Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: Implications for cervical carcinogenesis

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ABSTRACT   In many cervical cancers, human papillomavirus type 16 (HPV-16) DNA genomes are found to be integrated into the host chromosome. In this study, we demonstrate that integration of HPV-16 DNA leads to increased steady-state levels of mRNAs encoding the viral oncogenes E6 and E7. This increase is shown to result, at least in part, from an increased stability of E6 and E7 mRNAs that arise specifically from those integrated viral genomes disrupted in the 3′ untranslated region of the viral early region. Further, we demonstrate that the A+U-rich element within this viral early 3′ untranslated region confers instability on a heterologous mRNA. We conclude that integration of HPV-16 DNA, as occurs in cervical cancers, can result in the increased expression of the viral E6 and E7 oncogenes through altered mRNA stability.

Human papillomaviruses (HPVs) are small DNA viruses that infect epithelial cells. About 70 different genotypes have been identified to date, among which only a subset are associated with cervical cancers. More than 90% of cervical cancers contain these "high-risk" HPVs (HPV-16, -18, -31, and -33). In these cancers, two viral transforming genes, E6 and E7, are consistently expressed (1). The E6 and E7 proteins have been shown to interact with and inactivate the tumor-suppressor gene products p53 and pRb, respectively (34), and to immortalize human epithelial and fibroblastic cells as well as rodent fibroblasts (2–4). In transgenic mouse systems, expression of these genes leads to tumor formation (5–8). These studies have demonstrated the likely importance of E6 and E7 in cervical carcinogenesis.

The viral DNA genome of HPV-16 or -18 is often found integrated into the host chromosomes in cervical cancers (9–11). This viral DNA integration has been hypothesized to result in increased expression of E6 and E7 (12). To test this hypothesis, we have isolated a series of human cervical epithelial cell populations that harbor either extrachromosomal or integrated HPV-16 DNA (35). These cell populations were derived from a parental cell population, W12, that had been established from an HPV-16-positive cervical biopsy (13). Using these reagents, we have demonstrated that HPV-16 DNA integration correlates with increased expression of the viral E7 protein and with a selective growth advantage over cells harboring extrachromosomal HPV-16 DNA (35).

In the current study, we have sought to define a mechanism by which integration leads to increased expression of papillomaviral transforming genes. We demonstrate that the high levels of E7 protein seen in the integrated clones correlate with increased steady-state levels of E6- and E7-specific mRNAs, at least in part as a result of changes in their stability. This increased stability appears to be the result of the integrative

disruption of the viral 3′ untranslated region (UTR) which we demonstrate contains an mRNA instability element.

MATERIALS AND METHODS

Southern and Northern Hybridization Analyses. Southern and Northern hybridizations were carried out by standard methods. For Southern analysis, a full-length HPV-16 DNA probe was used. For Northern analysis either an HPV-16 E6/E7-specific probe, generated by PCR using primers complementary to HPV-16 nt 79–101 and nt 883–864, or an HPV-16 3′ UTR-specific probe, generated by digestion of plasmid pHPV-16 with *Stu* I and *Xcm* I, which cleave HPV-16 DNA at nt 3871 and nt 4466, respectively.

S1 Nuclease Mapping Analysis. The E6/E7-specific cDNA fragment, used as an S1 probe, was generated by PCR amplification of an HPV-16-specific cDNA with primers complementary to HPV-16 nt 831–850 and 4152–4136 and subcloned into plasmid pGEM-3Z (Promega) to derive the clone pSJ314.71. The cDNA was first generated by reverse transcription of total cellular RNA from clone 20850e by using the primer complimentary to HPV-16 nt 4152–4136. Digestion of pSJ314.71 with *Nco* I and *Nar* I gave rise to a 1000-bp-long E6/E7 cDNA-specific probe (probe 1). The HPV-16 genomic DNA probe (probe 2) was generated by digestion of pHPV-16 with *Taq* I and *Nar* I, which cleave at HPV-16 nt 505 and 1309. The probes were ^{32}P-labeled at the 3′ end by nucleotide fill-in reactions. S1 nuclease analysis was performed as described (14).

Actinomycin D Assay. Total cellular RNA was extracted 0, 1, 2, 6, and 12 hr after administration of actinomycin D (5 µg/ml; Pharmacia). Northern analyses were performed with the E6/E7 probe described above.

***fos* Promoter Expression System.** NIH 3T3 mouse fibroblasts were cotransfected by the calcium phosphate precipitation method (15) with either pSJfosGlob or pSJfosGlob16 and pSVneo (16), which confers Geneticin (G418) resistance. Cells were placed under selection for G418 resistance for 2 weeks and colonies were pooled and expanded. The pooled population was starved for 25 hr in Dulbecco's modified Eagle's medium (DMEM) containing 0.5% calf serum (completeness of synchronization was assessed by fluorescence-activated flow cytometry) and restimulated in DMEM with 10% calf serum. Digestion of pSJfosGLOB with *Hind*III and *Bam*HI gave rise to the β-globin-specific probe used for the Northern analyses. pSJfosGLOB was made by replacing the *Eco*RI–*Xcm* I fragment of pfos-βGLOB (human β-globin gene) (17) with that of pBBB3 (rabbit β-globin gene) (18), thus providing a unique *Bgl* II site in the β-globin 3′ UTR for cloning purposes. The 3′ UTR of HPV-16 was amplified by PCR using primers complementary to HPV-16 nt 4005–4025 and 4213–4195 and inserted into this unique *Bgl* II site of pSJfosGLOB, resulting in pSJfosGLOB16.

Abbreviations: HPV, human papillomavirus; UTR, untranslated region; ARE, A+U-rich element.
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RESULTS

To investigate the role that integration of HPV-16 DNA into human chromosome plays in cervical carcinogenesis, we have cloned and characterized cell populations harboring either extrachromosomal (e) or integrated (i) viral DNA (35) from the W12 cell population (13). A representative Southern analysis (Fig. 1) demonstrates that two of these clonal populations (20850e and 20863e) harbor ≈1000 copies of intact HPV-16 DNA, the majority of which were extrachromosomal as evidenced by the presence of the supercoiled form of HPV-16 DNA. Other clones exclusively harbor integrated viral DNA as judged by the absence of supercoiled DNA. Among these clones, two distinctly different patterns of integration were observed (Fig. 1); clones 20822 and 201402 gave rise to two junction-specific bands (type 1 integration), whereas clones 20831, 20862, and 20861 gave rise to both unique junction-derived fragments and unit-length 7.9-kb bands (type 2 integration).

To determine whether the heightened level of E7 protein expression in the integrated clones (35) was the consequence of increased levels of E7-specific mRNAs, we measured the steady-state level of HPV-16 E6/E7 mRNAs among the different clones by Northern analysis of total cellular RNA hybridized to an E6/E7-specific probe. A lower or similar level of E6/E7 mRNA expression was observed in the extrachromosomal clones compared with the integrated clones (Fig. 2). When corrected for differences in viral DNA copy number, a significantly higher level of E6/E7 mRNAs per viral genome copy accumulated in the integrated clones than in extrachromosomal clones (Table 1). When the same Northern blot was rehybridized to a radiolabeled probe specific for the 3' UTR of the HPV-16 early region, the E6/E7 mRNAs in the extrachromosomal clones, but not in the integrated clones, were detected. The lack of hybridization of the 3' UTR probe to E6/E7 mRNAs was unexpected in the case of the type 2
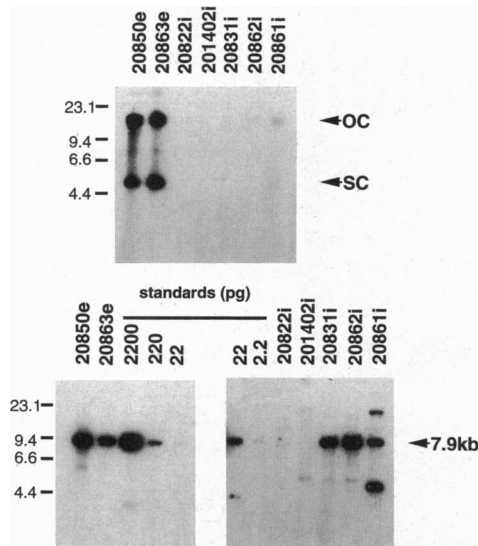

FIG. 1. State of HPV-16 DNA in the clonal populations derived from W12 cells. Southern analysis of both uncut sheared (*Upper*) and *Bam*HI-digested (*Lower*) total genomic DNA from clonal populations, hybridized with the full-length HPV16 probe. *Bam*HI cuts the HPV-16 genome once, at nt 6150. Because of the wide range of viral copy numbers among the cell populations, two different exposures of the blot of *Bam*HI-digested DNA are shown. Positions of open-circular (OC), supercoiled (SC), and 7.9-kb linearized HPV-16 DNA are indicated. Copy number of HPV-16 DNA was assessed in reference to the standards in which 2.2, 22, 220, and 2200 pg of cloned HPV-16 (corresponding to 1, 10, 100, and 1000 molecules of HPV-16 DNA per cell) were used in the reconstruction with DNA from HPV-negative Scc13ya cells.
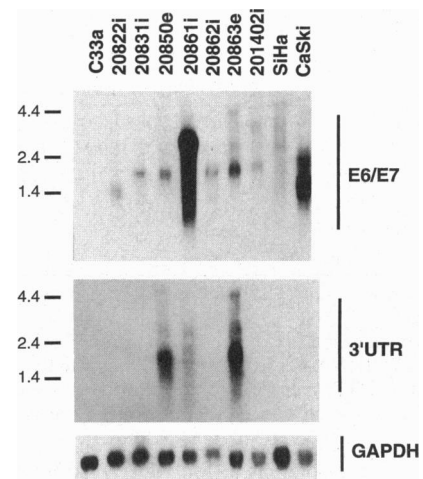

FIG. 2. Steady-state level of E6/E7 mRNA expression in the clonal populations. A Northern blot of total RNA from W12 clonal cell populations was sequentially hybridized with the probes specific to the HPV-16 E6/E7 region (*Top*), HPV-16 3' UTR (*Middle*), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (*Bottom*) (provided to account for sample load variations on the gel). The C33a human cervical cancer cell line, which is HPV-negative, was used as a negative control. SiHa and CaSki are HPV-16-positive human cervical cancer cell lines. Note that the strong hybridization to the E6/E7-region probe was not completely removed in the 20661i lane when it was reprobed with the 3' UTR probe. Additional experiments verified an absence of detectable hybridization to the 3' UTR probe for RNA obtained from clone 20861i (data not shown).

integrated clones 20831, 20861, and 20862, since a significant fraction of the viral genomes present in these cells are unit length and therefore should give rise to E6/E7 mRNAs that possess the viral 3' UTR. Interestingly, this result is consistent with what is observed in the cervical cancer cell line CaSki, which also has a type 2 integration event (19) (Fig. 2).

To more accurately characterize the structures of the E6/E7 mRNAs accumulating in our different clonal populations, we performed S1 nuclease mapping analysis using an E6/E7 cDNA probe (probe 1) (Fig. 3A). The 812-nt S1 product, indicative of the presence of E6/E7 mRNAs arising from intact viral genome (Fig. 3A), was detected only in the extrachromosomal clones (20850e and 20863e) (Fig. 3B). In contrast, S1 products of ≈380 nt were detected in the majority of integrated clones. These products were similar in size to that seen with CaSki RNA, suggesting that the integration events have led to the disruption of the viral sequences at nucleotide positions close to that in CaSki (20, 21). Consistent with this conclusion, we have cloned and sequenced the E6/E7 mRNAs arising from one of these clones, 20822i, and found the virus/cell junction to lie at HPV-16 nt 3732 (35). Two integrated clones, 20861i and 201402i, as well as the cervical cancer cell line SiHa, failed to give rise to any detectable S1 product with the E6/E7 cDNA probe, though one could detect E6/E7 mRNA with an E6/E7 genomic probe (probe 2) (Fig. 3B). The simplest interpretation of these results is that these E6/E7 RNAs utilize an alternative cell-specific 3' splice site. With probe 2, two S1 products were obtained with clone 20861i RNA, and both were slightly larger than the product generated from RNAs utilizing the HPV-16 5' splice signal at nt 880. This result suggests either that an alternative 5' splice signal close to HPV-16 nt 880 is utilized in clone 20861i or that the viral/cellular junction is at this location.

The above described RNA analyses indicate that the E6/E7 mRNAs that accumulate in the clonal cell populations harboring integrated viral DNA arise predominantly from the copies of the viral genome disrupted by the integration event even in the type 2 integrated clones. This result led us to

Table 1.   Properties of W12 clonal populations and cervical tumor cell lines

	Extrachromosomal	Integrated					
		Type 1			Type 2		
Cell population	20850/20863	20822	201402	SiHa	20831/20862	20861	CaSki
Viral copy number	1000	3	5	2	60	30	600
E6/E7 mRNA level*	0.2/0.4	0.2	0.3	0.3	0.2/0.3	2.3	1.0
	(0.12/0.24)	(40)	(36)	(94)	(2.0/3.0)	(48)	(1.0)
mRNA from intact HPV16[†]	+	−	−	−	−	−	−
E6/E7 mRNA half-life,[‡] hr	3	6	6	ND	6	>12	6

Quantitation was performed by PhosphorImager analyses (Molecular Dynamics). Data on viral copy number was adopted from elsewhere (35). ND, not done.

*Obtained from data in Fig. 2 corrected to that of GAPDH mRNA. Values shown are normalized to E6/E7 mRNA level in CaSki. In parentheses, level of E6/E7 mRNA per viral genome copy relative to CaSki is indicated.

[†]Results from 3' UTR-specific Northern blot (Fig. 2) and S1 nuclease mapping analysis (Fig. 3B) are summarized.

[‡]Calculated from actinomycin D mRNA-decay experiments (Fig. 4).

suspect that a possible cis effect was responsible for this selective accumulation of junction-derived E6/E7 mRNAs. The junction-derived E6/E7 mRNAs differ from the normal E6/E7 mRNAs in their 3' ends. Given the well-documented role of 3' UTRs in mRNA stability (22), we sought to determine whether the half-lives of E6/E7 mRNAs differed among the different clones. This was accomplished through the use of actinomycin D treatment, which blocks *de novo* RNA synthesis. The half-life of the E6/E7 mRNAs in the extrachromosomal clones 20850e and 20863e was 3 hr (Fig. 4). In comparison, the half-lives of E6/E7 mRNAs that accumulated in the integrated clones or cervical tumor cell lines were 6 to >12 hr. Thus, the actinomycin D experiments (summarized in Table 1) indicate that differences in mRNA stability might account in part for the selective accumulation of E6/E7 mRNAs from the junction copies of the viral genome.

Unstable mRNAs such as c-*fos* mRNA have been found to contain A+U-rich elements [AREs (18)] in their 3' UTR that contribute to mRNA instability (23). A region that is 80% A+U (HPV-16 nt 4005–4213) is present within the 3' UTR of E6/E7 mRNAs arising from intact viral copies. Importantly, the 3' UTRs of E6/E7 mRNAs transcribed from integrated, disrupted HPV-16 genomes are replaced by cellular sequences with a lower A+U content: for example, 55% in CaSki (20) and 51% in 20822i (35). To see whether the ARE in the HPV-16 early-region 3' UTR was sufficient to confer instability on an mRNA, the viral 3' UTR was inserted into β-globin gene, a gene that expresses a highly stable mRNA, under the transcriptional control of the serum-responsive c-*fos* promoter (17, 18, 24, 25). This inducible promoter creates a short pulse of mRNA synthesis in synchronized populations of NIH 3T3 cells upon serum stimulation, providing a means to measure
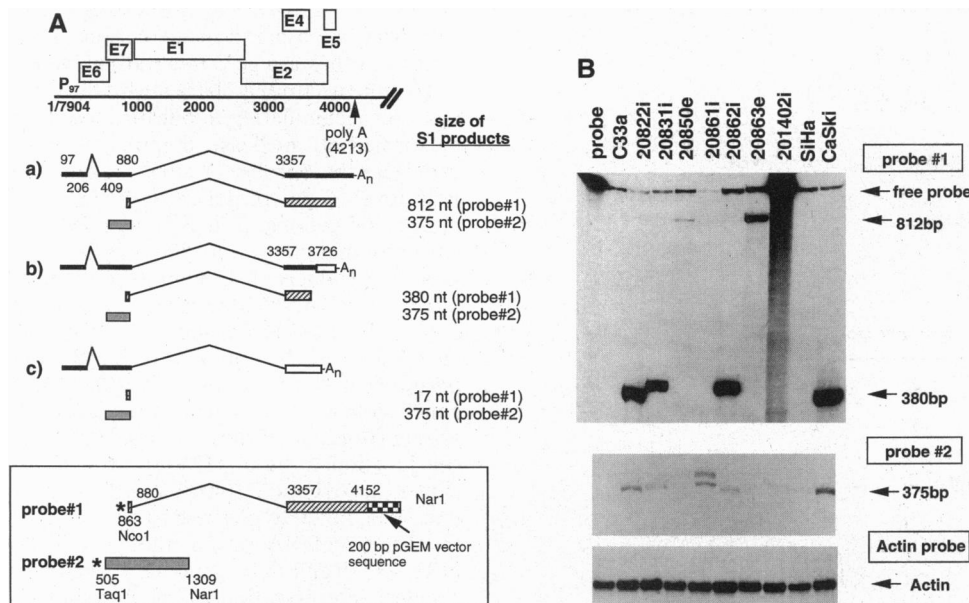

FIG. 3.   E6/E7 mRNA expression in extrachromosomal clonal populations vs. integrated clonal populations and tumor cell lines. (*A*) Schematic illustration of potential E6/E7 mRNA species (solid boxes, HPV-16 RNA; open boxes, cellular sequences). Below each species are indicated S1 nuclease products protected by either the E6/E7 cDNA-derived probe (probe 1) or the HPV-16 genomic DNA-derived probe (probe 2) shown at the bottom (hatched and stippled boxes, HPV-16 DNA). (*a*) Representative HPV-16 E6/E7 mRNA species. The presence of the splice between HPV-16 nt 880 and 3357 and the viral 3' UTR is common in all differentially spliced E6/E7 mRNAs from intact HPV-16 DNA. (*b*) The E6/E7 mRNA in CaSki cervical cancer cells. Splicing occurs between HPV-16 nt 880 and 3357 with fusion between viral and cellular sequences at nt 3726 (20). (*c*) The E6/E7 mRNA in SiHa cervical cancer cells. Splicing from HPV-16 nt 880 to a cellular 3' splice signal has been detected (21). (*B*) S1 nuclease mapping analysis using either probe 1 (*Top*), probe 2 (*Middle*), or an actin probe (*Bottom*). The human actin probe was used as a control for sample load variation. Note that the S1 digestion of probe 1 in the 201402i sample was incomplete in the experiment presented. In other experiments, complete S1 digestion demonstrated the absence of any probe 1-specific S1 product for this RNA similar to that seen with SiHa and 20861i. Also note that levels of 20850i HPV-16-specific S1 products were very low (correlating with low levels seen by Northern analysis in Fig. 1); however, upon longer exposure the 375-bp probe 2 S1 product was detected.
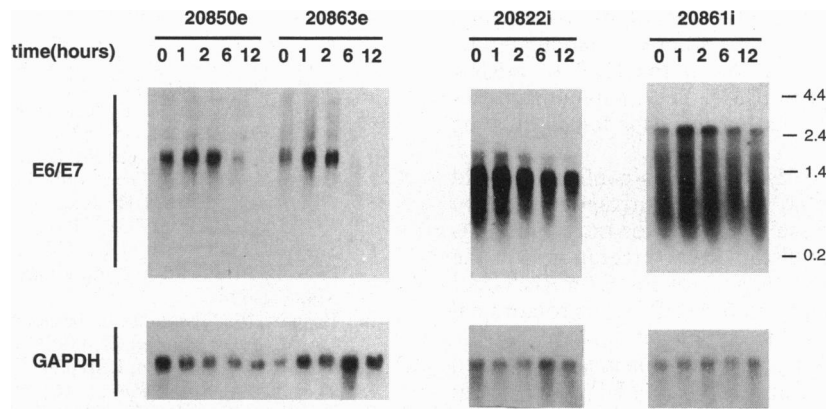
FIG. 4.   Stability of E6/E7 mRNAs in extrachromosomal vs. integrated clonal populations. Northern hybridization to an E6/E7-specific probe was carried out with total cellular RNA obtained from clonal populations (20850e, 20863e, 20822i, and 20861i) 0, 1, 2, 6, and 12 hr after blocking of *de novo* transcription with actinomycin D. To determine half-lives as reported in Table 1, levels of E6/E7 mRNAs were corrected for variation in loading by comparison with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA signal. The validity of the actinomycin D assay in our hands was determined by probing Northern blots with a c-*myc*-specific probe; the half-life of c-*myc* mRNA was reproducibly found to be 1 hr (data not shown). Actinomycin D experiments were performed four times and half-lives were reproducible (within 30%) among experiments.

the half-life of an mRNA. Using this approach, the ARE reduced the half-life of β-globin mRNA from >600 min to 90 min (Fig. 5). This reduction is similar to what has been observed with AREs from other short-lived mRNAs when assayed in the β-globin gene background (18, 19). Further, we found the E6/E7 mRNA, when expressed from the intact HPV-16 early region under the transcriptional control of the c-*fos* promoter, to have a half-life of only 20–40 min (data not shown). Based upon these findings, we conclude that the HPV-16 early-region 3' UTR contains an mRNA instability

FIG. 5.   Mapping the instability element present in the 3' UTR of HPV-16 E6/E7 mRNA. (*Upper*) Schematic illustration of the plasmids used (hatched box, HPV16e 3' UTR; stippled box, coding region of β-globin; open box, 3' UTR of β-globin). (*Lower*) Levels of β-globin message in NIH 3T3 cells harboring either pSJfosGLOB or pSJfos-GLOB16, plotted as a function of time after serum stimulation. The values were plotted relative to the peak levels of each specific RNA seen after serum stimulation. The time ($t_{1/2}$) at which the level of E6/E7 mRNA was half of the peak level is provided. Similar data were reproducibly obtained in two independent experiments.

element. Replacement of the viral 3' UTR in E6/E7 mRNAs arising from integrative disruption of the HPV-16 early region would therefore be predicted to stabilize the viral mRNAs.

DISCUSSION

In this report, we provide evidence that integration of HPV-16 DNA genomes in cervical epithelial cells leads to increased steady-state levels of viral mRNAs transcribed from the E6 and E7 oncogenes. Multiple mechanisms involving transcriptional derepression have been proposed previously to account for continued if not heightened expression of E6 and E7 oncogenes in cervical cancers (26, 27). We demonstrate that increased steady-state levels of E6/E7 mRNAs in cells harboring integrated viral DNA can result, at least in part, from an increase in mRNA stability through replacement of the HPV-16 early 3' UTR, containing an mRNA instability element, with cellular sequences. This mechanism for dysregulated expression of papillomaviral oncogenes can be likened to that proposed to occur with the cellular protooncogenes c-*myc* and c-*myb* as a consequence of genomic rearrangements in cancers (28, 29). That HPV DNA integration leads to the removal of a virally encoded mRNA instability element provides: (*i*) a simple explanation for the selective accumulation of viral mRNAs arising from the integrated, disrupted copies of the viral genome in cancer cells in which most copies are integrated intact, such as is found in CaSki cells: (*ii*) a rationale for why integrative disruption of the viral genome as seen in cervical cancers occurs upstream of the papillomaviral early region 3' UTR; and (*iii*) a potential explanation for why cellular sequences at the viral/cellular junction of an integrated copy of HPV-16 DNA in a particular cervical cancer were necessary for that HPV-16 DNA to confer efficient transformation of NIH 3T3 cells (30).

mRNA half-life is thought to be regulated through cis elements contained within the mRNAs. One such element controlling RNA stability is a specific sequence motif of the consensus sequence 5'-UAUUUAU-3' present in multiple copies within the A+U-rich 3' UTR of short-lived mRNAs (22). The ARE containing these sequence motifs within the HPV-16 early 3' UTR sequences was found to be sufficient to confer instability on the β-globin mRNA (Fig. 5). Other levels of posttranscriptional regulation are also known to occur. For example, the 3' UTR of the late genes of papillomiviruses has been implicated in modulating the steady-state levels of a distinct set of viral mRNAs that encode the viral structural (capsid) proteins (31, 32). Unlike the early 3' UTR which we
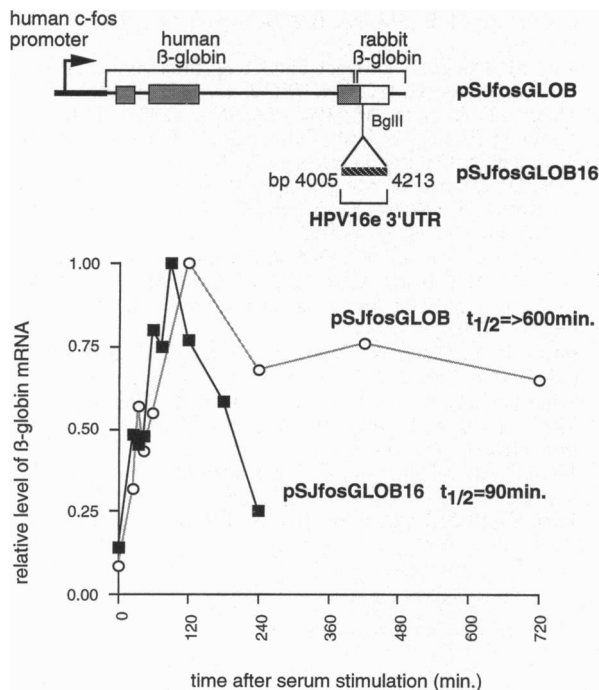
have demonstrated to function at the level of mRNA stability, the late-region 3' UTR has been found to modulate the steady-state levels of late mRNAs at the level of mRNA processing or nuclear transport (33). Thus, papillomaviruses use multiple posttranscriptional mechanisms for modulating expression of their genes.

We found the HPV-16 early 3' UTR to confer a >7-fold reduction in β-globin mRNA half-life. Correspondingly, we found a 2- to >4-fold increase in half-life for E6/E7 mRNAs arising from viral genomes disrupted by integration. Yet the differences in the relative abundance of E6/E7 mRNAs per viral genome copy in the integrated versus extrachromosomal clones ranged from 4- to 400-fold. Thus, it is likely that the additional levels of control of gene expression must be affected by integration. The hypermethylated state of HPV-16 DNA in the W12 integrated clones compared with that in the extrachromosomal clones (36) indicates that transcriptional activity of the integrated viral genomes is not heightened. Nevertheless, it has been proposed (27) that viral DNA integration, as found in cervical cancers, might cause derepression of E6/E7 mRNA synthesis, through the integrative disruption of the viral E2 gene, which encodes a transcriptional repressor of the E6/E7-specific P_{97} promoter. Since the integration events in our W12 clonal populations also result in the disruption of the E2 gene, derepression of the P_{97} promoter may contribute to the increased steady-state levels of E6/E7 mRNAs in our integrated clones in addition to the altered mRNA stability. An alternative mechanism of transcriptional derepression has been recently identified, in which deletion of binding sites for the cellular transcription factor, YY1, located upstream of the P_{97} promoter results in loss of YY1-mediated repression of E6/E7 mRNA synthesis (26). We failed to detect any deletions within the HPV-16 genomes present in our clones that would be indicative of the disruption of putative YY1 sites upstream of the P_{97} promoter. It is perhaps significant that the majority of independent integration events that we have characterized in the W12 cell clones result in viral/cellular junctions that are clustered in a short region of the viral genome >300 nt upstream of the 3' UTR. It is not known whether this region contains cis elements that affect steady-state levels of E6/E7 mRNAs independently of or in concert with the 3' UTR.

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