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ORIGINAL ARTICLE

The HPV-16 E7 oncoprotein is expressed mainly from the unspliced E6/E7 transcript in cervical carcinoma C33-A cells

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Abstract The HPV-16 E6/E7 early transcripts are first produced as bicistronic or polycistronic mRNAs, and about 90% of the original pre-mRNA is spliced to produce three new alternative mRNAs. HPV-16 spliced transcripts are expressed heterogeneously in tumors and cell lines. Our results suggest that suboptimal splicing acceptor sites in E6/E7 intron 1 and the differential expression of splicing factors are involved in the production of the heterogeneous splicing profile in cell lines. The unspliced pre-mRNA and the alternative spliced transcripts contribute differentially to the production of E7 in stably transfected C33-A cells. The highest level of E7 was produced from the least prevalent transcript, the unspliced E6/E7$^{pre\text{-}mRNA}$. The order of relative expression of E7 was unspliced E6/E7$^{pre\text{-}mRNA}$ > E6*I/E7 > E6*II/E7. Our findings suggest that E6/E7 alternative splicing may be a mechanism for differential expression of the E6 and E7 oncoproteins, which also affects the expression of their targets, the proteins p53 and pRb.

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Introduction

Human papillomavirus (HPV) infection of epidermal or mucosal tissues causes hyperproliferative lesions that can be transformed to malignant neoplasia. High-risk human papillomaviruses (hrHPVs), which include types 16 and 18, are detected in virtually every carefully analyzed cervical carcinoma sample [1]. HPV-16 is found in more than 60% of these tumors, suggesting that this virus is the major risk factor for cervical cancer development [2]. Additionally, it has been demonstrated that E6 and E7 oncoproteins, which are involved in the transformation and malignancy of epithelial keratinocytes, have a pivotal role in the development of cervical cancer [3–6]. The E6/E7 transcripts of the hrHPVs are first produced as bicistronic or polycistronic messages [7–9] and are subsequently spliced [10]; however, low-risk HPV E6/E7 transcripts are not spliced [11]. Furthermore, the E6/E7 bicistronic pre-mRNAs of the HPV-16 and HPV-33 [7, 8, 12, 13] are alternatively spliced. Notably, HPV-16 is the most frequently detected viral type in cervical carcinomas. The prevalence of HPV-16 correlates with the presence of a higher number of alternative splicing acceptor sites in its intron 1, resulting in more spliced transcripts in comparison with other hrHPVs. The intron 1 is branched at guanosine instead of at the usual adenosine [14]. It also contains one donor site and three suboptimal acceptor sites and thus produces three spliced messengers, as well as the remaining unspliced transcript [11]. As for other viruses, suboptimal splice sites and G- or U-branched introns provide a common mechanism that allows a small amount of the viral pre-RNA to

remain unprocessed; therefore, the coding potential of the pre-mRNA is not lost during alternative splicing [15, 16].

The HPV-16 E6/E7$^{pre-mRNA}$, which is a minor transcript that accounts for 2–10% of the E6/E7 transcripts encodes for both the E6 and E7 oncoproteins and is the only source of the E6 oncoprotein. Two spliced transcripts of HPV-16 E6/E7$^{pre-mRNA}$, E6*I/E7 and E6*II/E7, encode two short forms of E6 (E6*I and E6*II, respectively) and the E7 oncoprotein. E6*I/E7 is the more abundant of the two and accounts for approximately 80% of the E6/E7 spliced transcripts [17]. Three alternative bicistronic transcripts thus potentially encode for the E7 oncoprotein: the E6/E7$^{pre-mRNA}$, E6*I/E7 and E6*II/E7 mRNAs. Since second messages in bicistronic transcripts are inefficiently translated by eukaryotic ribosomes [18], it has been suggested that one advantage of splicing the E6/E7 RNAs is simply that it produces transcripts with increased ability to translate the second ORF, resulting in increased production of the E7 oncoprotein [19]. In HPV-16 and 18, the E6*I/E7 mRNA is considered the major contributor for the production of the E7 oncoprotein [20]; however, previous studies have also suggested that HPV-16 E7 is produced by the three E6/E7 mRNAs with a similar efficiency [21, 22]. One possible explanation for this discrepancy is that some of these data were obtained using different experimental approaches.

On the other hand, we and others have observed that the HPV-16 E6/E7 splicing profile is heterogeneous in premalignant lesions [23], tumor samples [24] and cell lines [14, 25]. We thus aimed to determine whether this heterogeneity resulted in the differential expression of E7 and the degree to which the three HPV-16 bicistronic E6/E7 messages contributed to E7 production. Initially, four HPV-positive and -negative cell lines (C33-A CaSki, HeLa and SiHa) where analyzed to explore the possibility that an intrinsic variation in the expression of splicing factors was involved in the heterogeneous splicing. This analysis showed that splicing factors were differentially expressed in these cells. We then transfected HPV-negative and -positive cervical carcinoma cell lines with green fluorescent protein (GFP) coupled to the bicistronic HPV-16 E6/E7 sequence. The heterogeneous transcript profile was reproduced in transfected cells, and this reproduction resulted in variations in the level of the E7 oncoprotein and its cellular target pRb in the cell lines. These findings suggested that variations in splicing factors and the presence of suboptimal splicing sequences in the E6/E7 intron 1 may drive the heterogeneity in the splicing profile and the E7 oncoprotein level in cervical carcinoma cells.

As an initial approach, we used this experimental model to investigate the role of the intron 1 splicing sequences in the heterogeneity of the splicing profile, by transfecting C33-A cells with several branch-point and acceptor mutants. The elimination of any of the acceptors impaired the expression of spliced transcripts. Moreover, the mutation of the splicing acceptor A (SA^{A}), which eliminated the expression of the major spliced mRNA, did not eliminate the production of the E7 oncoprotein, suggesting that the minor transcripts were the major contributor to E7 expression. To further confirm this point, an E6/E7 splicing donor mutant and constructs prepared using the E6*I/E7 and E6*II/E7 cDNAs were used to transfect C33-A cells. The results of these experiments confirmed that E7 expression was higher in cells expressing only the unspliced E6/E7$^{pre-mRNA}$ than in cells transfected with the others constructs.

Taken together, the results showed that the usage of suboptimal splicing acceptor sites in intron 1, together with a variation of splicing factors in cell lines, may result in the heterogeneity of the E6/E7 splicing profile. Additionally, the three HPV-16 E6/E7 bicistronic transcripts each contribute to the total level of E7, but the primary transcript (the E6/E7$^{pre-mRNA}$) is the major contributor. The data also suggest that, contrary to previous assumptions, the splicing of E6/E7$^{pre-mRNA}$ does not necessarily increase the levels of the E7 protein.

Materials and methods

Collection of tumor samples

Tumor tissues were obtained from the Department of Pathology at the Instituto Nacional de Cancerología-SSA in Mexico City. Samples were obtained from patients diagnosed with grade I or II invasive squamous cell carcinoma. Specimens were evaluated and characterized by experienced pathologists, and all were HPV-16-positive by PCR analysis performed as described previously [26]. All samples were obtained after informed consent from patients. Molecular biology reagents and primers were purchased from Invitrogen, USA.

Constructs

To construct the pE6/E7 EGFP-N1 vector, the HPV-16 E6/E7 bicistron (nt 83–855) was amplified by PCR from pE6/E7BSII [14] using the primer pair E6-begin*Hin*dIII and E7-end*Bam*HI to include new restriction sites and eliminate the stop codon. The E6*I/E7 and E6*II/E7 cDNAs were amplified by RT-PCR from total CaSki RNA using the same primer pair. Monocistronic E6 and E7 ORFs were constructed using the E6-begin*Hin*dIII and E6-end*Bam*HI and E7-begin*Hin*dIII and E7-end*Bam*HI primer pairs, respectively. Briefly, to obtain a proper connection between the E7 and the GFP, the stop codon sequence of E7 was changed (TAA/TT) to eliminate its function and subsequently cloned

in-frame in the *Bam* H1 site of the pEGFP-N1 vector (Clontech Laboratories, Inc). The E7 carboxy terminus was connected to the GFP protein by a seven-aa peptide that resulted from the translation of the linker sequence (21 nt) between the *Bam*H1 site and the start codon of GFP. All constructs contained fragments of 589 nt from the human cytomegalovirus (CMV) promoter and 112 nt from the 3′-untranslated region (3′ UTR) of SV40 virus. All constructs were transcribed with a 48-base RNA leader composed of 6 bp from CMV and 42 bp from multiple cloning site (MCS). Mutants were generated using a two-step PCR amplification procedure. The splice donor (E6/E7SD), and the acceptor B (E6/E7SA^{B}) were replaced with *Eco* RI enzyme restriction sites, and the acceptor A (E6/E7SA^{A}) was replaced with a *Not* I restriction site using the primer pair E6-begin*Hin*dIII and E7-end*Bam*HI in combination with any of the following primer pairs: RE6mut226*Eco*RI and FE6mut226*Eco*RI; 3BSSF*Eco*RI and 3BSSR*Eco*RI or 3ASSMR, and HPV53ASSM. The constructs were named E6/E7SD^{M}, E6/E7SA^{BM} and E6/E7SA^{AM}, respectively. The construct for E6/E7SD^{M} had two amino acid changes (R47E and E48F). Neither of these changes was located in the well-known binding sites for p53 or E6AP. Similarly, two branch-point (BP) mutants were generated by replacing the guanosine with adenosine or changing GTGTGA to the yeast consensus sequence UACUAAC [27] using the following primer pairs: E6-begin*Hin*dIII and BPMRW311 plus *Eco*RFW343 and E7-end*Bam*HI or E6-begin*Hin*dIII and BPCRW311 plus *Eco*RIFW343 and E7-end*Bam*HI. These constructs were named E6/E7BP$^{G/A}$ and E6/E7BP$^{G/y}$. An *Eco* RI restriction site was introduced in both mutants at the spliced sequence of the intron and possible amino acid changes were not analyzed because these constructs were not used for functional assays. Constructs were verified by sequencing using a Big Dye Terminator Ready Reaction Kit and an ABI PRISM 310 Genetic Analyzer System (Perkin-Elmer, Branchburg, NJ, USA).

Cell culture, transfection and isolation of stable cell lines

The cervical carcinoma cell lines HeLa, SiHa, CaSki and C33-A were obtained from the American Type Culture Collection. Cells were grown at 37°C in 5% CO_2, using MEM or RPMI (CaSki) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were stably transfected using the standard calcium phosphate co-precipitation method [28]. Briefly, cells were grown for 24 h, transfected with 10 μg of each DNA construct and selected in MEM medium supplemented with 500 μg/ml G418 (Invitrogen, Corp.). After 4 weeks of selection, G418 was removed for 4 weeks, GFP-positive cells were purified by FACS (FACS Calibur, Beckton-Dickinson Mountain View, CA, USA), and the cells were selected again for one to 2 months. The purity of the isolated transfected cell lines was about 96%. The transfected cells were not cloned, but each experiment was repeated in at least three different independent assays. Cell nuclei were counterstained using 1 μg/ml of 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) dissolved in PBS. Cells were analyzed by fluorescence microcopy using an Olympus IX7 confocal microscope. Images were analyzed using Fluoview 4.3 software. A display of ten slices of 0.2 μM is shown in the figures.

Detection of E6/E7 spliced transcripts and RNA-protein binding by UV-cross-linking assays

Cells were grown to 80–85% of confluence, harvested and rinsed twice with PBS. Total RNA was extracted from tumors or cell lines using the Tripure technique following the manufacturer's instructions (Boheringer Roche), treated with DNase I, and then reverse-transcribed using oligo-dT primers. The RT-PCR reaction was performed as described previously [14], using 0.5 μM of each of the following primer pairs: E6-begin and E7-end for endogenous E6/E7 transcripts, E6-begin and GFP-N1A for E6/E7 exogenous transcripts, CMV5′UTR and GFP-N1A for GFP fused transcripts, and the actin sense and actin antisense primer pair for loading control. Oligonucleotide sequences are listed in Table 1. To amplify the poorly detected transcripts further, a nested PCR was performed for some of the RT-PCR assays. For these transcripts, the first round of RT-PCR was performed using 1 μl of the cDNA mixture and 0.5 μM of CMV5'UTR and GFPNIA primers. Nested PCR was performed using 1 μl of the RT-PCR mixture, 0.5 μM of primer HPVRTFW, 0.5 μM of primer HPVRTRV and 1 μl of Platinum Taq polymerase. The PCR protocol consisted of 25 cycles of the following steps: 30 s pre-incubation at 92°C followed by 35 cycles of 15 s at 92°C, 15 s at 45°C, 60 s at 72°C and then a final step of 7 min at 72°C. Transcripts were resolved by electrophoresis in 2% agarose gels stained with ethidium bromide. QRT-PCR experiments were performed by using the Power SYBR Green PCR Master Mix and a Real Time ABI-PRISM 7000 SDS (Applied Biosystems). For detection of the total E6/E7 transcripts, E6/E7$^{pre\text{-}mRNA}$ and the E6*I/E7 or the E6*II/E7, the forward primers E7-begin, E6*I (226/409) and E6*II (226/526), respectively, were used in combination with the reverse primer C1-HPV16. The amplification protocol was 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 45 s at 61°C for annealing, and 45 s at 72°C for elongation. Samples were analyzed in triplicate, and negative and positive controls were included in each reaction [29, 30]. For RT-PCR coupled to Southern blot, the agarose gels were transferred to nylon membranes and hybridized with an E6/E7 [α-32P] CTP-labeled DNA probe.

Table 1 Nucleotide sequences of oligonucleotide primers

Primer name		Sequence	Nt position
RT-, QRT-, and nested- PCR primers			
1	E6-begin	5′-CCACCATGCACCAAAAGAGAACTGCAA-3′	83–104
2	E6-end	5′-CAGCTGGGTTTCTCTACGTG-3′	556–537
3	E7-begin	5′-CCACCATGCATGGAGATACACCTACAT-3′	562–583
4	E7-end	5′-TGGTTTCTGAGAACAGATGGG-3′	855–835
5	Actin sense	5′-ATGGATGATGATATCGCCGCG-3′	103–122
6	Actin antisense	5′-CCACTCACCTGGGTCATCTTC-3′	619–642
7	CMV5′UTR*	5′-TCAGATCCGCTAGCGCTACCG-3′	583–603
8	GFP N1A*	5′-CTCCTCGCCCTTGCTCACC-3′	699–681
9	C-1HPV16	5′-CATTAACAGGTCTTCCAAAGTACG-3′	813–789
10	E6*I (226/409)	5′-CGACGTGAGGTGTATTAA-3′	218–226//409–417
11	E6*II (226/526)	5′-CGACGTGAGATCATCAAG-3′	218–226//526–534
12	HPVRTFW	5′-ACAGGAGCGACCCAGAAAGTT-3′	118–138
13	HPVRTRV	5′-TGCCCATTAACAGGTCTTCCA-3′	787–767
Construct primers			
12	E6-begin*Hin*dIII	5′-aagcttCCACCATGCACCAAAAGAGAACTGCAA-3′	83–104
13	E6-end*Bam*HI	5′-ggatccAACAGCTGGGTTTCTCTACGTG-3′	556–537
14	E7-begin*Hin*dIII	5′-aagcttCCACCATGCATGGAGATACACCTACAT-3′	562–583
15	E7-end*Bam*HI	5′-ggatccAATGGTTTCTGAGAACAGATGGG-3′	855–835
16	RE6mut226ECORI	5′-gaattcTCGCAGTAACTGTTGCTTGCA-3′	220–200
17	FE6mut226ECORI	5′-gaattcGTATATGACTTTGCTTTTCGG-3′	227–247
18	3ASSMR	5′-tttgcggccgcAATTAACAAATCACACAACGGTTTG-3′	406–382
19	HPV53ASSM	5′-tttgcggccgcAACTGTCAAAAGCCACTGTGTC-3′	416–437
20	3BSSFECO	5′-gaattcTCATCAAGAACACGTAGAGAAACC-3′	527–550
21	3BSSRECO	5′-gaattcACAAGACATACATCGACCGGTC-3′	520–499
22	BPMRW311	5′-gaattcTGTCTATATTCACTAATTTTAGAATA-3′	336–311
23	ECOFW343	5′-gaattcTTATAGTTTGTATGGAACAACATTAGAACAGC-3′	343–374
24	BPCRW311	5′-gaattcTGTCTATGTTAGTAAATTTTAGAATA-3′	336–311

The GenBank accession numbers for actin and HPV-16 sequences are NM_001101 and U89348, respectively. Lowercase letters indicate the sequence of restriction enzyme sites included in the oligonucleotide primers

* Nucleotide numbering according to plasmid EGFP-N1

Splicing reactions were performed at 30°C according to the manufacturer's instructions (Promega Co). For binding assays, 7 µg of HeLa nuclear protein extract and 5 ng of labeled E6/E7 RNA probe were incubated for 20 min at 4°C in 10 µl splicing buffer supplemented with 2.5 mg/ml yeast tRNA and 2 mg/ml heparin. RNA-protein mixtures were irradiated for 10 min using a 254-nm UV lamp located 5 cm above the samples. Samples were subsequently digested with 2 mg/ml of RNAase A, boiled for 10 min in Laemmli loading buffer containing 200 µg heparin, and fractionated on a 12% SDS-PAGE gel [31]. Gels were dried and exposed to X-ray film for 2–8 days. RNA probes were synthesized in vitro from the plasmid pE6/E7BSII using T7 RNA polymerase and 50 µCi of [α-32P] UTP according to the manufacturer's instructions (Riboprobe Combination System, Promega). All protein complexes detected in the UV-cross-linking assays were reproducible; however, each experiment was repeated at least four times.

Western blot analysis

Cells were grown to approximately 80–85% confluence, harvested, rinsed twice with PBS, scraped off of culture dishes, and lysed using the ProteoJET™ mammalian cell lysis reagent (Fermentas, K0301). Total protein (30 µg) was mixed with Laemmli sample buffer, boiled, separated by 12% SDS-PAGE, and transferred onto a PVDF membrane (Amersham Pharmacia Biotech) as described previously [26]. The commercial antibodies used were anti-GFP, anti-U2AF65, anti-PTB, anti-HPV16 E7 (Zymed); anti-pRb (Cell Signaling); anti-hnRNP A1, anti-hnRNPC1/C2, anti-ASF/SF2, anti-p53 and anti-U170k (Santa Cruz Biotechnology). The secondary antibodies were anti-mouse

HRP antibody, anti-rabbit-HRP antibody (Zymed) or anti-goat-HRP antibody (Rockland). Membranes were stripped and reprobed using anti-actin antibody or up to five different splicing factor antibodies. Finally, immunocomplexes were visualized using the ECL chemiluminescence system (Amersham Pharmacia Biotech).

Results

The profile of early HPV-16 spliced transcripts is heterogeneous and may be reproducible in cell lines

The alternative usage of the acceptor sites in the HPV-16 intron 1 produced three spliced transcripts (Fig. 1a). After analysis by RT-PCR coupled to Southern blot, it was repeatedly observed that E6/E7 splicing profiles were heterogeneous in the HPV-16+ tumor samples. Furthermore, a small amount of the unspliced transcript (E6/E7$^{\text{pre-mRNA}}$) remained in several samples. Four transcripts were thus produced from the E6/E7 ORFs of HPV-16, but the levels of the four transcripts were variable (Fig. 1b). Heterogeneity in the E6/E7 splicing profile was observed previously in the HPV-16+ CaSki and SiHa cell lines [14]. Since heterogeneity of the E6/E7 splicing profile may result in variation in E7 oncoprotein levels, careful analysis of this splicing profile is merited.

We hypothesized that the heterogeneous splicing profile may be the result of an intrinsic variation in the expression of splicing factors in these cell lines. To address this possibility, we performed RNA-protein UV-cross-linking experiments to analyze the profile of nuclear proteins bound to E6/E7 synthetic transcripts using nuclear extracts from the cell lines C33-A (HPV−), CaSki (HPV-16+), HeLa (HPV-18+) and SiHa (HPV-16+). These experiments revealed a heterogeneous profile of E6/E7-transcript-bound protein factors in the cell lines (Fig. 1c). We next analyzed the relative level of expression of splicing factors using western blots and found that some factors were differentially expressed in these cervical cell lines. The relative expression levels of the recognition factors U170k and U2AF65, and the splicing regulatory proteins ASF/SF2 and PTB varied among the cell lines, but little or no significant variation was observed in the expression of the heteronuclear ribonucleoproteins hnRNPA1 and hnRNPC1/C2 (Fig. 1d). The four cells lines were thus stably transfected with the CaSki sequence using the E6/E7-GFP reporter system. The stable cell lines had a heterogeneous splicing profile similar to that observed in the tumor samples (Fig. 2a). Contrary to our expectations, only the stably transfected CaSki and C33-A cells expressed the four mature transcripts, while SiHa cells expressed three transcripts, and HeLa cells expressed only two (Fig. 2b). Due to their low expression levels, the minor E6/E7 mRNAs species (E6*II/E7 y E6^E7) were clearly detected only by a nested RT-PCR assay. This last experiment also ruled out the possibilities that SiHa cells expressed undetectable level of the E6^E7 transcript or that the stably transfected HeLa cells expressed the E6*II/E7 and E6^E7 RNAs. In this experiment, the accuracy of the relative expression levels of each of the transcripts was sacrificed to rule out the possibility that low levels of the aforementioned transcripts were not detected by simple RT-PCR assays.

These data suggested that the splicing profile heterogeneity may result in variation of the E7 oncoprotein in these cell lines. Indeed, E7-GFP was differentially expressed in the stably transfected cell lines (Fig. 2f). Remarkably, the endogenous levels of E7 were very different in CaSki and SiHa cells (Fig. 2d). The expression level of the E7 oncoprotein in HeLa cells was not analyzed because the commercial antibody used for western blots was specific for HPV-16 E7. To further assess E7 expression in these cell lines, we examined the expression levels of pRb, which are a direct measure of the level of E7 oncoprotein expression. As expected, the four cell lines showed different levels of endogenous pRb expression; the lowest level was observed in CaSki cells and the highest was in SiHa cells (Fig. 2c). This result was consistent with the endogenous E7 expression levels observed in these cells (Fig. 2d). Stable transfection with E6/E7-GFP severely diminished pRb expression in the four cell lines. In stably transfected HeLa and CaSki cells, but not C33-A and SiHa cells, pRb expression was completely eliminated (Fig. 2e). These results also indicate that the E7-GFP was functional.

Taken together, these findings suggest that the variations in splicing factors in the cell lines affect the recognition of the intron 1 splicing sequences, resulting in heterogeneity in the HPV-16 E6/E7 spliced mRNAs and subsequent variation in the expression of the E7 oncoprotein.

Heterogeneity may be produced by the use of suboptimal splicing acceptor sites

Elucidating the importance of the variation in the levels of the splicing factors for the recognition of the splicing sequences in the E6/E7$^{\text{pre-mRNA}}$ requires an enormous knowledge of each of these sites and its mechanism of alternative splicing. We performed initial characterization of these splicing sequences and found that the HPV-16 intron 1 contains a BP at a guanosine instead at the usual nucleotide adenosine [14]. We suggested that this guanosine-based weak BP favored the differential usage of splicing acceptor sites, thus contributing to a heterogeneous splicing profile. To address this possibility, the intron 1 BP was mutated by replacing the guanosine with an adenosine,

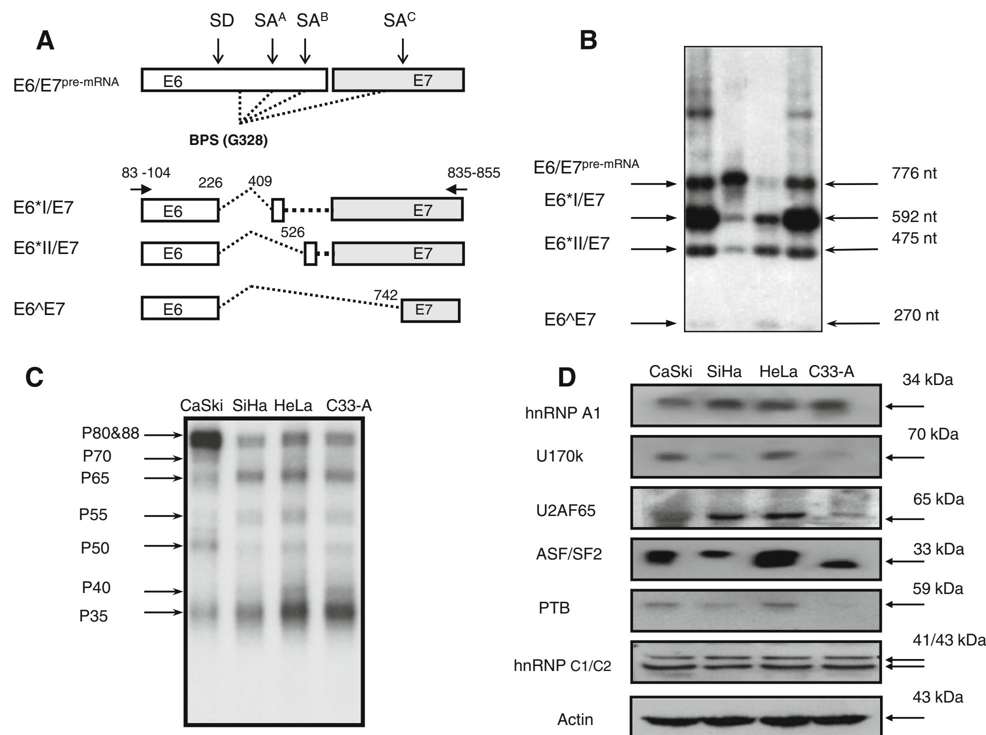

Fig. 1 The HPV-16 E6/E7 alternatively spliced transcripts are expressed heterogeneously. **a** Genomic organization of the HPV-16 E6/E7 genes. Intron 1 has one splice donor (*SD*) site at nt 226 and three acceptor sites at nts 409 (SA^{A}), 526 (SA^{B}) and 742 (SA^{C}), respectively. The *dashed line* between *boxes* represents the alternatively spliced sequences. *Open boxes* represent the total or partial E6 ORF, while shaded boxes represent E7. The position of the primer pair used for RT-PCR is indicated by *horizontal arrows*. **b** E6/E7 alternative splicing profiles as determined by RT-PCR using total RNA from four squamous cell carcinoma samples (grade I–II). Transcripts were detected by Southern blot using a radioactively labelled E6/E7 probe. **c** Nuclear factors binding to the E6/E7 bicistronic synthetic RNA as detected by RNA-protein UV-cross-linking assays using nuclear extracts from the four cell lines. **d** Relative expression level of splicing factors as detected by western blot. Membranes were stripped and reprobed with the different splicing factor antibodies

and the mutant construct (pE6/E7BP$^{G/A}$) was used to transfect C33-A cells. RT-PCR analysis of the stable transfected cells did not reveal a significant change in the splicing profile; however, the adenosine-based BP moderately favored the production of the E6*I/E7 transcript (Fig. 3a). Moreover, SYBR green Q-RT-PCR analysis failed to detect significant changes in the expression of the E6/E7$^{pre\text{-}mRNA}$ and E6*I/E7 transcripts (data not shown). Furthermore, replacing the weak BP with a yeast consensus sequence (pE6/E7BP$^{G/y}$), which has been suggested to be a strong BP in eukaryotic cells, did not result in significant changes in the E6*II/E7 and E6^E7 transcript levels. These data were confirmed by nested-PCR (data not shown), which showed that the levels of the poorly detected transcripts were not significantly changed. We next analyzed the impact of each of the splicing acceptor sites on the splicing profile. The elimination of either acceptor A or acceptor B by mutation resulted in the impairment in the usage of distal acceptor sites and loss of expression of the products of these sites. For example, the elimination of the acceptor A (E6/E7SA^{AM}) resulted in the loss of all of the spliced products; only E6/E7$^{pre\text{-}mRNA}$ was detected. The elimination of the acceptor B (E6/E7SA^{BM}) inhibited the expression of E6*II/E7 but did not increase the expression of the other mRNA species (Fig. 3b).

Analysis of the E7 oncoprotein levels by western blot yielded surprising results. For example, stable transfection with the E6/E7SA^{BM} mutant, which produced a low level of the E6/E7$^{pre\text{-}mRNA}$ but a high level of the E6*I/E7 transcript, resulted in very low amount of E7 oncoprotein, while stable transfection with the E6/E7SA^{AM} construct, which produced mainly E6/E7 $^{pre\text{-}mRNA}$, resulted in a high level of E7. In addition, stable transfection with the wild-type E6/E7 construct, which produced all three bicistronic transcripts, resulted in expression of E7 protein at around 30% of the level of the E6/E7SA^{AM} construct. Conversely, stable transfection with the E6/E7SA^{BM} mutant, which produces the major transcript E6*I/E7 and very little E6/E7$^{pre\text{-}mRNA}$ resulted in a lower level of E7-GFP (Fig. 3c). Monocistronic E6 constructs contain the first two splicing acceptor sites, and the elimination of the entire E7 ORF only eliminates the acceptor C. Transfection with the

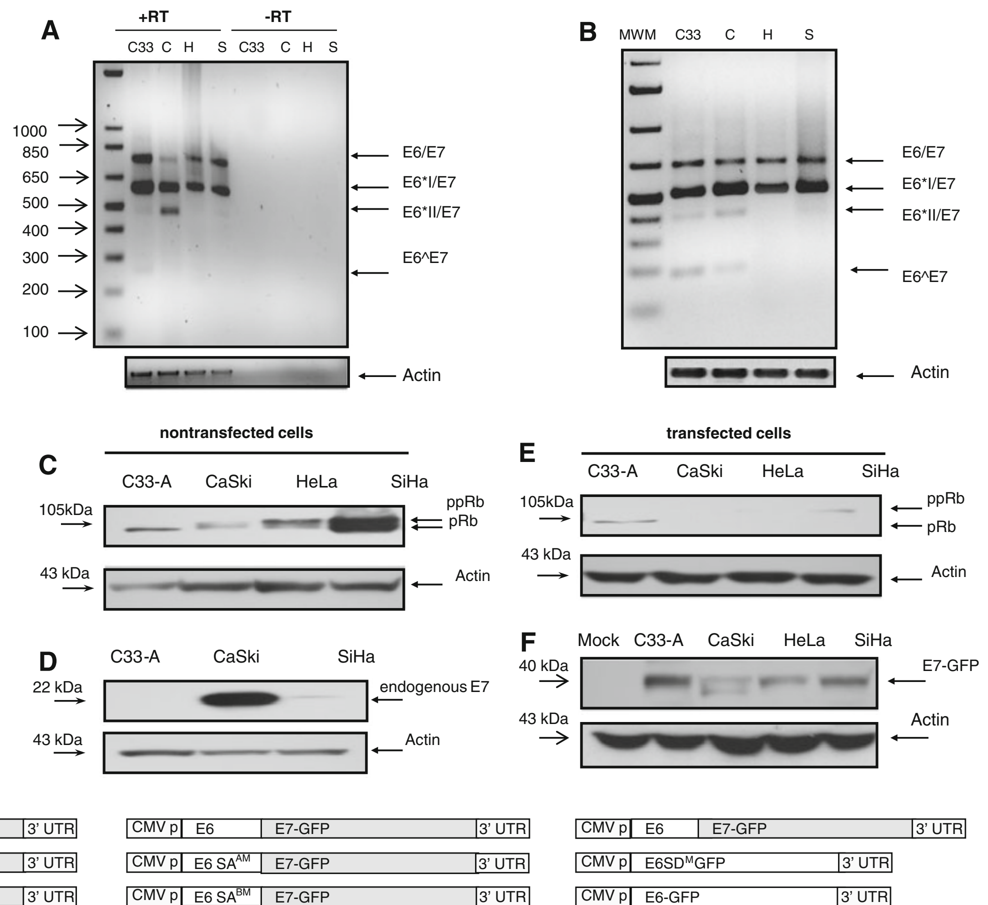

Fig. 2 The heterogeneous splicing profile may be reproduced in cell lines. **a** C33-A (*C33*), CaSki (*C*), HeLa (*H*) or SiHa (*S*) cells were transfected with the E6/E7 sequence isolated from CaSki DNA. Transcript profiles are shown with their respective–RT controls. **b** The respective nested PCR round for the four stable transfected cell lines is shown. **c** The relative expression level of endogenous pRb is shown. **d** The relative expression level of endogenous E7 detected in CaSki or SiHa nontransfected cells is shown. **e** The relative expression level of pRb in E6/E7 in stable cells lines and **f** the relative expression level of E7-GFP is shown. Western blot membranes were stripped and reprobed using an anti-actin antibody. *Right arrows* indicate the transcript or protein name and *left arrows* indicate molecular weights

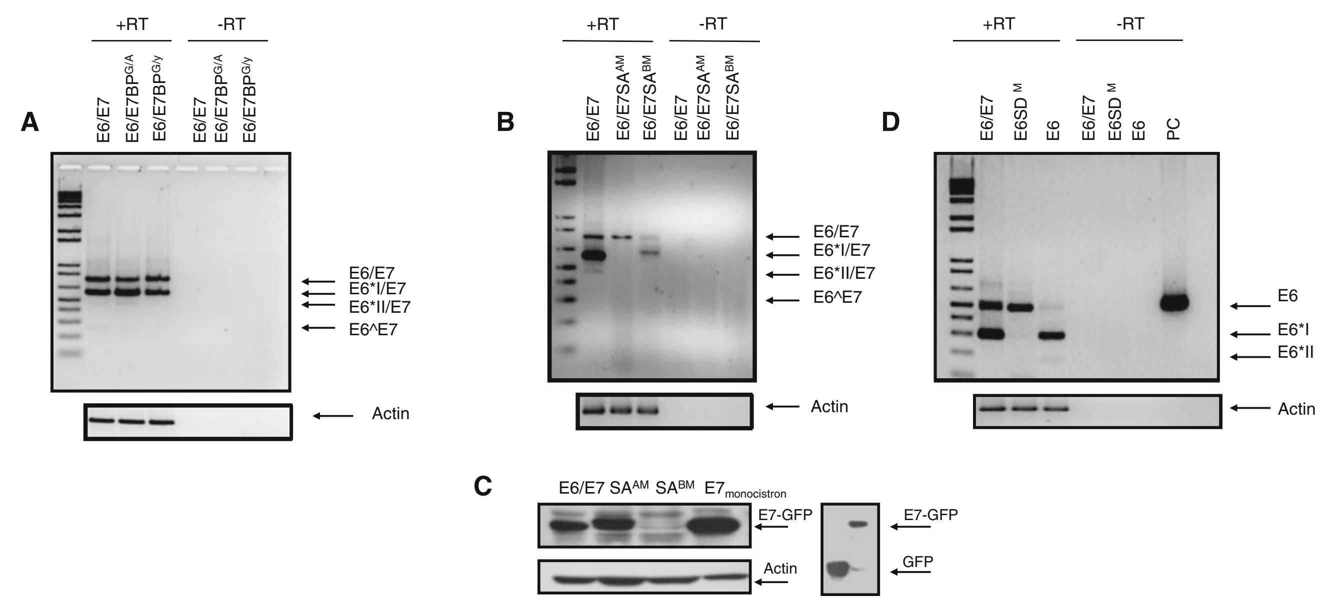

Fig. 3 Elimination of either of the splicing acceptors impairs the alternative splicing of intron 1 **a** The profile of spliced transcripts detected by RT-PCR in C33-A cells stably transfected with the E6/E7, E6/E7BP$^{G/A}$ or E6/E7BP$^{G/y}$ constructs. The respective–RT negative controls are shown. **b** Splicing profile of transcripts detected by RT-PCR in C33-A cells stably transfected with the E6/E7, E6/E7SA^{AM} or E6/E7SA^{BM} constructs and the respective–RT negative controls are shown. **c** Expression of the E7-GPF protein in C33-A cells stably transfected with the acceptor mutants and wild-type E6/E7 constructs as detected by western blot with an anti-GFP antibody; the relative migration of GFP and E7-GFP is also showed in the blot on the right. **d** E6 spliced transcripts detected by RT-PCR in C33-A cells stably transfected with E6/E7, E6SD^{M} or E6 and their respective–RT negative controls are shown. PC shows an E6 positive control. The E6/E7-GFP and mutated constructs used for stable transfection and isolation of the C33-A cells are represented above the RT-PCR panel

monocistronic E6 construct resulted in a more frequent usage of the acceptor A, producing more E6*I transcript, but it did not alter the usual low levels of the E6$^{pre\text{-}mRNA}$ and E6*II transcripts. The level of unspliced E6$^{pre\text{-}mRNA}$ in stably transfected cells with a splicing donor mutant control (E6SD^{M}) was similar to that observed in cells transfected with the bicistronic E6/E7 construct (Fig. 3d).

Taken together, all of these results suggested that the acceptor sites are more important than the branch point sequence in determining the heterogeneous HPV-16 E6/E7 splicing profile. The findings also suggest that the unspliced transcript is responsible for most of the E7 oncoprotein expression.

The E6/E7$^{pre\text{-}mRNA}$ produces more E7 than the E6*I/E7 and E6*II/E7 transcripts

To compare the production of E7 by each of the unspliced and spliced E6/E7 transcripts, C33-A cells were transfected separately with the constructs E6/E7SD^{M}, E6*I/E7 and E6*II/E7. Semi-quantitative RT-PCR and western blots revealed that cells containing the E6*I/E7 or E6*II/E7 constructs produced similar levels of their respective transcripts but a low level of E7-GFP, whereas cells containing E6/E7SD^{M} produced a very low level of E6/E7$^{pre\text{-}mRNA}$ but higher levels of E7-GFP (Fig. 4a, c). Quantitative SYBR green RT-PCR assays revealed that the number of copies of the E6/E7SD^{M} transcript was tenfold lower than that of the E6/E7, E6*I/E7 or E6*II/E7 transcript (Fig. 4b). The mean mRNA copy numbers detected in nine experiments were as follows: E6/E7SD^{M}, 44,367 ± 6269; E6/E7, 428,227 ± 43,965; E6*I/E7, 403,773 ± 54, 903 and E6*II/E7, 477,804 ± 44, 822. These data confirm the results of the endpoint RT-PCR experiments. Conversely, the expression of the E7-GFP was tenfold higher in cells stably transfected with the E6/E7SD^{M} construct than in cells transfected with the E6*I/E7 or E6*II/E7 construct. The relative expression levels of E7-GFP in cells stably transfected with the three bicistronic transcripts were as follows: the E6/E7$^{pre\text{-}mRNA}$ > E6*I/E7 > E6*II/E7. Cells stably

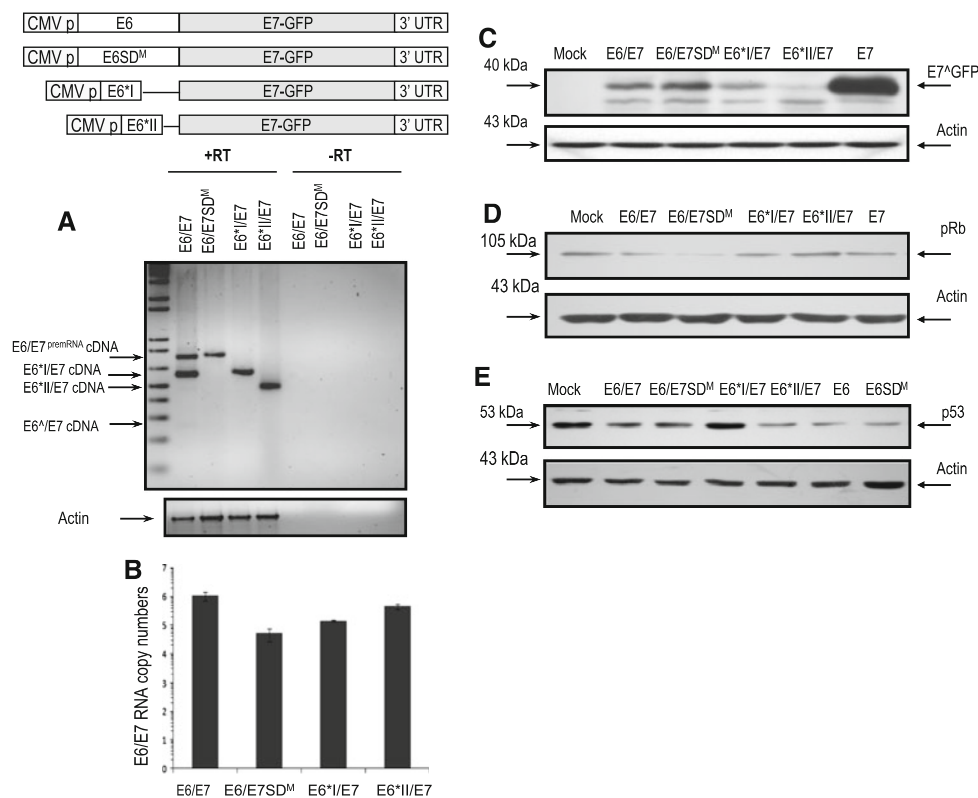

Fig. 4 The E6/E7 bicistronic transcripts produce the E7 oncoprotein with different efficiencies. **a** Expression products detected by RT-PCR of C33-A cells stably transfected using the constructs E6/E7, E6/E7SD^{M}, E6*I/E7 and E6*II/E7 cDNAs and their respective–RT negative controls are shown. Molecular weight markers are shown. **b** The relative copy numbers in 125 ng of total RNA determined by SYBR green QRT-PCR analysis of the same transfected cells is shown. **c** The relative expression of the E7-GPF constructs was analysed by western blot using an anti-GFP antibody; negative (Mock) and monocistronic E7 controls are shown. **d** pRb or **e** p53 detected in C33-A cells stably transfected with the bicistronic contructs and compared to that from E7 or E6 monocistronic constructs, respectively. Mock-transfected cells are C33-A cells stably transfected with the empty pEGFP-N1 vector. *Arrows* indicate the identity of spliced transcripts in RT-PCR assays or proteins in western blot assays. The E6/E7-GFP constructs used for stable transfection and isolation of the C33-A cells are represented above of each RT-PCR panel

transfected with the monocistronic E7 control expressed 50-fold more E7 than cells stably transfected with the E6*I/E7 construct (Fig 4c). The relative protein expression levels were assessed by western blots of dilutions of protein extracts of the stably transfected cells (data not shown). All of the experiments were performed using pools of stably transfected cells and not individual clones; however, we did three independent transfection assays to rule out the possibility that highly expressing cells were selected or pooled during the transfection process. However, no significant differences were observed (data not shown).

To confirm these results, the expression levels of pRb in stably transfected cells were evaluated. The results showed that low levels of pRb were observed in cells stably transfected with E6/E7SD^{M} or with a monocistronic E7 construct. Stable transfection with the E6*I/E7 construct, which resulted in a low level of E7, also resulted in decreased pRb expression. This probably indicates that the low level of E7-GFP expressed from this transcript was sufficient to abate most of the expression of pRb. The expression of pRb was not diminished in cells stably transfected with the E6*II/E7 construct, which was consistent with its low E7 expression level (Fig. 4d). We were unable to detect the E6 oncoprotein or the short proteins produced by the E6 splicing products by using commercial E6 antibodies or anti-E6 rabbit or mouse antibodies prepared against *E. coli* rE6 protein. This may be due to the low specificity of the E6 antibodies (data not shown). We thus analyzed the levels of the p53 protein as an indirect measurement of the activity of E6. The E6 monocistronic constructs (E6 and E6SD^{M}) had a more evident effect on p53 levels than the E6/E7 bicistronic construct, which suggested that the splicing of the E6/E7$^{pre\text{-}mRNA}$ also reduced the expression of the E6 oncoprotein (Fig. 4e), as was observed for E7. Surprisingly, the expression of E6*II in the absence of the complete E6 protein (E6*II/E7 message) decreased the level of p53, whereas the E6*I/E7 construct did not affect p53 expression.

Fluorescence confocal microscopy showed that E7-GFP was expressed in both the nucleus and the cytoplasm in cells stably transfected with all constructs, although nuclear expression was poor (fine and scarce punctuated pattern), and most of the fluorescence was observed as an intense punctuated pattern in the perinuclear/cytoplasmic region (Fig. 5).

The relative fluorescence of the stable cell lines was not quantified; however, the fluorescence observed in most of these cells was representative of the E7-GFP expression observed in western blot assays.

In summary, our data show that suboptimal acceptor sites and differential expression of splicing factors are important in producing the heterogeneous splicing profile that may result in variation of the E6 and E7 oncoprotein levels.

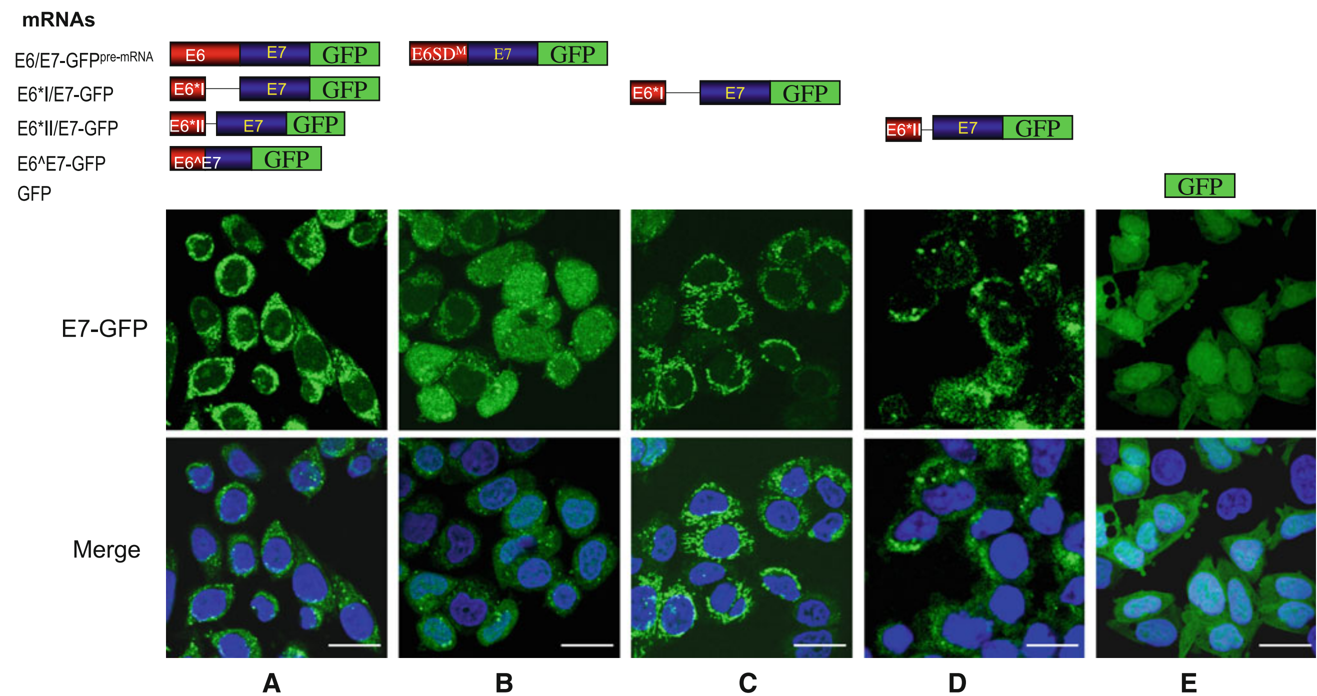

Fig. 5 The unspliced E6/E7 messenger produces more of the E7-GFP protein. C33-A cells were stably transfected using cDNAs from each of the unspliced or spliced transcripts. The relative fluorescence observed by confocal microscopy is shown in the images for each construct: E6/E7, E6/E7SD^{M}, E6*I/E7, E6*II/E7 and pEGFP-N1 (**a**, **b**, **c**, **d** and **e**, respectively). The E6/E7-GFP or GFP messages expressed by each construct are represented above each panel. *White bars* correspond to 20 µM. Nuclei were counterstained with DAPI

Discussion

The HPV-16 E6/E7 splicing profile is heterogeneous in tumors and cell lines [14, 24, 32, 33]; however, this fact is only important if each spliced transcript contributes at a different level to the expression of E7. Alternative splicing of the E6/E7$^{pre-mRNA}$ produces four transcripts. The first of these, E6*I/E7 which is the major spliced product, is a bicistronic mRNA with a structure formed by a region coding for the E6 short isoform (named E6*I), a 149-nt intercistronic spacer region and the complete E7 ORF. The second, E6*II/E7 mRNA, has a structure similar to that of E6*I/E7, but its intercistronic spacer region is only 18 nt [32]. The third, the E6^E7 mRNA, is a minor monocistronic transcript formed by an in-frame fusion of the E6 amino terminus and the E7 carboxyl terminus that potentially produces a hybrid protein between E6 and E7 [9, 14, 24, 34]. The fourth, E6/E7$^{pre-mRNA}$, which corresponds to the remaining unspliced E6/E7 mRNA, accounts for 2–10% of the total early transcripts, depending on the tumor sample or cell line [17, 32]. Certainly, the highly expressed E6*I/E7 mRNA prevails in most tumor cells, suggesting that it is the major contributor to the expression of the E7 oncoprotein [20]. Therefore, the potential consequences of the heterogeneous splicing profile observed in cervical carcinoma cells have been ignored.

In this work, we used an E6/E7-GFP system in four cell lines that have an intrinsic variation in the level of recognition and regulatory splicing factors to model a HPV-16 E6/E7 heterogeneous splicing profile in cervical cancer cells. The variation in splicing factors in many cancer [35] and leukemia [36] cells may be ubiquitous and inherent to the oncogenic process. For example, PTB is up-regulated in cervical cancer cells but is down-regulated in superficial cervical epithelial cells [37]. Additionally, the expression level and the degree of phosphorylation of ASF/SF2 increase during the differentiation of W12 cells [38]. Our results also showed that the expression levels of E7-GFP were variable in transfected cells. Similar to the findings of other groups [39], the endogenous expression of the E7 oncoprotein was different in CaSki and SiHa cells. Notably, after reintroduction of the E6/E7 sequence, CaSki cells produced all four spliced transcripts, but SiHa cells produced only three transcripts, and HeLa cells produced only two. Interestingly, the endogenous HPV-16 sequence drives expression of only three transcripts in SiHa cells; therefore, the number of transcripts seems to depend not only on the HPV sequence but also the cell line. This result suggested that the heterogeneity in the transcripts may be produced by microenvironmental differences inherent to the cell lines acting on the splicing sequences of the E6/E7$^{pre-mRNA}$. As an initial approach to understanding the importance of these splicing sequences, we analyzed the BP sequences in the intron 1 of the HPV-16 E6/E7. This intron contains a guanosine-based BP [14]. It was previously suggested that this weak BP could provide an opportunity for increased use of the suboptimal distal acceptor sites in intron 1. We addressing this point by replacing the G with A in the BP; however, our results were not conclusive, likely due to the high expression of E6*I/E7, which made it difficult to observe any additional changes. On the other hand, the elimination of the distal splicing acceptor B or acceptor C impaired the usage of the two most proximal acceptor sites, and this resulted in decreased production of E6*I/E7 and E6/E7$^{pre-mRNA}$. These results suggested that splicing acceptor sites were more important in producing the heterogeneous profile than the BP sequence. It is thus reasonable to suggest that the presence of these distal acceptors is necessary for proper recognition of the proximal splice sites.

The E7 ORF theoretically has the potential to be translated from three E6/E7 bicistronic mRNAs; however, several experimental models have shown that the second message in the bicistronic transcripts is translated inefficiently [18]. Although, the HPV-16 E6/E7 bicistronic mRNAs have been scrutinized using different experimental models to determine whether these transcripts contribute to the overall expression of E7, no conclusion has been reached because in vitro assays and varying experimental approaches have resulted in contradictory findings. The published results can be summarized in two ways: first, the three transcripts (E6/E7$^{pre-mRNA}$, E6*I/E7 and E6*II/E7) are translated and contribute equally to E7 oncoprotein expression [17, 19, 21]. Second, only one of the bicistronic messengers (E6*I/E7) accounts for all E7 oncoprotein expression [20]. In contrast, our results, which were obtained using stable transfection of C33-A cells with two different E7-GFP constructs (E6/E7SD^{M} and E6/E7SA^{AM}), showed that the E6/E7$^{pre-mRNA}$ message, which was expressed at a tenfold lower level than E6*I/E7, produced the largest amount of the of E7-GFP protein. In addition, we found that the spliced and unspliced bicistronic HPV-16 messages (E6/E7$^{pre-mRNA}$, E6*I/E7 and E6*II/E7) were differentially translated to produce the E7 oncoprotein. Furthermore, the levels of pRb observed in transfected cells were consistent with the expression levels of E7-GFP. The differences observed between our results and those of others may reflect the use of cell lines from different tissue sources. Importantly, the E6/E7 splicing profile varies among cervical cell lines [14, 24, 33], suggesting that variation may also occurs in other tissues.

Our data suggest that, in addition to driving the variation of E7 oncoprotein expression levels, the spliced products may exert a role in cancer development that is not yet understood. Currently, it is not clear whether the short forms of E6 (E6*I and E6*II ORFs) are actually translated in vivo.

Previous reports have suggested that these short proteins are difficult to detect or not detectable at all [40]; however, most of the hrHPVs process their pre-mRNAs by splicing. Interestingly, HPV-16, which is the most prevalent virus in cervical carcinoma, also possesses the greatest number of splicing acceptor sites and produces four products. The other hrHPVs, which are less prevalent, have fewer splice acceptor sites. HPV-33 bears two acceptors and yields three transcripts, E6/E7$^{pre\text{-}mRNA}$, E6*I/E7 and E6*II/E7 [13], while HPV-18, -31, -35, -39, -45, -51, -56 each have one acceptor [10] and produce only two messengers, E6/E7$^{pre\text{-}mRNA}$ and E6*I/E7.

Our findings are also consistent with a differential expression of the E6 oncoprotein, as demonstrated by a large decrease in the p53 level in cells transfected with constructs that yield high levels of E6 expression (E6 and E6SD^{M}). Our data suggest that the mutations in E6/E7SD^{M} did not alter the ability of E6 to degrade p53. The low levels of p53 and pRb were consistent with increased expression of E6 and E7 in cells stably transfected with the unspliced E6/E7$^{pre\text{-}mRNA}$. These data thus confirm a possible role of HPV-16 E6/E7 splicing in driving differential expression of oncoproteins, and in turn, of their protein targets; however, a differential contribution of the spliced transcripts due to different positional effects in the HPV-16 integration sites or the HPV-16 copy numbers present in each carcinoma cell cannot be ruled out. Differential expression of the E7 and E6 oncoproteins [39, 41] or the pRb and p53 proteins [42] has been observed in CaSki and SiHa cells. Finally, the expression of the E6*II/E7 construct, surprisingly, diminished the level of the p53 protein. This finding suggests that, in contrast to reports, E6*I can bind to p53 but cannot participate in its degradation [43], p53 may be degraded in vivo by E6*II protein in the absence of the full-length E6. These data suggests that the E6 short isoforms may have different functions, and this hypothesis will be explored in more detail in the future. The E6 monocistronic construct expressed low levels of E6$^{pre\ mRNA}$ and E6*II but high levels of the E6*I transcript, as was shown in the results. Although the expression of the E6*I and E6*II proteins has not been fully demonstrated in vivo, the full-length E6 protein and the E6 short isoforms may be co-expressed from the E6 monocistronic constructs. Therefore, it is important to analyze their expression in order to define more precisely the oncogenic effect of each one of them. The ectopic expression of E6*I in CaSki cells increased the level of p53 [43]; it also exerted an anti-proliferative effect on HPV+ cell lines because E6*I interacted with E6AP and the HPV-18 E6 full-length protein, regulating its expression [44]. HPV-18 E6*I may also participate in down-regulating the expression levels of Akt, Dlg and Scribble in the absence of the full-length HPV-18 E6 protein [45]. In addition, HPV-18 E6*I seems to stabilize caspase 8, while E6 promotes its degradation [46]. These data suggest that E6* products may participate in regulating the expression levels of p53 and its other E6 targets. Whether the variation in the levels of E6 and its short isoforms contributes to selective advantages in survival or development of tumor cells remains to be determined.

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