. 1).

The results clearly demonstrated distinct requirements for viral gene products at each of the two steps of the immortalization assay (Table 4). As often occurs in experiments on primary cultures, absolute efficiencies varied between batches of REF cells and batches of serum. All the values measured within one experiment were, however, always consistent, and the pVV2-only controls were always negative. These points are illustrated in Table 4, which shows the extent of variation observed in a total of eight independent experiments.

For each full-length BPV1 genome carrying a single mutation in an early ORF, formation of G418-resistant colonies was observed, without any reproducible decrease in efficiency, suggesting that distinct viral functions are separately sufficient. The alternative hypothesis that none of these mutations inactivates the essential gene cannot be ruled out *a priori*, since we do not yet know all possible splices in the transforming region. This possibility seems less plausible because we used mutations in every early ORF from E1 through E7. In fact, the first hypothesis could be shown to be correct by experiments with isolated ORFs expressed under LTR control (Table 5). The $E2^-E5^-$ double deletion (pXH800) was negative, whereas colonies were produced with constructs expressing either E2 only or E5 only.

Expression of E6 alone did not result in the appearance of $neo^r$ colonies (Table 5), which, conversely, was not prevented by the $E6^-$ mutant 775 (Table 4). This mutant was, however, the only one for which the frequency of establishment of permanent cultures from the $neo^r$ colonies was severely affected. The latter result was reproducibly obtained in three different experiments. The low background seen in each case may represent a distinct, as yet unidentified, immortalization pathway. It was not seen in experiments performed with LTR-driven isolated genes (Table 5). Long-term immortalization was observed with none of the constructs or combinations of constructs that induced colony formation in G418 medium but did not express E6. This result indicates that, beyond the initial growth stimulation produced by either E2 or E5, the E6 gene product is required to promote continuous growth. Cooperation between E6 and E2 or between E6 and E5 could be directly demonstrated by cotransfer of the proper LTR constructs (Table 5, two of three independent experiments are shown).

Table 3. Immortalization by HPV16

| Plasmids | Coding capacity | Colonies in G418 medium* | Established cultures |
|---|---|---|---|
| p142-6 + pVV2 | BPV1, $neo^r$ | 50 | 6/12 |
| p142-6 + pVEJB | BPV1, $neo^r$, *ras* | 50 | 4/6 |
| pVH16 | HPV16, $neo^r$ | 33 | 6/6 |
| pVH16 + pVEJB | HPV16, $neo^r$, *ras* | 65 | 6/6 |

*Colonies per $10^6$ cells; average of six to eight plates.

Table 4. Effect of point mutations on immortalization potential

| Plasmid | Genotype | Colonies in G418 medium* | Established cultures Exp. 1 | Established cultures Exp. 2 |
|---|---|---|---|---|
| pVV2 | $neo^r$ | 0 (0) | — | |
| p142-6 | BPV1 wt | 18 (1) | 6/12 | 4/6 |
| p760 | $E1^-$ | 100 (0.7–5) | 6/12 | |
| p709 | $E2^-$,$E3^-$,$E4^-$ | 33 (0.6–1.8) | 10/22 | |
| pE2am7 | $E2^-$ | 66 (0.7–3.6) | | |
| p793 | $E2^-$ | 45 (0.4–2.5) | 4/6 | |
| pE5XL2 | $E5^-$ | 27 (0.3–1.5) | 4/6 | 6/6 |
| p775 | $E6^-$ | 90 (0.4–5) | 2/6 | 1/6 |
| p797 | $E7^-$ | 90 (0.5–5) | 4/6 | |

wt, Wild type.
*Colonies per $10^6$ cells; average of six to eight plates. The values in parentheses indicate the range of variation observed in eight independent experiments, results being normalized to 1 for wild-type BPV1 DNA in the same experiment.

## DISCUSSION

There is no definition of immortalization at the molecular level. At the cellular level, it is broadly defined as a function(s) that allows long-term growth under the highly artificial conditions of cell culture. Although dependent on a series of experimental conditions (culture medium, serum),

Table 5. Immortalization by isolated BPV1 ORFs

| Plasmid DNA* | Coding capacity | Colonies in G418 medium† | Established cultures |
|---|---|---|---|
| | Experiment 1 | | |
| pVV2 | $neo^r$ | 0 | |
| pHLB1 | $E2^+$,$E3^+$,$E4^+$,$E5^+$ | 15 | 0/18 |
| pHLB1-500 | $E2^+$,$E3^+$,$E4^+$,$E5^-$ | 24 | 0/6 |
| pHLB1-709 | $E2^-$,$E3^-$,$E4^-$,$E5^+$ | 39 | 0/6 |
| pHLB1-793 | $E2^-$,$E3^+$,$E4^+$,$E5^+$ | 12 | NT |
| pXH800 | $E6^+E7^+$ | 0 | |
| pXH875 | $E6^-E7^+$ | 0 | |
| pXH997 | $E6^+E7^-$ | 0 | |
| pHLB1 + pXH800 | $E2^+E5^+E6^+E7^+$ | 90 | 3/6 |
| | Experiment 2 | | |
| pVV2 | $neo^r$ | 0 | |
| pSVc-myc-1 | *myc* | 8 | 2/5 |
| pHLB1 + pXH997 | $E2^+E5^+E6^+$ | 10 | 3/5 |
| pHLB1 + pXH875 | $E2^+E5^+E7^+$ | 3 | 0/3 |
| pHLB1-500 + pXH997 | $E2^+E5^-E6^+$ | 9 | 3/7 |
| pHLB1-709 + pXH997 | $E2^-E5^+E6^+$ | 8 | 2/4 |

NT, not tested.
*Cotransfected with pVV2.
†Colonies per $10^6$ transfected cells; average of six to eight plates.

as well as on the cell type and species, it has provided useful biological assays for the characterization of cellular and viral oncogenes and of their interactions with growth controls.

Its complexity is again illustrated by our findings that, unlike polyomaviruses and adenoviruses, which encode a single protein capable of complete immortalization, BPV1 induces a similar phenotype by a cooperative effect involving three viral genes—E2, E5, and E6. The E6 function is required for long-term growth in culture. The observation that it is not, however, sufficient to confer this ability on REF cells leads us to assume that a first event, or series of events, is required. This function, exerted by *myc*, E1A, or T antigen, but not by E6, can be, on the other hand, fulfilled by either E2 or E5 (but not by *ras*). Its physiological or molecular identification will be of interest but will obviously require further work.

One of the limitations of our current approaches of the molecular biology of papillomaviruses is the lack of information on their multiple gene products. With only a fraction of the mRNAs being identified, it is already clear that several of the early ORFs participate in the synthesis of distinct proteins. Two different functions are encoded by ORF E2, a transcriptional activator and a repressor (24, 25). Further studies would be necessary to ascertain their respective roles in immortalization, since with the present set of constructs we could not assay the mutants that discriminate between them (793 and 709) in the absence of E5 expression. Nevertheless, our results show that E2 may have direct effects on cell physiology, in addition to indirect effects via regulation of other BPV1 genes.

The protein product of ORF E5 is as a small hydrophobic polypeptide associated with the membranes of transformed cells (26). Genetic evidence indicates that it can transform established cell lines (8–11). The fact that the same oncogene may act either at the immortalization stage or in terminal transformation, although not usual, is reminiscent of previous reports on overexpression of *ras* (27). Its activity in promoting cell growth up to the formation of isolated colonies is likely to be related to the induction of cellular DNA synthesis, which was recently demonstrated following expression of the gene (28) or microinjection of the protein (29).

Only one protein product of ORF E6 has until now been identified biochemically, localized both in nuclear and membrane fractions (30). It exhibits characteristic repeats of a Cys-Xaa-Xaa-Cys motif, suggesting that it could be a metal binding protein. The protein synthesized in bacteria has recently been shown to bind zinc (31) and some nonspecific DNA binding in low salt has been reported (32). The existence of a second gene product has been inferred from the demonstration that the most 5′ part of E6 can be joined to the 3′ part of E7 by a splice junction between nucleotides 304 and 527 (Fig. 1) (18, 19). The differential effects on immortalization of mutations 775 (nucleotide 445), which would affect the E6 but not the E6–E7 mRNA, and 797, within the E6–E7 fused ORF, suggest that the protein required for immortalization is the complete translation product of E6. The same gene is also necessary for transformation of the established cell line C127. In rat FR 3T3 and in mouse NIH 3T3 cells, it is not absolutely required, but it plays a role in the establishment of the transformed state in suspension culture (refs. 8–11; unpublished data).

No meaningful comparison can yet be made between the immortalizing functions of BPV1 and of the only human papillomavirus that has thus far been shown to participate in the immortalization of the rat cells, HPV16 (23). In contrast to what has commonly been found with other oncogenes, it appears from a comparison of our results with those of Matlashewski *et al.* (23) that the two most frequently used rat primary cell culture systems, REF and baby rat kidney cultures, react in different manners to the expression of HPV16 genes. Baby rat kidney cells were immortalized only by combining high level expression of HPV16 from a heterologous promoter and coexpression of an activated *ras* oncogene. REF cells could be immortalized efficiently by expressing HPV16 under control of its own promoter–enhancer region and coexpression of *ras* had only a limited effect. Experiments using the same LTR-driven HPV16 constructs as in the published experiments should be informative on this point.

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