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The Viral Transcriptional Regulatory Region Upstream of the E6 and E7 Genes Is a Major Determinant of the Differential Immortalization Activities of Human Papillomavirus Types 16 and 18

HELEN ROMANCZUK,[1]* LUISA L. VILLA,[2] RICHARD SCHLEGEL,[3] AND PETER M. HOWLEY[1]

Laboratory of Tumor Virus Biology, National Cancer Institute, Bethesda, Maryland 20892[1]; Ludwig Institute for Cancer Research, Sao Paulo, Brazil[2]; and Department of Pathology, Georgetown University Medical School, Washington, D.C. 20007[3]

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The human papillomavirus types 16 (HPV-16) and 18 (HPV-18) can immortalize primary human keratinocytes. The region of the viral genome responsible for this function maps to the E6 and E7 genes and their respective upstream transcriptional regulatory sequences, the long control regions (LCRs). The HPV-18 LCR/E6/E7 is more efficient in this immortalization function than the analogous region of the HPV-16 genome, resembling the difference in the immortalization potentials of the two full-length viral genomes. This study was designed to examine the basis for the difference in HPV-16 and HPV-18 immortalization efficiencies. The E6 and E7 genes of either HPV-16 or HPV-18, when expressed from the same heterologous promoter, immortalized primary human keratinocytes with the same low efficiency, suggesting that the difference in immortalization activities was not due to the different E6 or E7 genes themselves but rather to a difference in the transcriptional regulatory regions upstream of these genes. The analysis of a series of chimeric HPV-16 and HPV-18 LCR/E6/E7 constructs confirmed this observation and further mapped the viral element responsible for the major difference in immortalization efficiency to the transcriptional regulatory region upstream of the E6 and E7 genes.

Human papillomaviruses (HPVs) are associated with certain human anogenital cancers and with their precursor intraepithelial neoplastic lesions. A subgroup of HPVs, including type 16 (HPV-16) and type 18 (HPV-18), has been detected in over 70% of the invasive squamous carcinomas of the cervix that have been examined (25). Benign lesions containing these HPV DNAs are considered preneoplastic and at risk for progression to carcinomas. Accordingly, HPV-16 and HPV-18 have been grouped with the HPV types associated with a "high risk" for carcinogenic progression. Analyses of lesions positive for HPV-16 or HPV-18 DNAs have shown HPV-16 to be prevalent in both intraepithelial neoplasias and invasive carcinomas, whereas HPV-18 is more prevalent in invasive carcinomas and only rarely found in preneoplastic lesions (3, 4, 13). It has therefore been suggested that HPV-18 might be associated with the development of more rapidly progressing cervical cancers (3, 13). An etiologic role for HPV-16 and HPV-18 in human carcinogenesis is supported by their in vitro immortalization properties. HPV-16 and HPV-18 DNA can each immortalize primary human keratinocytes in culture, resulting in a resistance to stimuli for terminal differentiation. In vitro raft cultures established with HPV-16 or HPV-18 DNAs develop morphologic alterations characteristic of in vivo lesions containing those DNAs (6, 10, 14, 18, 21). HPV-18 DNA is more efficient than HPV-16 DNA in the immortalization of epithelial cells (2, 21, 23), providing some experimental support for the suggestion that it behaves more aggressively in vivo.

Together, the E6 and E7 genes of either HPV-16 or HPV-18 are necessary and sufficient for the efficient immortalization of human squamous epithelial cells (8, 9, 11, 15). Subgenomic fragments containing the HPV-16 or HPV-18 long control regions (LCRs) and the E6 and E7 genes immortalize cells with different degrees of efficiency, resembling the activities of the respective full-length genomes (23). HPV-18 is approximately 10- to 50-fold more efficient than HPV-16 in its immortalization potential (2, 21, 23). The difference in immortalization capacity of the LCR/E6/E7 subfragments of HPV-16 or HPV-18 is a general characteristic of these viruses, a fact confirmed by the analysis of viral subgenomic fragments isolated from individual cancers harboring those DNAs (23). Those earlier studies mapped the genetic determinants for HPV-16 or HPV-18 immortalization efficiency to the E6 and E7 transforming genes and to the transcriptional regulatory regions in the LCRs controlling their expression. The studies presented in this report were designed to further delineate the regions of the HPV-16 and HPV-18 LCR/E6/E7 subgenomic fragments responsible for the difference in immortalization capacity and to determine whether this difference could be accounted for by some property inherent to the transforming genes or the transcriptional regulatory sequences of the respective viruses.

In order to compare the relative biological activity of each of the LCR/E6/E7 regions of the HPV-16 and HPV-18 genomes with that of their full-length viral genome, the LCR/E6/E7 regions were cloned into a pUC-derived vector, as previously described (23) (Fig. 1A). In these constructs, the HPV-16 LCR directs the transcription of the HPV-16 E6 and E7 genes, and the HPV-18 LCR directs the transcription of the HPV-18 E6 and E7 genes. Each of these DNAs was analyzed for its ability to immortalize primary human foreskin keratinocytes by using a transfection procedure previ-

* Corresponding author.

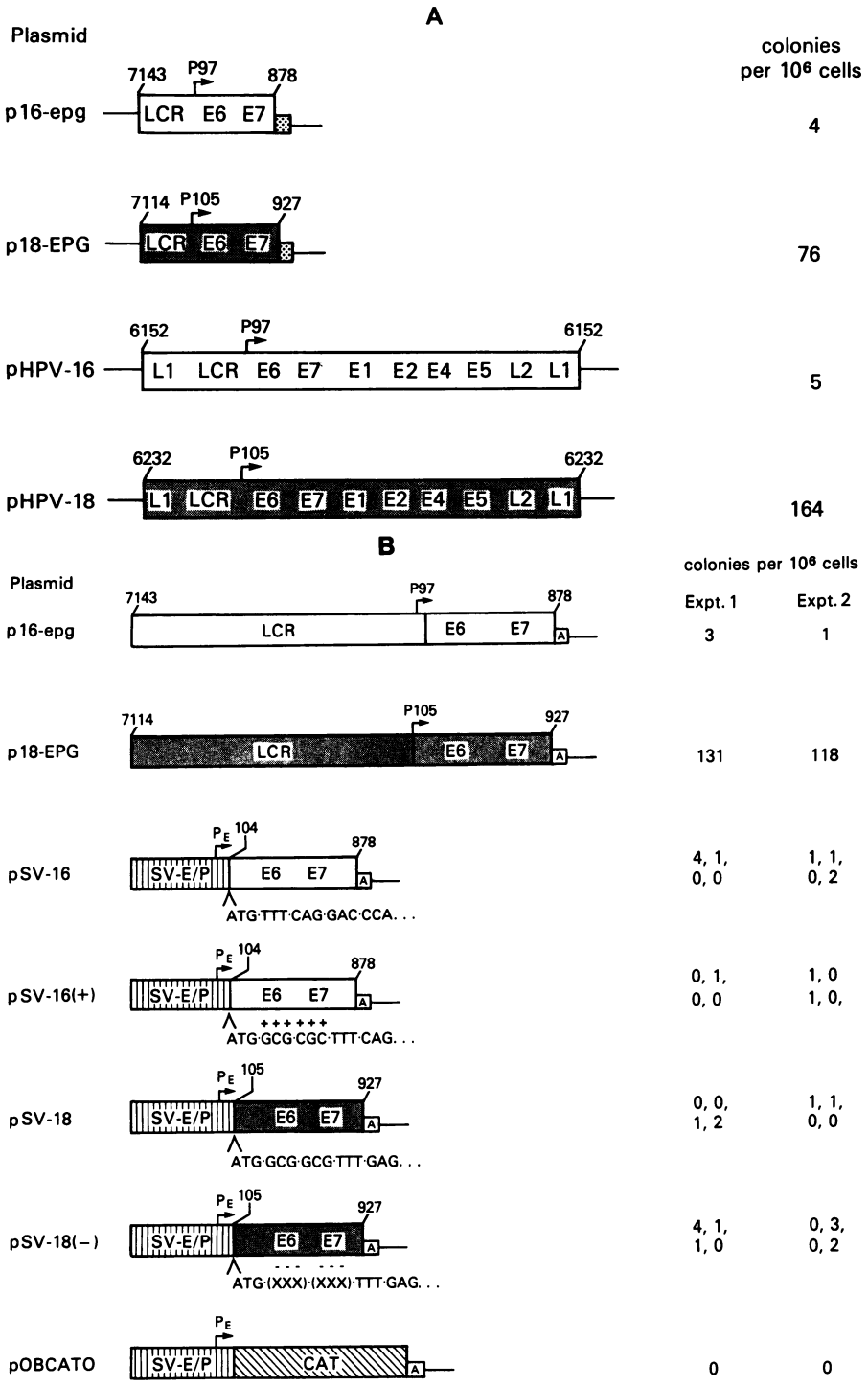

FIG. 1. (A) Immortalization efficiencies of full-length HPV-16 and HPV-18 genomes and the LCR/E6/E7 subgenomic fragments. The LCR/E6/E7 subgenomic fragments of HPV-16 and HPV-18 (4, 7) were cloned in a pUC-derived vector (1), as previously described (23). In p16-epg, the P_{97} promoter directs the transcription of the HPV-16 E6 and E7 genes. In p18-EPG, the P_{105} promoter directs the transcription of the HPV-18 E6 and E7 genes. In the nomenclature used, the uppercase letters refer to HPV-18 sequences and the lowercase letters refer to HPV-16 sequences. The letters E, P, and G refer to enhancer, promoter, and gene sequences, as further described in the legend to panel B. The full-length HPV-16 genome differs from the original HPV-16 isolate (7) in that it contains an uninterrupted E1 open reading frame. A total of 5 μg of each DNA was used for transfection of primary human keratinocytes by lipofection, as previously described (21). Prior to transfection, the full-length HPV-16 and HPV-18 genomes were separated from vector sequences by digestion with *Bam*HI (HPV-16) or *Nco*I (HPV-18); plasmids p16-epg and p18-EPG were linearized with *Hin*dIII, which cuts at the 5′ boundary of each LCR. The colonies were counted 6 weeks after transfection on the basis of their resistance to serum- and calcium-induced differentiation. Open boxes denote HPV-16 sequences, shaded boxes denote HPV-18 sequences, and dotted boxes denote SV40 polyadenylation sequences. Positions of promoters are indicated by arrows. The HPV nucleotides included in each construct are indicated above each diagram. (B) Immortalization

ously described (21). As shown in Fig. 1A, the HPV-18 full-length genome was 30-fold more efficient than the full-length HPV-16 genome in its immortalizing activity. This difference was also observed with the LCR/E6/E7 regions of the respective viruses (p16-epg and p18-EPG), in agreement with a previous study (23).

In order to directly compare the biological activities of the E6 and E7 gene products of HPV-16 and HPV-18, the corresponding genes were cloned in an analogous position downstream of the simian virus 40 (SV40) early promoter in pOBCAT0 (1) (Fig. 1B). The constructs pSV-16 and pSV-18 contain the SV40 enhancer and early promoter upstream of the E6 and E7 genes of HPV-16 and HPV-18, respectively. The HPV sequences in each construct begin with the ATG of E6, downstream of a strong translation initiation consensus sequence (12). The plasmid pSV16(+) contains six additional nucleotides (GCGCGC) downstream of the HPV-16 ATG, and the plasmid pSV-18(−) has six nucleotides (GCGCGC) deleted downstream of the HPV-18 ATG. These modifications were included to compare the HPV-16 and HPV-18 E6 and E7 immortalization efficiencies by using constructs in which the E6 proteins have similar amino termini (5, 22). Thus, pSV-16(+) has two additional amino acids at the amino terminus of E6 and is therefore comparable to the wild-type HPV-18 E6 protein. Sequences encoding these two additional amino acids are deleted in pSV-18(−), and it is therefore comparable at its amino terminus to the E6 protein of HPV-16. Each chimeric construct contains identical nucleotide sequences upstream of the E6 ATG. The junctions between the regulatory regions and the E6 and E7 genes were verified by sequence analysis. Each of these hybrid plasmids was then tested for its ability to immortalize primary human keratinocytes compared with p16-epg and p18-EPG. As shown in Fig. 1B, each of the plasmids pSV-16, pSV-16(+), pSV-18, and pSV-18(−) was similar and inefficient at immortalization. None of these hybrid constructs could transform cells with an efficiency comparable to that of the HPV-18 E6 and E7 genes downstream of the HPV-18 LCR (p18-EPG). The fact that the HPV-16 and HPV-18 proteins had similar, although low, immortalization properties when expressed from the same enhancer-promoter suggested that the intrinsic viral transforming potentials of the different E6 and E7 proteins were not responsible for the 10- to 50-fold difference in biological activity observed in the keratinocyte assays using HPV regulatory regions. The low level of activity obtained with an SV40 enhancer-promoter is a phenomenon observed with every heterologous enhancer-promoter analyzed to date (15, 16). These data support the hypothesis that the HPV upstream regulatory regions are major determinants in the different immortalization activities of the HPV-16 and HPV-18 genes. Therefore, we compared the regulatory regions upstream of the E6 and E7 genes for different activities.

The HPV-18 P_{105} promoter is more active than the HPV-16 P_{97} promoter in primary human keratinocytes (19). In order to determine whether differences in promoter strength, in the context of a full viral LCR, could account for differences in immortalization efficiency, a series of chimeric plasmids was constructed in which analogous regions from one virus were substituted in the other, as shown in Fig. 2A and B. The DNA segments exchanged were the enhancer-promoter region upstream of the TATA box (designated "E" or "e"), the proximal promoter region extending from the TATA box to the E6 translational start site (designated "P" or "p"), and the E6 and E7 coding region (designated "G" or "g") (Fig. 2A and B).

The chimeric HPV-16 and HPV-18 LCR/E6/E7 constructs were assayed for their abilities to immortalize primary human keratinocytes. The results, presented in Fig. 2B, revealed that the HPV-18 LCR upstream of the TATA box was responsible for a high efficiency of immortalization as long as the downstream promoter was intact. Only those constructs containing the HPV-18 LCR enhancer region (Fig. 2B, p18-EPG, pc-EpG, and pc-Epg, where "c" denotes a chimeric construct) could immortalize primary cells with high degrees of efficiency. In contrast, those plasmids containing the analogous HPV-16 LCR enhancer region (p16-epg, pc-ePg, pc-epG, and pc-ePG) had lowered immortalization activities. The chimeric construct pc-EPg, which contains the HPV-18 LCR and the HPV-18 proximal promoter upstream of the HPV-16 E6 ATG, had a lowered efficiency in this immortalization assay, probably because of the alteration of the E6 translational start site. In this construct, the E6 and E7 genes of HPV-16 were substituted for those of HPV-18 at the ATG of each E6 gene, resulting in the deletion of apparently necessary sequences between the transcriptional and translational start sites of HPV-16 E6 (Fig. 2A). The substitution of only the HPV-18 proximal promoter upstream of the HPV-16 E6 and E7 genes did not increase the immortalization efficiencies of those genes (pc-ePg). The substitution of the HPV-16 proximal promoter segment for the HPV-18 proximal promoter (pc-EpG) resulted in a transformation efficiency similar to that of the wild-type HPV-18 construct (p18-EPG). These data suggest that although the exact sequences of the HPV-16 and HPV-18 proximal promoters are not invariable, there is a minimum requirement for the spacing between the TATA box and the E6 ATG. Furthermore, the data show that the HPV-16 and HPV-18 transcriptional regulatory regions upstream of the respective E6 ATGs are major determinants of the different immortalization activities of the E6 and E7 genes.

The data presented here demonstrate that sequence elements of the HPV-16 and HPV-18 LCRs constitute the major determinants that discriminate between the biological activities of the respective viruses. Those constructs containing the HPV-18 LCR upstream of either the HPV-16 or HPV-18 E6 and E7 genes induced colonies which appeared two

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by the E6 and E7 genes of HPV-16 and HPV-18 expressed from the SV40 early promoter. The LCRs of p16-epg and p18-EPG, up to the ATG of the respective E6 genes, were replaced by the SV40 enhancer and early promoter of pOBCAT0 (1). The junction between the SV40 sequences and the respective HPV E6 genes was modified by the addition of the synthetic oligonucleotides CCACC immediately upstream of the ATG to create a good translational initiation site (12). Each construct has the identical 77 nucleotides downstream of the SV40 promoter to the start of each E6 ATG. A total of 5 μg of each plasmid was linearized at the *Hin*dIII site upstream of each enhancer, prior to transfection of primary human keratinocytes by lipofection. The number of differentiation-resistant colonies was determined at 6 weeks. The results shown are based on two experiments using two different keratinocyte cultures. Open and shaded boxes are as in panel A; vertically hatched boxes denote SV40 enhancer and promoter sequences, and diagonally hatched boxes denote CAT sequences. "A" denotes the SV40 early polyadenylation site. Positions of promoters are shown by arrows. The sequences downstream of each E6 ATG are shown for comparison; nucleotides deleted (− − −) or added (+ + +) by oligonucleotide reconstruction are shown. P_E, Early promoter.

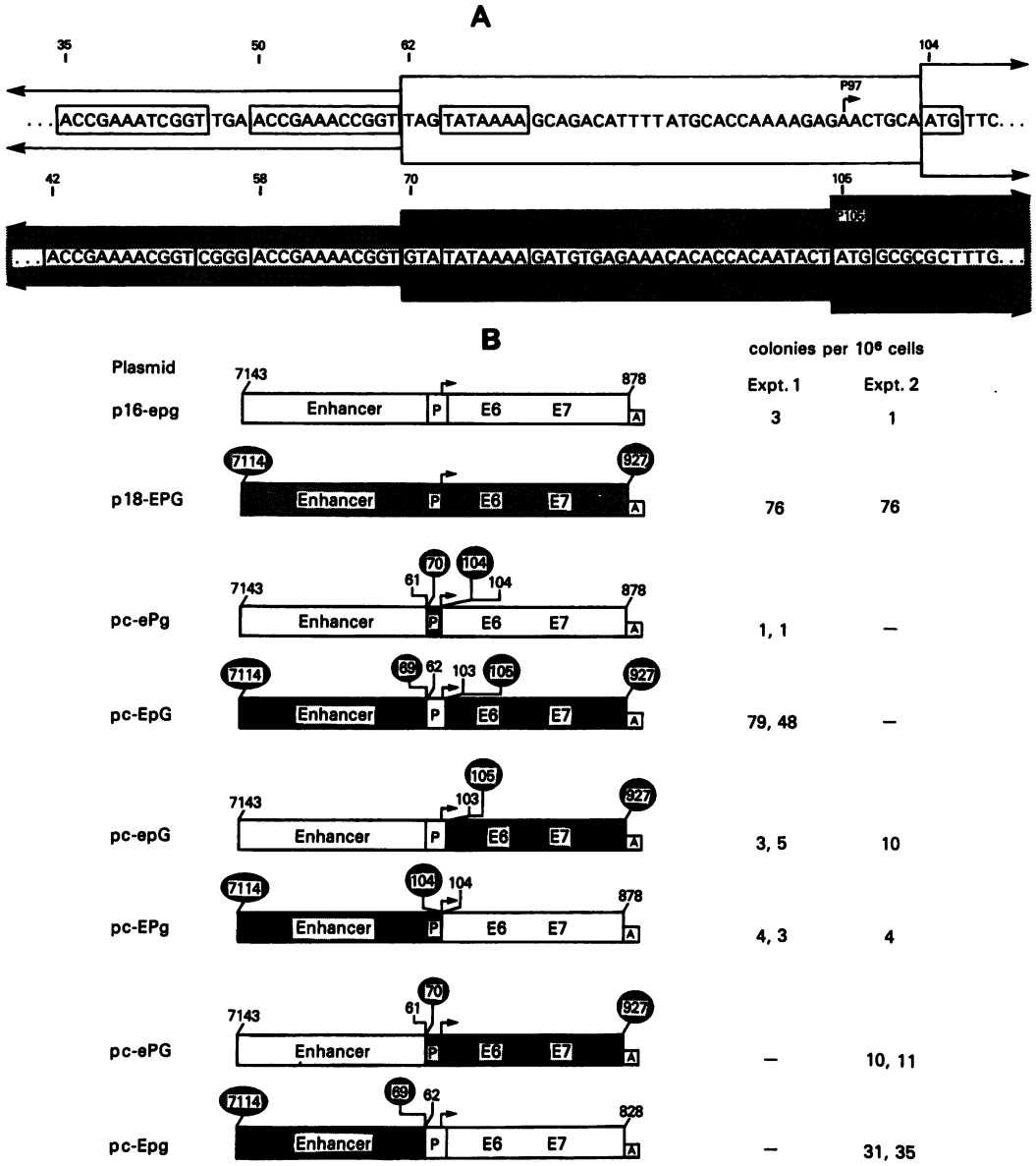

FIG. 2. (A) Subgenomic HPV-16 and HPV-18 regions used for generating chimeric constructs. The LCR/E6/E7 regions of HPV-16 and HPV-18 were subdivided as shown schematically by each boxed set of nucleotides for construction of chimeric plasmid DNAs. HPV-18 sequences are shaded to distinguish them from HPV-16 sequences. Uppercase letters (E, P, G) refer to HPV-18 sequences; lowercase letters (e, p, g) refer to HPV-16 sequences. The positions of the P_{97} and P_{105} promoters of HPV-16 and HPV-18 are designated by arrows. The ATG of each E6 gene, the TATA boxes, and the proximal E2 binding sites ($ACCN_6GGT$) are boxed. (B) Immortalization activities of chimeric HPV-16 and HPV-18 LCR/E6/E7 constructs. Chimeric LCR/E6/E7 regions were generated with the subfragments shown in panel A. Each chimeric construct contains an enhancer segment, the 3′ end of which is the promoter proximal E2 binding site; a proximal promoter (P), whose 3′ boundary is the nucleotide upstream of the E6 ATG; and the E6 and E7 genes, the 5′ boundary of which is the ATG of E6. HPV-18 sequences are shaded to distinguish them from HPV-16 sequences. The HPV-16 and HPV-18 nucleotides contained in each construct are indicated. A total of 5 μg of each construct was linearized by *Hin*dIII, which cuts at the 5′ boundary of each LCR, prior to transfection. Results shown are from two separate experiments using two different keratinocyte cultures.

weeks earlier than constructs containing the HPV-16 LCR, and the constructs were more efficient in immortalizing primary human keratinocytes in culture, as shown in Fig. 3. In the flask of cells transfected with p16-epg, only a single small colony was visible 6 weeks after transfection (Fig. 3a). In contrast, a flask of keratinocytes transfected with p18-EPG contained numerous colonies which grew to form a confluent sheet of cells within the same time after transfection (Fig. 3b). Chimeric constructs containing the HPV-18 enhancer upstream of the HPV-16 E6 and E7 genes gave rise to numerous colonies which formed a near-confluent sheet of cells (Fig. 3d and f), while the substitution of the HPV-16 enhancer in place of the analogous HPV-18 enhancer resulted in a decreased immortalization efficiency (Fig. 3c).

In summary, we have shown that the transcriptional regulatory regions upstream of the HPV-16 and HPV-18 E6

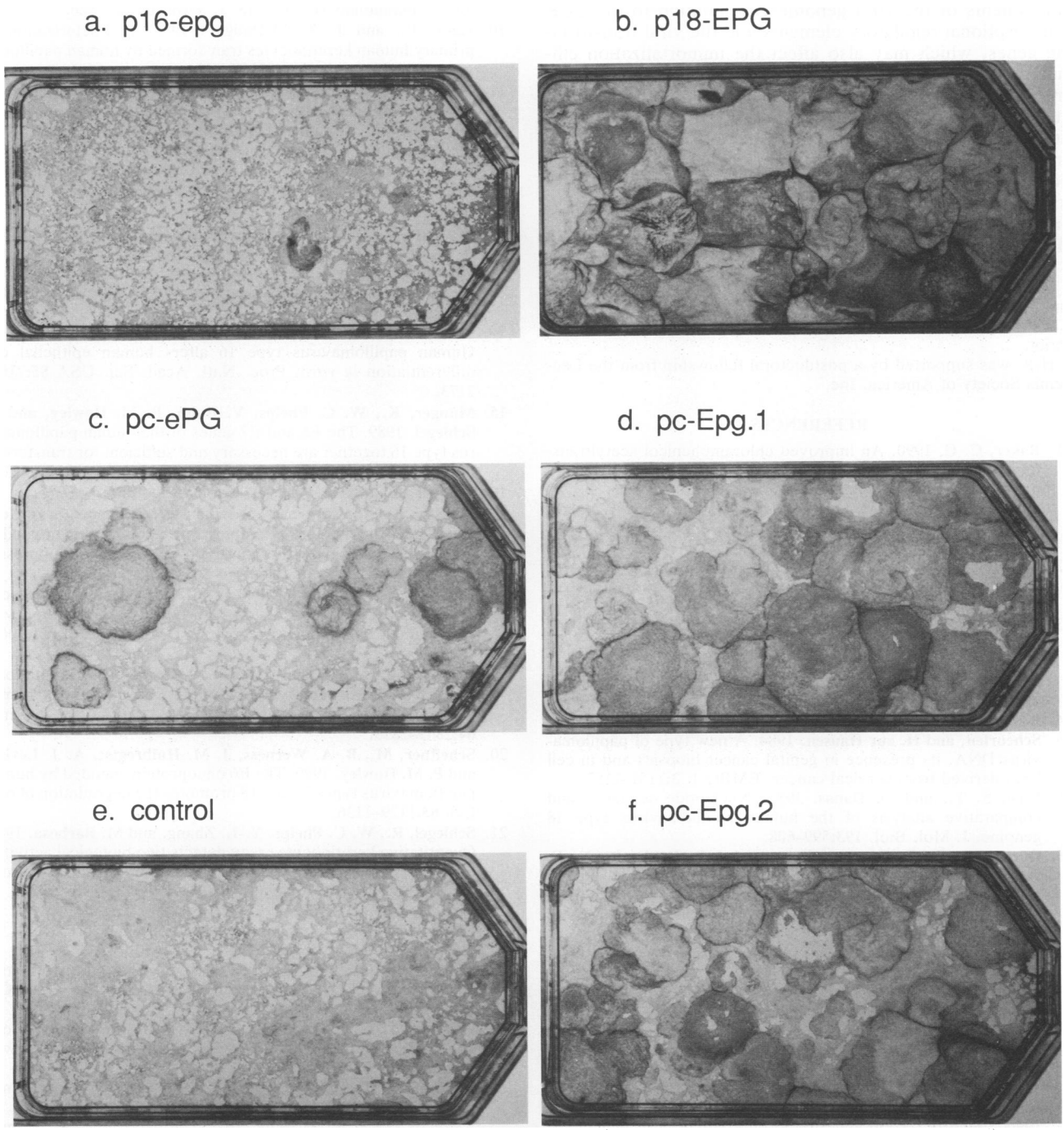

FIG. 3. Immortalization of primary human keratinocytes by HPV-16 and HPV-18 LCR/E6/E7 chimeric DNAs. A total of 5 μg of the indicated plasmid DNA was linearized prior to transfection of primary human keratinocytes by using lipofection. Cells were fixed and stained 6 weeks after transfection, as previously described (21). DNAs used for transfection are indicated. The control, p1318, is a plasmid containing a human β-actin promoter in a plasmid vector (15). Epg.1 and Epg.2 are independent chimeric constructs.

and E7 genes are important in specifying the differential immortalization activities of the respective viral genomes. The region of the LCR responsible for the majority of this difference in activity maps upstream of the E6 ATG of HPV-16 and the E6 ATG of HPV-18. The 10- to 50-fold difference in immortalization capacity observed between HPV-16 and HPV-18 cannot be accounted for by the E6 and E7 genes themselves. The HPV-16 and HPV-18 E6 and E7 genes downstream of the SV40 early promoter had similar low activities in these immortalization assays and appeared at similar times after transfection. These results are not unexpected considering the biochemical similarities between the E6 proteins and the E7 proteins of HPV-16 and HPV-18 (17, 20, 24). It is possible, however, that there are other

components of the viral genomes, in addition to the LCR transcriptional regulatory elements and the viral transforming genes, which may also affect the immortalization efficiencies of the viral genomes and that have not been uncovered by the studies presented here. The importance of the nucleotide spacing between the transcriptional and translational start sites suggests that message translatability may be an important determinant for viral protein activity. In addition, sequences in the 3′ portion of the complete viral early regions could have important effects on mRNA stability, providing additional differences between HPV-16 and HPV-18 transforming potentials.

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REFERENCES

1. **Baker, C. C.** 1990. An improved chloramphenicol acetyltransferase expression vector system for mapping transcriptional and post-transcriptional regulatory elements in animal cells, p. 75–86. *In* K. K. Alitalo, M.-L. Huhtala, J. Knowles, and A. Vaheri (ed.), Recombinant systems in protein expression. Elsevier Science Publishers B.V., Amsterdam.
2. **Barbosa, M., and R. Schlegel.** 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. Oncogene **4**:1529–1532.
3. **Barnes, W., G. Delgado, R. J. Kurman, E. S. Petrilli, D. M. Smith, S. Ahmed, A. T. Lorincz, G. F. Temple, A. B. Jenson, and W. D. Lancaster.** 1988. Possible prognostic significance of human papillomavirus type in cervical cancer. Gynecol. Oncol. **29**:267–273.
4. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3**:1151–1157.
5. **Cole, S. T., and O. Danos.** 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. J. Mol. Biol. **193**:599–608.
6. **Dürst, M., R. T. Dzarlieva-Petrusevska, P. Boukamp, N. E. Fusenig, and L. Gissmann.** 1987. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene **1**:251–256.
7. **Dürst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
8. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8**:3905–3910.
9. **Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laimins.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J. Virol. **64**:519–526.
10. **Kaur, P., and J. K. McDougall.** 1988. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. J. Virol. **62**:1917–1924.
11. **Kaur, P., J. K. McDougall, and R. Cone.** 1989. Immortalization of primary human epithelial cells by cloned cervical carcinoma DNA containing human papillomavirus type 16 E6/E7 open reading frames. J. Gen. Virol. **70**:1261–1266.
12. **Kozak, M.** 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**:8125–8148.
13. **Kurman, R. J., M. H. Schiffman, W. D. Lancaster, R. Reid, A. B. Jenson, G. F. Temple, and A. T. Lorincz.** 1988. Analysis of individual human papillomavirus types in cervical neoplasia: a possible role for type 18 in rapid progression. Am. J. Obstet. Gynecol. **159**:293–296.
14. **McCance, D. J., R. Kopan, E. Fuchs, and L. A. Laimins.** 1988. Human papillomavirus type 16 alters human epithelial cell differentiation *in vitro*. Proc. Natl. Acad. Sci. USA **85**:7169–7173.
15. **Münger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63**:4417–4421.
16. **Münger, K., and R. Schlegel.** Unpublished data.
17. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. **8**:4099–4105.
18. **Pirisi, L., S. Yasumoto, M. Feller, J. Doniger, and J. A. DiPaolo.** 1987. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J. Virol. **61**:1061–1066.
19. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J. Virol. **64**:2849–2859.
20. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63**:1129–1136.
21. **Schlegel, R., W. C. Phelps, Y.-L. Zhang, and M. Barbosa.** 1988. Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. EMBO J. **7**:3181–3187.
22. **Seedorf, K., G. Krämmer, M. Dürst, S. Suhai, and W. G. Röwekamp.** 1985. Human papillomavirus type 16 DNA sequence. Virology **145**:181–185.
23. **Villa, L. L., and R. Schlegel.** Differences in transformation activity between HPV-18 and HPV-16 map to the viral LCR/E6/E7 region. Virology, in press.
24. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248**:76–79.
25. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in human anogenital cancer, p. 245–263. *In* N. P. Salzman and P. M. Howley (ed.), The papovaviridae. Plenum Publishing Corp., New York.