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LETTER

HPV18 DNA replication inactivates the early promoter P_{55} activity and prevents viral E6 expression

Dear Editor,

Human papillomaviruses (HPV) are a large group (> 200 genotypes) of small double-stranded DNA viruses (https://pave.niaid.nih.gov/). Although infections by most HPV types are asymptomatic, persistent infections in cervical and ano-genital epithelia by high-risk HPV can lead to the development of cervical and ano-genital intraepithelial neoplasia and invasive carcinoma. Of 15 known high-risk HPV types, HPV16 and HPV18 are the two most common types inducing the development of cervical, anal, and a small portion of oropharyngeal cancers and are found in ~70% of cervical cancer cases worldwide (Munoz et al., 2003).

Active HPV18 DNA amplification depends on keratinocyte differentiation and requires viral DNA helicase E1 and E2, an accessory factor for E1 binding to the viral replication origin (Ori) (Demeret et al., 1995). We recently constructed a full transcription map of HPV18 from raft cultures with productive HPV18 infection and identified two major transcription start sites (TSS), one at nt 55 (TSS-55) and the other at nt 102 (TSS-102), for transcription of viral early transcripts (Wang et al., 2011). Analyses of the region 5′ to each TSS showed a TATA box (a eukaryotic core promoter motif) 27 bp upstream of the TSS-55 and 25 bp upstream of the TSS-102 (Figure 1A). We designated the sequence upstream of each TSS, respectively, as the promoter P_{55} and the promoter P_{102} (Wang et al., 2011). Interestingly, the P_{55} promoter is positioned in a core Ori region (Demeret et al., 1995) which contains three E2 binding sites (Figure 1A). The identified TATA box for the promoter P_{55} is within the A+T-rich region of the core Ori, whereas the TATA box for the promoter P_{102} is outside of the core Ori (Demeret et al., 1995; Demeret et al., 1998). Thus, we are assuming that the viral DNA replication might prevent the transcription of P_{55} promoter. Using poly A^+ total RNA isolated from either day 8 or day-16 rafts derived from HPV18-infected human foreskin keratinocytes (HFK18) for TSS assays by 5′- rapid amplification of cDNA ends (5′ RACE), a powerful method to specifically amplify the 5′ end of a given transcript (Wang et al., 2011; Wang and Zheng, 2016), we discovered that RNA transcription from both P_{55} and P_{102} promoters were equally active in the day 8 rafts, but this transcription from the P_{55} promoter faded in the day 16 rafts where the P_{102} promoter activity remained unchanged (Figure 1B). It has been demonstrated that active HPV18 DNA replication is associated with keratinocyte differentiation and becomes robust after day 10 in the raft culture (Wang et al., 2009). To further correlate the cell differentiation to the blockade of P_{55} promoter activation, we examine human foreskin keratinocytes immortalized with HPV18 infection (HFK18 cells) (Meyers et al., 1997; Mclaughlin-Drubin and meyers, 2005) either in the low calcium or in high calcium condition for transcription activities of both P_{55} promoter and P_{102} promoter by 5′ RACE (Wang et al., 2011; Wang and Zheng, 2016). Calcium has been routinely used to induce keratinocyte differentiation in HPV field (Jones et al., 1997; Moody and laimins, 2009). Under high calcium condition, a notable reduction of the transcription activity was found for the P_{55} promoter, but not for the P_{102} promoter in the HFK18 cells (Figure 1C), similar to what we observed in HPV18-infected day-16 rafts. These data suggest that human keratinocyte differentiation and HPV18 DNA replication inhibits RNA transcription from the P_{55} promoter effectively.

The promoter P_{102}-derived early transcripts contain an extremely short 5′ untranslational region (UTR), with only three nucleotides upstream of the viral E6 open reading frame (ORF) in which its first translation initiation codon ATG starts at nt 105 position in the HPV18 genome (Figure 1A). This short 5′ UTR structure of the P_{102} transcripts would be impossible for recruiting ribosome binding to initiate E6 translation. In contrast, the promoter P_{55}-derived RNA transcripts have a much longer 5′ UTR (51 nts) upstream of the E6 ORF (Figure 1A) and should have enough space for ribosome recruiting and binding to initiate E6 translation. Thus, the P_{55} promoter-derived RNA transcripts would be the RNA transcripts to translate E6 protein, although both transcripts derived from the P_{55} promoter and P_{102} promoter are capable for translation of E7 and other viral early proteins after alternative RNA splicing (Tang et al., 2006). If this assumption is true, the reduced transcrip-

tion from the P_{55} promoter in the differentiated keratinocytes with active viral DNA replication would decrease E6 expression and lead to stabilize host p53, because viral E6 interacts with p53 and induces p53 degradation via E6-mediated ubiquitination (Scheffner et al., 1993). As expected, we found that the p53 level in the HFK18 cells was increased by ~84% in the high calcium condition over that in the low calcium condition by Western blot-

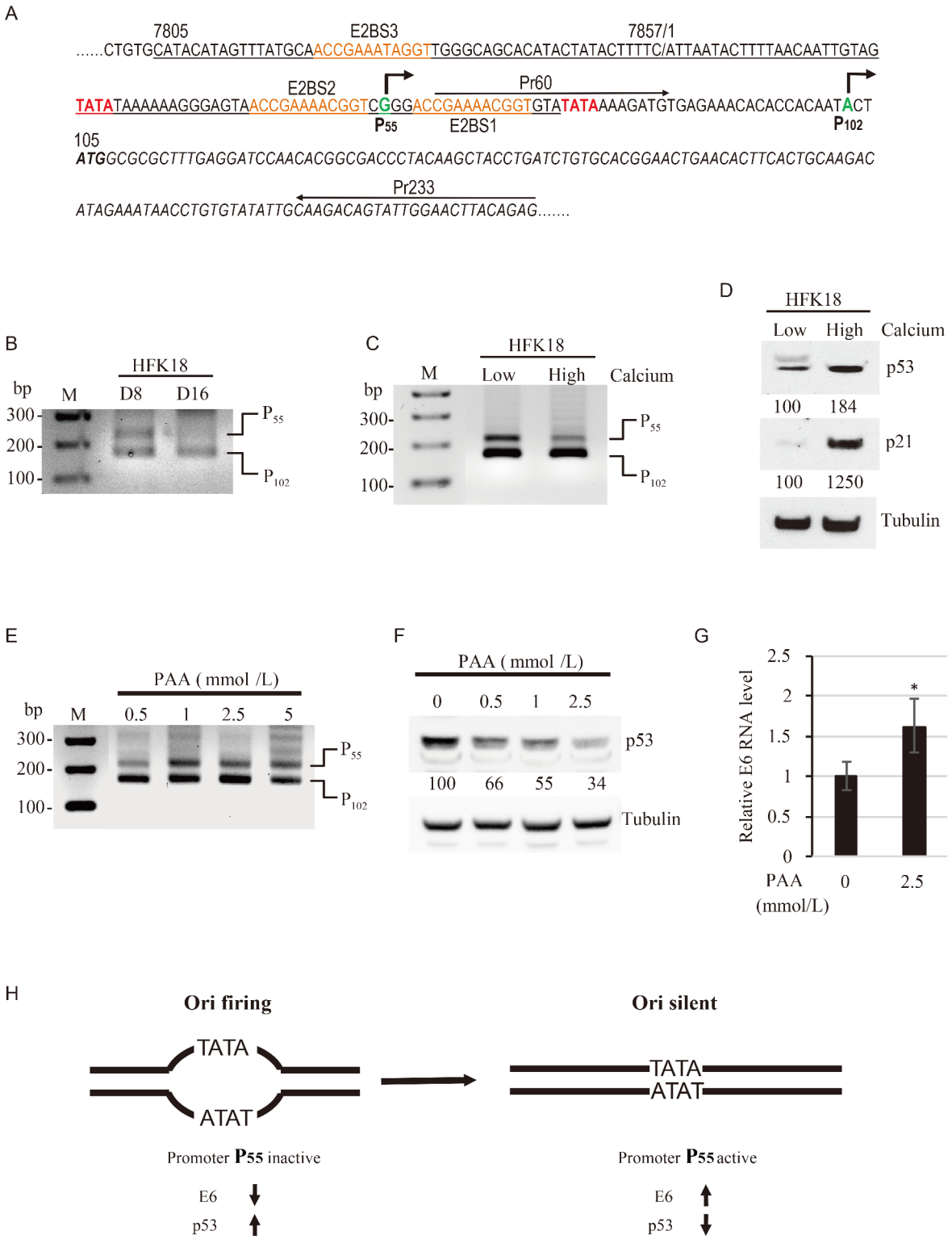

Figure 1. Viral DNA replication inactivates the transcription from HPV18 P_{55} promoter and prevents viral E6 expression. (A) HPV18 genome sequence from its replication origin to the E6 ORF region, with the sequence underlined for HPV18 replication origin (Ori), orange letters for three E2 binding sites (E2BS), red letters for two TATA boxes, green letters for transcription start sites (TSS) at nt 55 and nt 102 in the virus genome, and italic letters for E6 ORF with translation initiation codon ATG bolded. Shown above the HPV18 sequence are the positions of the sense primer Pr60 and antisense primer Pr233 used in this study and named by its 5′ end nucleotide position in the HPV18 genome. (B) Late stage of HPV18 infection blocks transcription from the P_{55} promoter, but not the P_{102} promoter. 5′ RACE with the antisense Pr233 primer was performed on poly (A)-selected total RNA from HPV18-infected human foreskin keratinocyte (HFK18) raft tissues collected at early (day 8 [D8]) or late stage (day 16 [D16]) during productive viral infection. The RACE products were then resolved by electrophoresis in a 1.5% agarose gel. M, 100-bp DNA ladders. (C) Calcium-mediated keratinocyte differentiation decreases the P_{55} promoter transcription. 5′ RACE were performed on total RNA from HFK18 keratinocytes grown in calcium-free (low) EpiLife Medium (Invitrogen, Carlsbad, CA) or high (2 mmol/L) calcium medium for 24 h. (D) Inactivation of P_{55} promoter in the differentiated HFK18 keratinocytes enhances p53 accumulation and transactivates p21 expression. HFK18 cells with low or high calcium treatment described in (C) were lysed and blotted for p53 and p21 expressions. β-tubulin served as an internal control. Relative p53 or p21 level (%) of HFK18 cells in high calcium condition, after normalizing to tubulin, is showed using the p53 and p21 level in low calcium condition set to 100%. (E and F) Inhibition of DNA polymerase activity increases the P_{55} promoter transcription and decreases p53 expression. 5′ RACE (E) with the primer Pr233 and Western blotting (F) were conducted, respectively, on total RNA (E) and on total protein (F) prepared from HFK18 cells with high calcium culture condition and the indicated doses of phosphonoacetic acid (PAA) for 24 h. Relative p53 protein level in the cells with PAA over the cells without PAA was calculated as in the panel D. (G) Inhibition of HPV18 DNA replication by PAA enhance P_{55}-derived E6 transcription. Total RNA extracted from HFK18 cells grown in high calcium (2 mmol/L)-containing F-12 medium (Invitrogen) in the presence or absence of 2.5 mmol/L PAA were analyzed specifically for the P_{55}-derived E6 RNA by RT-qPCR using an HPV18-specific primer set of Pr60 and Pr233 shown in (A) and an HPV18 E6-specific TaqMan probe oMA410 (nt 205–174, 5′-FAM-TATACACAGGTTATTTCTATGTCTTGCAGTGA-TAMRA-3′) synthesized by Integrated DNA Technologies (Coralville, IA, USA). A TaqMan gene expression assay for GAPDH (Hs02758991_g1, Thermo Fisher Scientific) served as an internal control. *, $P < 0.05$ by two tailed t-test. (H). Proposed Model of how HPV18 DNA replication affects viral promoter P_{55} activity and viral E6 expression. Since the TATA box in the P_{55} promoter is overlapped with the HPV18 core Ori, initiation of DNA replication leads to Ori firing and unwinding the double-stranded DNA. Consequently, the Ori firing makes TATA box being single-stranded, thereby preventing TATA-binding protein (TBP) from binding to the double-stranded TATA box and reducing the P_{55} transcription and viral E6 expression, or vice versa when the Ori becomes silent. As a result, the level of p53 protein, a downstream target for E6-mediated degradation, could either increase with the Ori firing for viral DNA replication or decrease with the Ori in a silent state.

ting using an anti-p53 antibody (Ab-6, Calbiochem, San Diego, CA, USA), accompanied by ~12-fold increase of p21, a p53-mediated transcription product (Figure 1D).

To determine if viral DNA replication directly inactivates the P_{55} promoter activity, we added phosphonoacetic acid (PAA), a chemical compound that inhibits DNA polymerases (Allaudeen and Bertino, 1978), to inhibit HPV18 DNA replication in calcium-differentiated HFK18 cells. As shown in Figure 1E and 1F, PAA at 1 mmol/L or above did induce a much higher level of RNA transcription from the P_{55} promoter, but had little effect on the P_{102} promoter (Figure 1E), accompanied by reduction of p53 protein level in a dose-dependent manner (Figure 1F), although PAA at 5 mmol/L might exhibit some toxicity to the cells. Consistent with the increased P_{55} promoter transcription and p53 level reduction, we saw a greater production of the P_{55} promoter-derived E6 RNA in the HFK18 cells treated with PAA (Figure 1G). Altogether, these data provide strong evidence that transcription of the HPV18 P_{55} promoter is directly regulated by viral DNA replication and is responsible for viral E6 production to modulate host p53 stability during virus infection.

HPV18 DNA replication requires an A+T-rich Ori in the long control region (LCR) and the characterized core Ori (Demeret et al., 1995; Demeret et al., 1998) contains the binding sites for two viral DNA-binding proteins, E1 as a DNA helicase and E2 as an accessory factor to E1 (Demeret et al., 1995; Kuo et al., 1994). In addition to its role in viral DNA replication, E2 has been described as a transcriptional activator or repressor to regulate viral early promoter activity through consensus E2-binding sites (Romanczuk et al., 1990), upstream of the viral early promoter. 5′ RACE is a powerful method to specifically amplify the 5′ end of a transcript to map the transcription start sites and efficiency of the individual promoter usage (Wang et al., 2011; Wang and Zheng, 2016). By using this method in this study, we demonstrated that HPV18 viral DNA replication inactivates RNA transcription from viral early promoter P_{55}, but not

the P_{102} promoter. Our data thereby provides the direct evidence that HPV18 DNA replication contributes to another layer of the control for viral E6 expression.

FOOTNOTES

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