The Promoter of the Human Proliferating Cell Nuclear Antigen Gene Is not Sufficient for Cell Cycle-Dependent Regulation In Organotypic Cultures of Keratinocytes

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Running Title: Regulation of the human \textit{PCNA} gene
Summary

The proliferating cell nuclear antigen (PCNA) is essential for DNA replication of mammalian cells and their small DNA tumor viruses. The mechanism of the cell-cycle dependent regulation of the human PCNA promoter is not clear despite extensive investigations. In this report, we employed organotypic cultures of primary human keratinocytes, which closely resemble native skin comprising both proliferating and postmitotic, differentiated cells, to examine the cell-cycle dependent regulation of the human PCNA gene (hPCNA) in the absence or presence of the human papillomavirus type 18 (HPV-18) E7 protein. HPV-18 E7 promotes S phase re-entry in post-mitotic differentiated keratinocytes by abrogating the transcription repression of E2F transcription factors by the retinoblastoma susceptibility protein, pRb. We demonstrated that E7 reactivated the transcription of the endogenous hPCNA in differentiated keratinocytes. In contrast, with or without E7, the expression of a transduced hPCNA promoter-driven reporter did not correlate with that of the endogenous hPCNA gene in either proliferating or differentiated cells. Moreover, in CHO and L-cells, HPV E7 and the adenovirus E1A protein repressed the transduced hPCNA promoter, but both activated an extended promoter construct spanning the first intron. Mutations of two E2F sites in the intron reduced the basal activity and abolished the response to E7 or E1A. Promoter repression or activation required the CR2 domain of E7, and to a lesser extent, CR1 as well. However, in organotypic cultures, this extended promoter construct failed to recapitulate the cell cycle-dependent regulation of the endogenous hPCNA gene. Only when a full-length myc-tagged hPCNA spanning the 5’ promoter, all exons and introns was used, was the native pattern of expression largely restored, indicative of the complexity of its regulation.
**Introduction**

The proliferating cell nuclear antigen (PCNA) is a conserved highly acidic 29-kDa nuclear protein that functions as a sliding clamp to enhance the processivity of the DNA polymerase δ and is crucial for eukaryotic DNA synthesis (1-3). PCNA protein synthesis peaks in early S phase of the cell cycle (4) and is comparatively low in quiescent cells (5,6). This differential expression is thought to be achieved by both transcriptional and posttranscriptional mechanisms (7-11). The human PCNA (hPCNA) transcript spans 4961 bases and consists of 6 exons (12). The region from nucleotide -1265 to +61 of hPCNA, relative to the transcription initiation site, has been considered the promoter region of the gene and is designated here as hPCNAp. The fragment from -249 to +61 is critical to achieve high levels of expression in HeLa cells and has been examined extensively in transiently transfected cell lines (13). Many of the studies were conducted in conjunction with the expression of adenovirus E1A protein, which inactivates the tumor suppressor protein pRb and derepresses adenovirus early promoter 2 factor (E2F)-controlled genes. The family of E2F/DP heterodimeric transcription factors is known to control many genes necessary for S phase entry and cell cycle progression (14). However, hPCNAp does not contain binding sites for E2F (12,13) but nevertheless is activated by adenovirus E1A in HeLa cells (15,16).

hPCNAp activation by E1A is mediated through an element termed PERE that contains binding sites for transcription factors ATF/CRE and RFX1 (13,17-19). p300/CBP and p107, a pRb-related protein, interact indirectly with these sequences. The ability of E1A proteins to bind both these cellular proteins is thought to be responsible for the observed activation (20,21). However, this E1A activation of the PCNA promoter-driven reporter is dependent on the cell lines used. In nontransformed cells such as baby rat kidney (BRK) cells or cloned rat embryo
fibroblasts (CREF), repression rather than activation is observed (22). In a reporter assay, the responsiveness of the hPCNA promoter to overexpression of E2F-1 and DP-1 transcription factors was attributed to two consensus E2F binding sites in intron 1 (23). These sites were occupied by E2F in a cell cycle-dependent manner both in vivo and in vitro (11), but it is still unknown whether they are sufficient to confer proper cell cycle-dependent regulation to the gene.

PCNA is also required by small DNA tumor viruses to replicate their genomes (24,25), including the human papillomaviruses (HPVs) (26). In particular, vegetative HPV DNA amplification and progeny virus packaging take place in postmitotic, differentiated cells in a squamous epithelium (27). However, PCNA is normally undetectable in these strata in uninfected epithelia (28). To re-establish an S phase milieu in these cells, the HPV E7 protein, which shares structural and functional similarities with adenovirus E1A protein, binds and abrogates pRb functions. Based on sequence similarities, two conserved regions (CR) are defined in E7. Conserved region 1 (CR1), which spans residues 1 to 15, contains the target for ubiquitination (29), and also plays a role in the destabilization of pRb (30,31). Conserved region 2 (CR2), which spans residues 16 to 36, contains the pRb binding domain (32), as well as a casein kinase II phosphorylation site (33). The integrity of both CR1 and CR2 is required for the transformation and transactivation activities of E7 (34-37).

The native HPV enhancer-promoter (URR, for upstream regulatory region) responsible for E7 transcription is activated upon differentiation in vivo (27) and in organotypic cultures of primary human keratinocytes (PHKs) (termed hereafter as raft cultures) (38). When acutely transduced via a recombinant retrovirus into PHKs, the HPV-18 URR-driven E7 induces PCNA protein in the differentiated cells of raft cultures. This activity requires the presence of both the
pRb binding motif and the CKII phosphorylation site in CR2 (39). When assayed by immunofluorescence, PCNA protein is detected in all the differentiated cells, whereas the less sensitive immunohistochemistry reveals PCNA protein in only a subset of these cells (40,41). Moreover, HPV-18 URR-E7 promotes S phase reentry in some but not all postmitotic, differentiated cells in raft cultures due to intriguing interactions involving the cyclin E/cdk2 complex and its inhibitors, p27kip1 and p21cip1 (42,43).

In the raft culture system, PHKs are seeded onto a dermal equivalent composed of collagen and fibroblast feeder cells. When placed at the liquid-air interface, PHKs proliferate, stratify, and differentiate into a squamous epithelium over a period of 1 to 2 weeks. At the histologic and molecular levels, the tissue developed closely resembles the three-dimensional architecture of the epidermis of the skin. Above the collagen, there are one layer each of basal and parabasal cells. Only cells in these strata are capable of cell division and are positive for PCNA while in S phase. Above them are daughter cells that have exited the cell cycle and are undergoing successive squamous differentiation, generating spinous cells, granular cells, and finally squames. These cells no longer replicate their DNA and are negative for PCNA (44) (see Fig. 1D, left panels). This culture system, in conjunction with the differentiation-dependent expression of HPV-18 E7 from the URR promoter, provides an exceptional opportunity to examine the mechanisms by which the cell controls the expression of genes necessary for S phase entry.

Here, by using this culture system, we investigated the regulation of a transduced hPCNA promoter and a transduced hPCNA gene and compare it to the endogenous counterpart in the absence or in the presence of E7. We also contrasted the results with those obtained from cell lines in submerged cultures. We show that the regulation of this gene is complex. The promoter alone does not confer the cell-dependent regulation and, rather than being upregulated, it is
repressed by E7 in cells lines. However, E7 transcriptionally activates an extended hPCNA promoter. The activation is dependent on two E2F sites in the first intron as well as the E7 CR2 and, to a lesser extent, CR1. However, in the absence or in the presence of E7, only an ectopic hPCNA gene, which spans the promoter, all exons, all intron and 3' untranslated region, exhibits a proper cell cycle-dependent regulation in both the proliferating and the differentiated cell strata of raft cultures. The promoter alone or the extended promoter did not. This study highlights the importance of using an appropriate system in studying the regulation of S phase genes that are active in cycling cells.

**Materials and Methods**

**Plasmids and retrovirus vectors.** pBACAT reporter plasmid carrying the full-length hPCNAp promoter driving chloramphenicol acetyl transferase (CAT) was kindly provided by Michael B. Mathews (13). The human DNA polymerase α p180 subunit promoter reporter construct was a gift of Theresa Wang (45). HPV-18 E7, E7 mutations, and the adenovirus E1A 289R genes were expressed from the cytomegalovirus IE promoter in the pcDNA3.1 plasmid (Invitrogen, Carlsbad, Calif.) or from the adenovirus major late promoter in the pMTX plasmid (46), a derivative of pMT2.

The recombinant Moloney murine leukemia virus (MLV) containing HPV-18 URR-E7 (pLC-18URR-E7) or the dDLLC mutation has been described (39,41). These viruses also express the bacterial neomycin resistance gene from the simian vacuolating virus 40 (SV40) early promoter located downstream of URR-E7. The H2P mutation of HPV-18 E7 was constructed by using M13 and T7 primers in conjunction with a reverse-strand mutagenic primer: 5'-609 gttgccttaggctcaGgcataatcaagc577-3' (with the complement of the E7 start codon
underlined and the mutation in uppercase letter). H2P was then introduced into the same MLV retrovirus vector as the wild type HPV-18 E7 as previously described (39). The pBabe\textsuperscript{Puro}-URR-E7 was constructed by excising the HPV-18 URR-E7 from the pLC-18URR-E7 with \textit{Bam}HI and \textit{Sall} and ligating to equivalent positions of the pBabe\textsuperscript{Puro} vector which has has a puromycin-resistant gene under the control of the SV40 early promoter (47).

To construct the retrovirus carrying the \textit{LacZ} reporter driven by the hPCNA\textsubscript{p} (pLN-hPCNA\textsubscript{p}-LacZ), we excised the hPCNA\textsubscript{p} fragment from the pBACAT vector (13) by using \textit{XhoI} and \textit{HindIII} (partial digestion). The fragment was cloned by blunt-ended ligation at the \textit{NruI} site of pLN-LacZ (38). This virus expresses the neomycin-resistance gene from the LTR. The pLN-K14-LacZ, in which the 2.4-kb human K14 promoter drives the reporter, has been described (48). Amphotropic producer cells of a recombinant MLV retrovirus, in which a 2.5-kb involucrin promoter in a pBabe\textsuperscript{Puro} backbone drives the LacZ reporter gene, were a gift from Joseph Carroll and Lorne Taichman (49).

An extended \textit{hPCNA} promoter-driven LacZ was constructed by fusing the 5’ portion of exon 2 of the \textit{PCNA} gene to the LacZ gene. The pEcoRV full-length \textit{hPCNA}, kindly provided by Renato Baserga, contains 789 bp of the 5’-flanking sequence, six exons, five introns, and 400 bp of the 3’ untranslated region in a pGEM3 backbone (8). We performed a polymerase chain reaction (PCR) amplification using a Sp6 primer, which anneals in the pGEM3 backbone, and the reverse primer 5’-GGGATCCGAATCCGTAACtactagcgaagg-3’, complementary to the sequence from positions +1160 to +1173 in the second exon of \textit{hPCNA} (lowercase letters). The primer also contains 18 bases from the LacZ gene (uppercase letters) corresponding to the complement of the amino-terminus minus the first methionine. A \textit{Bam}HI site in this sequence (underlined) was used for in-frame fusion. The PCR product was digested with \textit{NdeI}, at position
-234, and BamHI, generating a 1417-bp fragment (from position -234 to +1173 of hPCNA). This fragment was swapped into the hPCNAp (–1265 to +61) to give the hPCNAep (–1265 to +1173). Finally, hPCNAep was cloned into pBluescript SK+ (Stratagene, La Jolla, Calif.) to give pBS-hPCNAep. To make the pBS-hPCNAep:LacZ, the LacZ gene was excised from pLN-LacZ with BamHI and NheI and cloned into pBS-hPCNAep using BamHI and XbaI. The clone encodes a fusion protein consisting of the first 74 residues of the PCNA protein fused in frame to an intact βgal without its first methionine. The poly A signal 3’ end of the LacZ gene was not removed.

The mutations of the E2F binding sites in intron 1 of hPCNA were generated by a two-stages PCR amplification using a combination of two non-mutagenic primers and a mutagenic primer which spans the two E2F binding sites: 5’-624 cggaaaaacacctgttggctagcaccggctttgtgacttttacgatatagaagcaggtcge-3’. The E2F sites were altered by creating an NheI site and a ClaI site (underlined). The mutated fragment was swapped into pBS-hPCNAep:LacZ using NcoI and BamHI.

To generate the lentiviral vector pPC-hPCNAep:LacZ, the hPCNAep:LacZ fusion was excised from the pBS-hPCNAep:LacZ with HindIII and XhoI, and introduced into the vector pPC-eGFP (50) replacing eGFP. This vector contains the two LTRs, the packaging signal, rev responsive element, the central polypurine tract, and central termination site of HIV-1. pPC(SIN)-myc-hPCNAREV, a self-inactivating (SIN) lentiviral vector containing the myc-tagged full-length hPCNA was generated as follows. The fragment +171 to +5198 of the genomic clone (12) was excised from pEcoRV full-length hPCNA with AscI and SacI, and swapped into the equivalent sites on pBS-hPCNAep:LacZ giving pBS-hPCNA. This constructs spans from -1265 to +5198 of the hPCNA genomic clone. The myc-epitope consists of six tandem repetitions of the undecapeptide EQKLISEEDLN. This epitope was amplified by PCR from the pCS2-m-
cyclin E (51) using the pair of primers: 5’-AATGGCGCGCagaattagatgacg-3’ and 5’-ATTGGCGCGCCTcgcctctctctgttcatgg-3’ (introduced AscI sites are underlined, regions of homology to the myc-epitope fragment are in lowercase). The 294-bp PCR product was cloned into the pBS-hPCNA at position +171 using AscI, generating pBS-myc-hPCNA. In the resulting fusion protein, the 98-residue myc-epitope was inserted between residues 5 and 6 of the native hPCNA protein. The myc-hPCNA gene was then introduced in the opposite orientation to the 5’ LTR of a self-inactivating (SIN) lentivirus vector (see Fig. 2C), which is essentially identical to pPC-eGFP except that the 3’ LTR has been inactivated (52). To generate pPC(SIN)-myc-hPCNAREV, the 6.7-kb restriction fragment containing myc-PCNA was inserted between SacI (blunted) and XhoI, replacing eGFP. The identities of all the described clones were confirmed by sequencing.

**Retroviruses, lentiviruses, and organotypic raft cultures.** Amphotropic recombinant MLV was generated as described (38,41). Trans-lentiviruses were generated by transfecting 293T cells with four plasmids: 5 µg packaging plasmid pCMV-gag-pro, 1.5 µg pLR2P-vpr-RT-IN (trans-enzyme expression plasmid), 2 µg the VSV-G expression plasmid (53), and 5 µg gene transfer vector (pPC-hPCNAep:lacZ or pPC(SIN)-myc-hPCNAREV) using the CaPO₄ precipitation method as described previously (50). Supernatants were harvested after 60 h, clarified by low-speed centrifugation, filtered through 0.45-µm pore-size filters, and concentrated by ultracentrifugation (SW28, 23,000 rpm, 4°C, 90 min). The pellets were resuspended in DMEM, aliquoted and frozen at –80°C. The particle number was estimated from p24 antigen concentration (50).

PHKs recovered from neonatal foreskins were grown in serum-free medium (Life Technologies, Inc., Rockville, Md.) and acutely infected with recombinant MLV, selected for 2
days with 300 µg/ml of G418 (Life Technologies). The bulk, selected cultures were allowed to recover for 2 to 4 days and then used to seed the epithelial raft cultures as previously described (38,41). PHKs infected with the trans-lentiviruses at a multiplicity of approximately 5 particles per cell were not selected and were subsequently super-infected with recombinant MLV as described above, or used directly to prepare epithelial raft cultures. Raft cultures were harvested after 9 days at the medium-air interface by fixation in 10% buffered formalin, embedded in paraffin, and then cut into 4-µm sections. To mark cells in S phase, the cultures were incubated in raft culture medium containing 50 µg/ml bromodeoxyuridine (BrdU) for 12 h before harvest. The exposure to BrdU at this concentration does not affect the growth and differentiation of PHKs in raft cultures (54). Prior to embedding, raft cultures were probed for βgal activity with X-gal (Gold Biotechnology Inc., St. Louis, Mo.) as previously described (55).

**Transient transfection and reporter assays.** Chinese hamster ovary (CHO) cells were maintained in Ham’s F-12 supplemented with 10% fetal bovine serum. 293, COS-7 and mouse L cells were maintained in DMEM media containing 10% fetal bovine serum. L-cells were transfected using a mammalian transfection kit (Stratagene) based on the CaPO$_4$ method (56). Electroporations of 293, CHO and COS-7 cells were performed essentially as described (57) with a pulse of 180V (COS-7, 293 cells) or 230V (CHO cells) and a capacitance of 960 µF using a Gene Pulser (Bio-Rad, Hercules, Calif.). In addition to 50 µg sheared herring sperm DNA (Life Technologies), empty expression vector was used to equalize the total amount of transfected plasmid DNA. To control for transfection efficiency, 1 µg of the plasmid pGL3-Control (Promega, Madison, Wis.), which expresses the firefly luciferase gene from the SV40 early promoter, was included in all the reactions. The cells were then plated with 5 ml of fresh culture media in a 60-mm culture dish, media changed 12 h later and harvested 48 h post
transfection. For CAT assays, the cells were lysed by freeze-thaw cycles in 250 mM Tris (pH 8).
For βgal assays, the cells were lysed in the plate with Reporter Lysis Buffer (Promega). Protein concentrations from total cell extracts were determined by the Bio-Rad protein assay. Equal amounts of proteins were used in reporter assays. CAT assays were performed by the fluor-diffusion method (58) with 14C-Acetyl CoA (Amersham, Piscataway, New Jersey). The luciferase assay was performed using the Luciferase Assay System (Promega). The βgal assay was performed as described (59) and the activities were normalized against those of the luciferase. All assays were performed in duplicate or triplicate.

**GST-Rb coprecipitations and immunoblots.** COS-7 cells transfected with an E7 expression vector were lysed in 500 µl cold RIPA buffer (150 mM NaCl, 10 mM phosphate buffer [pH 7.3], 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 2 mM EDTA). Total protein content was determined as above. For the immunoblots, 30-100 µg total protein extracts were separated by electrophoresis through sodium dodecyl sulfate-10% or -15% polyacrylamide gels, transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham), and probed with a 1:2,000 dilution of a polyclonal antibody against HPV-18 E7 (60), a monoclonal antibody against hPCNA (clone PC10; Dako Corp., Carpinteria, Calif.), or a monoclonal antibody against c-myc epitope (clone 9E12; Zymed Laboratories, San Francisco, Calif.). The GST-Rb pocket domain protein was expressed in E. coli (61), and E7 binding assays were performed as described (39).

**Immunohistochemistry and RNA in situ hybridization.** PCNA or BrdU immunohistochemistry was performed using the monoclonal antibodies anti-PCNA (Dako Corp.; 1:100 dilution) or anti-BrdU (Zymed Laboratories; 1:50 dilution) in conjunction with the Histostain-SP broad-spectrum kit following the manufacturer’s instructions (Zymed
Laboratories). The sections were then counterstained lightly with hematoxylin. Images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, Mo.) attached to an Olympus BH-2 microscope. For hPCNA RNA in situ hybridization, hPCNA cDNA, a gift from Bruce Stillman, was cloned into pGEM1 and in vitro transcribed using T7 (antisense probe) or SP6 (sense probe) in the presence of \( \alpha^{35}\text{S}\)-UTP (>1,000 Ci/mmol, 10 mCi/ml; Amersham Pharmacia Biotech). The probe was applied at 50% saturation as described (41). For myc-hPCNA RNA in situ hybridization, the 294-bp fragment coding for the myc epitope was labeled with biotin by PCR using Detector PCR DNA biotinylation kit (KPL Inc., Gaithersburg, Md.). Hybridization and washings were performed at 37°C in the presence of 50% formamide as described (43). The slides were then blocked with 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1% casein and probed with streptavidin-HRP. Detection was carried out by deposition of fluorescein-labeled tyramide (NEN Life Science Products, Boston, Mass.). The slides were mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, Calif.) and photographed with an Olympix 2000 digital camera (Life Sciences Resources, Cambridge, United Kingdom) attached to an Olympus Provis AX70 microscope. The images were digitally processed using Photoshop 6.0 (Adobe Systems, San Jose, Calif.).

**PCR Amplification.** PHKs were harvested and genomic DNA was purified using the DNeasy System (Qiagen, Valencia, Calif.). 500 ng of template were used in a PCR amplification reaction with the following primers: forward, 5’-gacagcgtggtgacgtcg-3’ (nucleotide number indicates the position in the hPCNA gene), and reverse, 5’-aacgacggactccgaatc-3’, which anneals to the 5’ end of the LacZ gene. The approximate positions of these primers are shown in Fig. 2B.
Results

HPV-18 E7 reactivates the transcription of hPCNA in differentiated keratinocytes. E7 is known to induce the accumulation of proteins, such as p21cip1, via a posttranscriptional mechanism (40,43). Therefore, we first asked whether the distribution of hPCNA mRNA corresponded to that of PCNA protein in the raft culture in the presence and in the absence of E7. We performed in situ hybridization with $^{35}$S-labeled, strand-specific RNA probes to detect hPCNA message and immunohistochemical staining for the PCNA protein in adjacent sections in raft cultures transduced with either the vector-only retrovirus (pLC) or the retrovirus containing HPV-18 URR-E7. In the absence of E7, the antisense-strand probe detected a weak signal only in the proliferating basal cells and parabasal cells where the protein is also found (Fig. 1A, top right and 1D, bottom left). Neither protein nor mRNA was detected in the differentiated cells. However, in HPV-18 URR-E7-transduced raft cultures, PCNA mRNA was additionally detected in the suprabasal strata in a diffused pattern with signal intensity comparable to that in the basal layer (Fig. 1A, bottom right). As before, the PCNA protein was also detected by immunohistochemistry in some of the differentiated keratinocytes (Fig. 1D, bottom middle). Sense-strand riboprobes produced no signals in either specimen (Fig. 1A, left panels). Thus, if antisense RNA exists as reported previously (11), its level must be very low and has therefore escaped our detection. In addition, our results suggest that E7 induces PCNA protein mainly by increasing its mRNA.

The induction of PCNA by E7 in differentiated keratinocytes requires an intact E7 CR2 domain, which binds to pRb (39). The CR1 domain has also been implicated in the transformation activity of E7 (62). To evaluate the importance of the CR1 domain for PCNA activation, we prepared a point mutation of histidine 2 to proline in HPV-18 E7 (H2P). E7 H2P
protein was detected at similar level as the wild-type E7 protein in transfected COS-7 cells (Fig. 1B) and was able to bind to GST-pRb pocket domain fusion protein in vitro (Fig. 1C). However, in raft cultures, the HPV-18 URR-E7 H2P induced PCNA only in the lowest spinous layers and the induction was weaker when compared to the wild-type E7. Moreover, it failed to induce S phase reentry in differentiated keratinocytes, as evidenced by a lack of BrdU incorporation. In parallel experiments, E7 induced PCNA and S phase reentry in differentiated cells, whereas in the control raft culture transduced by vector-only virus, PCNA- and BrdU-positive cells were only observed in basal and parabasal strata (Fig. 1D, compare right to center and left columns).

A similar mutation in HPV-16 E7 abolishes its ability to transform BRK cells (62) and to induce S phase in suprabasal cells of raft cultures when expressed from the LTR of a retrovirus (63).

**HPV-18 E7 and adenovirus E1A 289R repress the hPCNAp in cell lines.** To investigate how E7 activates the hPCNA gene, we began by examining the regulation of the hPCNA promoter (Fig. 2A) in cultured cell lines using CAT as a reporter (13). Surprisingly, in CHO cells, expression of the HPV-18 E7 from the adenovirus major late promoter down-regulated hPCNAp (Fig. 3A). We then repeated the experiments in L-cells along with the adenovirus E1A 289R (also referred as 13S) driven by the same promoter as a reference. Both expression vectors caused significant repression of hPCNAp (Fig. 3B). In contrast, both viral proteins weakly trans-activated the promoter of the p180 subunit of the human DNA polymerase α (pol α p180) (Fig. 3B) in agreement with our previous observation in raft cultures (39). This latter promoter contains an E2F binding site (45,64,65), which very likely mediated the E7 transactivation.

To verify that the hPCNAp repression was associated with E7 expression and also to examine the domains involved in repression, we tested two HPV-18 E7 mutations, H2P, which
was characterized above, and dDLLC, which has a deletion of four amino acid residues in the pRb-binding motif LxCxE. A retrovirus containing HPV-18 URR-E7dDLLC induces neither PCNA protein nor pol α p180 RNA in differentiated strata of raft cultures (39). The dDLLC and H2P mutations retained, respectively, about 50% and 25% of the repression activity of the wild-type E7 (Fig. 3B). When tested on the pol α p180 promoter, neither mutation was able to elicit any transactivation. These data confirm that E7 and E1A can repress hPCNAp, that repression requires CR2, and that CR1 also appears to contribute to this activity.

**Exogenous hPCNAp does not recapitulate the cell cycle-dependent regulation of the endogenous PCNA gene in epithelial raft cultures.** The above transient reporter assays in proliferating cells suggest that the hPCNAp does not contain all the necessary cis elements that respond to E7 expression and, by inference, cell cycle-dependent regulation. To verify this interpretation, we cloned the hPCNAp upstream of the *E. coli* *LacZ* gene (Fig. 2A) and then into a retrovirus vector (pLN-hPCNAp-LacZ). The recombinant retrovirus was used to infect PHKs that were then selected for G418 resistance for 2 days. The bulk-selected cells were used to develop raft cultures without further expansion. As controls, the promoterless *LacZ* reporter gene (38), and the reporter driven by the promoter of the human keratin 14 (*K14*) gene (66) or of the human *involucrin* gene (49) were similarly transduced into PHKs. After harvest, the cultures were stained with X-gal to assess βgal activities.

As reported previously (48), *LacZ* expression from the *K14* promoter was limited to the basal and parabasal layers of the cultures raft, as expected from K14 expression in native tissues (67), whereas the activity from the *involucrin* promoter was only detected in cells in the upper strata, where involucrin is found (68). In the negative control, the promoterless pLN-LacZ retrovirus
(38) yielded no signal in raft cultures or in submerged cultures (Fig. 4A, B). Surprisingly, hPCNAp:LacZ generated βgal activities only in some of the suprabasal layers where the endogenous hPCNA is inactive, but not in basal or parabasal cells (Fig. 4A) where the endogenous PCNA is normally expressed (Fig. 1D). In contrast, βgal was observed in a fraction of proliferating, submerged PHK cultures (Fig. 4B). These observations are consistent with the conclusion from our studies of HPV URR LacZ expression that the cellular milieu in proliferating cells of submerged cultures may resemble a state of wound healing and is somewhat different from the maintenance mode in the basal stratum of an established epithelium (38,55).

We then tested the response of the hPCNAp to E7 in raft cultures. PHKs acutely infected with pLN-hPCNAp-LacZ were superinfected with high titers of HPV-18 URR E7-containing retroviruses (pBabe^Puro-URR-E7) or empty retroviruses (pBabe^Puro) and selected with puromycin. The bulk-selected PHKs were then developed into raft cultures. The expression pattern of hPCNAp did not change significantly in the presence of E7, and the reporter activity was similar to that obtained with the empty vector (Fig. 4C, blue staining). In contrast, the endogenous PCNA protein remained exclusively basal when the empty vector was used but was additionally detected in suprabasal strata in the presence of E7, as revealed by immunohistochemistry on the same sections (Fig. 4C, red staining). Collectively, these results demonstrate that hPCNAp by itself is not sufficient to control the proper expression of the gene in either proliferating or differentiating cells. Furthermore, it does not respond to E7 induction in the differentiated cells, as the endogenous gene does.

Two E2F binding sites in the first intron are required to confer transactivation by E7 and E1A in cell lines. To examine the possibility that the two E2F sites in intron 1 may confer
cell cycle-dependent regulation to the hPCNAp and hence are necessary for E7 transactivation, we prepared a reporter construct with an ‘extended promoter’ (hPCNAep) of 2438-bp in length. pBS-hPCNAep:LacZ spanned position -1265 to +1173 to include the 5’ promoter region, the first exon (380 bp), the first intron (708 bp), and 85 nucleotides of the second exon (to position +1173), which was then fused in frame to the βgal coding region (Fig. 2B). This plasmid was cotransfected with an E7 or an E1A expression vector into CHO cells. Reporter activities increased in a dose-dependent manner up to fourfold with E7 and 10-fold with E1A in this experiment (Fig. 5A). The E7 mutations dDLLC and H2P were then tested, along with the wild type (Fig. 5B). Interestingly, H2P, which binds pRb in vitro (Fig. 1C), maintained a significant transactivation activity, but dDLLC, which is unable to bind pRb, did not. These results demonstrate that the intron 1 of the hPCNA gene is required for E7-mediated activation. In addition, this activation depends on the ability of E7 to target the pRb-E2F pathway.

To substantiate the interpretation that the two E2F sites of the first intron mediate the E7-dependent activation, we site-mutated both sites in the reporter construct and tested its activity in transiently transfected CHO cells. As shown in Fig. 5C, the mutations reduced the basal activity by 70%. Furthermore, the construct was stimulated by E7 or the adenovirus E1A to a much lesser extent than did the wild-type promoter construct (compare Fig. 5C to 5A). These observations implicate the E2F binding sites in intron 1 as important elements for high basal activities and as targets for E7 and E1A transactivation.

The hPCNAep spanning the first intron is insufficient to confer cell cycle-dependent regulation in raft cultures. To test whether the hPCNAep:LacZ (Fig. 2B) is sufficient to provide cell cycle-dependent regulation in raft cultures, as does the endogenous hPCNA, we
cloned this reporter cassette into a trans-lentivirus vector, which have a reduced probability of mRNA splicing when compared with that of MLV (69,70) and, hence, an increased chance of retaining the intron in the integrated provirus. PHKs were transduced via acute infection. After harvesting, the raft cultures were stained for the expression of LacZ. A typical image is shown in Fig. 4D. Although the βgal signal intensities were stronger than those elicited by pLN-hPCNAp:LacZ, they remained confined to the differentiated strata, and no activity was detected in the basal cells where the endogenous hPCNA gene is expressed. Moreover, as with hPCNAp (Fig. 4C), coinfection with an HPV-18 URR-E7 retrovirus had little effect on either signal strength or signal distribution (data not shown).

To determine whether our culture contained the unspliced provirus reporter construct, we performed PCR with genomic DNA of the transduced keratinocytes using primers specific for the transduced hPCNAep:LacZ construct, spanning the intron to the LacZ gene (Fig. 2B). A product of 1250-bp indicates the presence of the intron, whereas one of 542 bp signifies the absence of the intron. Both forms of the provirus were detected in the infected PHKs (Fig. 4E). The relative abundance of the unspliced form is likely somewhat underestimated relative to the spliced form because amplification of a longer product is probably less efficient than that of a shorter product. In any case, had the intron been able to direct the expression of the reporter gene to the proliferating cells, some of the basal and parabasal cells would have been positive for the reporter gene. This was clearly not the case. We did not detect a response to E7 in the differentiated strata, due at least in part to the inappropriate but high levels of expression in these cells in the absence of E7. Therefore, the presence of the first intron in the construct is not sufficient to confer proper cell cycle-dependent regulation in either tissue compartment.
Proper cell cycle-dependent regulation of \textit{hPCNA} expression in raft cultures is largely achieved when the entire gene is included. The results described above indicate that additional regulatory sequences are required for a proper regulation of \textit{hPCNA}. These sequences may reside elsewhere in the cloned gene. To test this possibility, we examined the regulation of the \textit{hPCNA} gene spanning positions -1265 to +5198. It includes the 1.2 kb 5’ promoter, 6 exons, 5 introns, and the 3’ untranslated region including the polyA site. The gene was tagged with the myc-epitope near its amino-terminus to distinguish it from the endogenous one and was denoted \textit{myc-hPCNA}. To verify the expression of the clone, we electroporated pBS-\textit{myc-hPCNA} into 293 cells and detected the fusion protein by using myc-epitope and hPCNA antibodies. Both antibodies detected a band with an apparent molecular weight of 77 kDa, which increased in intensity in parallel with the amounts of the transfected \textit{myc-hPCNA} DNA (Fig. 6A). The expected molecular weight of the myc-hPCNA fusion is 42 kDa. We attribute this discrepancy largely to the presence of the 6 copies of the myc epitope that further exacerbated the slightly aberrant migration exhibited by the native PCNA protein (71). The differences in the intensities of myc-hPCNA and the endogenous hPCNA bands may reflect in part to the fact that not all the cells were transfected.

The \textit{myc-hPCNA} gene was then cloned into a self-inactivated (SIN) lentivirus vector to generate pPC(SIN)-myc-hPCNA$^{\text{REV}}$ in the opposite orientation relative to the strong 5’ LTR promoter (Fig. 2C). This strategy avoids removal of the introns via mRNA splicing during the production of the recombinant lentivirus. As the 5’ LTR of the SIN lentivirus becomes inactivated upon integration of the provirus, no antisense myc-hPCNA transcripts would be generated. Antisense RNA, when present, could inhibit the translation of both exogenous and endogenous PCNA proteins, affecting cell survival. The trans-lentivirus produced was used to
infect primary keratinocytes. As a negative control, we used the same lentivirus vector carrying eGFP under the control of the CMV promoter.

A fraction of the infected cells were used directly to make raft cultures. The presence of the myc:PCNA protein was detected in crude protein extracts using a myc-epitope antibody. The band corresponding to the myc:PCNA fusion was only detected in the myc-hPCNA lentivirus-infected rafts but not in those infected with the lentivirus expressing GFP (Fig. 6B). The endogenous PCNA protein is shown as a loading control. As in Fig. 6A, the intensities of the myc-hPCNA bands were weaker than the endogenous hPCNA bands probably because the infection and trans-gene expression efficiencies are not at an efficiency of 100% due to the lack of a selection step after exposure to the lentivirus.

The remaining keratinocytes infected with the recombinant myc-hPCNA lentivirus were then acutely re-infected with the MLV recombinant retroviruses carrying HPV-18 URR-E7 or the empty pLC control virus. The cells were selected for 2 days with G418 for the MLV viruses. The raft cultures obtained were subjected to RNA in situ hybridization using a myc-epitope specific biotinylated probe. RNA signals were detected in cultures infected with the myc-hPCNA lentivirus but not in those infected with the CMV-eGFP lentivirus (Fig. 6C, compare top to bottom panels). Although the expression levels were heterogeneous, the signal was mainly restricted to the basal cells in the absence of E7. The signals were additionally found in a fraction of suprabasal cells when E7 was also expressed (Fig. 6C, compare lower left and right panels, and Fig. 6D) and only in the cultures infected with the myc-hPCNA lentivirus (compare top and bottom panels on the right). The presence of active E7 was confirmed by the incorporation of BrdU in the suprabasal cells (data not shown). These patterns of expression closely resemble those observed for the endogenous hPCNA (Fig. 1A). We conclude that
sequences beyond the extended promoter and first intron in the *hPCNA* gene play a critical role on its cell cycle-dependent regulation.

**Discussion**

We have taken advantage of our observation that HPV-18 URR-E7 induces PCNA and S phase reentry in postmitotic differentiated keratinocytes in raft cultures to examine the cell cycle-dependent regulation of the *hPCNA* gene. Our results show that the endogenous *hPCNA* is primarily if not exclusively regulated at the RNA level because PCNA transcripts are detected only in proliferating basal and parabasal cells but not in postmitotic, differentiated keratinocytes. The HPV-18 E7 induces PCNA protein in differentiated keratinocytes by reactivating the transcription of the *hPCNA* (Fig. 1A). Most importantly, our results demonstrate that the regulation of this gene is rather complex and that the promoter alone or in conjunction with the E2F sites in the first intron are not sufficient to confer cell cycle-dependent regulation. Additional regions inside the gene and perhaps additional surrounding sequences are needed for proper regulation of the *hPCNA* gene.

We show that, in the absence or in the presence of HPV-18 URR-E7, the activity from the 1.2-kb long, well-characterized promoter hPCNAp, 5’ to the coding region, differed from that of the endogenous gene both in proliferating and in differentiated cells of raft cultures. Although hPCNAp is active in proliferating cells in submerged cultures (Fig. 4B), it is completely inactive in the basal and parabasal proliferating strata of the raft culture. Rather, it is active in the postmitotic, differentiated strata in the absence of E7 (Fig. 4A), and fails to respond to further E7 transactivation (Fig. 4C). These patterns of expression are in total contrast to the endogenous *hPCNA* (Figs. 1A, 1D and 4C), demonstrating that the promoter region alone is not sufficient to
confer cell cycle-dependent regulation to the gene. It was previously suggested that sequences outside the promoter are required for proper growth-dependent regulation of the gene in serum-deprived cells (72).

Unexpectedly, hPCNAp was repressed by E7 and E1A in mouse L cells and CHO cells that do not harbor HPV (Fig. 3). Our observation was in agreement with a repression by adenovirus E1A in CREF and BRK cells (22). The repression was attributed to the combined presence of E1A and p53 in the same cell and their interaction with CBP (22,73). However, no definitive interaction between E7 and CBP has been described to date (74). In fact, HPV E6 but not E7 can modify the properties of CBP (75,76). In contrast, the expression of E7 does provoke an increase in the levels of p53 (40,77-79). One of the main functions of p53 is to inhibit cell proliferation upon DNA damage. In this regard, high concentrations of p53 are able to inhibit the expression of hPCNA via a p53 binding site in the promoter (80,81). We suspect that the repression of hPCNAp by E7 is also mediated via p53. In this regard, the H2P and dDLLC mutations, which do not induce the p53 protein (data not shown but see 77), repressed hPCNAp less effectively (Fig. 3B).

In contrast to the observations made with the conventional 1.2-kb hPCNA promoter (Fig. 2A), we have shown that, in submerged cultures, both E7 and E1A can activate an extended hPCNA promoter, hPCNAep, which additionally includes contiguous downstream sequence up to 85 bp into exon 2 (Fig. 2B). Site-directed mutagenesis demonstrates that this promoter transactivation is mediated at least in part via two E2F binding sites located in the first intron at positions +546 to +556 and +579 to +590 (11,23) (Fig. 5C). Consistent with this observation, E7 dDLLC mutation, which contains a deletion of the pRb-binding motif in CR2, fails to induce this promoter (Fig. 5B). Previously, we have shown that this mutation does not bind pRb or p107 in
vitro and does not induce PCNA protein in the differentiated strata of raft cultures (39). Thus, we conclude that \textit{hPCNAep} transactivation by E7 requires E2F sites in intron 1, in agreement with the known role of E2F transcription factors. We also infer that the repression attributed to E7-induced p53 is overcome by the transactivation mediated by the E2F sites. We note that, to date, \textit{hPCNA} is the only case among the conserved \textit{PCNA} genes (human, mouse, \textit{Drosophila melanogaster}, and rice) (82,83) in which putative E2F binding sites are located not in the 5’ promoter but in an intron. Also important, we suggest that the induction of \textit{hPCNAep and myc-PCNA} gene (see below) by E7 is not equivalent to the transactivation of the conventional \textit{hPCNA} promoter by adenovirus E1A in HeLa cells. HeLa cells constitutively express HPV-18 E6 and E7 oncogenes (17,20,84). E1A and E7 share functional and sequence similarities in CR1 and CR2 (74). As does HPV E7, E1A targets the pRb-E2F pathway and promotes S phase re-entry in postmitotic differentiated myotubes (85). It would seem that the transactivation of the conventional \textit{hPCNA} promoter without the first intron by E1A in HeLa is mediated by mechanisms beyond the cell-cycle dependent regulation.

Our analysis also revealed that CR1 of E7 is implicated in this induction, as well. The H2P mutation, which has an intact pRb binding motif and binds the pRb pocket domain in vitro (Fig. 1C), and is able to transactivate the extended promoter construct in submerged cell cultures (Fig. 5B). But it does not fully activate the endogenous gene in raft cultures (Fig. 1D). The reduced transactivation activity in vivo could be due to reduced protein stability in the differentiated keratinocytes but, alternatively, might be construed to implicate additional transcription factors besides E2F. Indeed, a number of E7 mutations that cannot be phosphorylated by casein kinase II (CKII) but are fully capable of binding pRb and p107, do not induce PCNA in postmitotic, differentiated keratinocytes in raft cultures (39). These mutations also induced the extended
promoter in CHO cells much less efficiently (data not shown). In contrast, the CKII recognition site mutation, which is no longer phosphorylated, is fully capable of inducing the transcription of the DNA pol α p180, a gene regulated by E2F (65). Whether the CKII mutations and the H2P mutation affect the same pathway remains to be investigated.

The most intriguing aspect of our study is that the extended hPCNA promoter containing the intron 1 with E2F sites still fails to behave in manners similar to those of the endogenous gene in raft cultures (Fig. 4D). Qualitatively, hPCNAep is regulated as hPCNAP is (Fig. 4A and D). Thus, the presence of E2F binding sites is not sufficient for activation in basal and parabasal cells, or for repression in postmitotic, differentiated cells. We do not think the presence of the LTR in our retroviral or lentivirus vector constructs has much bearing on these results, because the similarly transduced K14 promoter, the involucrin promoter (Fig. 4A), and the HPV URR promoter (38,55) were all regulated properly as in vivo. Furthermore, E7 activates the E2F-controlled adenovirus E2a promoter in raft cultures (our unpublished observations). Collectively, our results suggest that neither of the two hPCNA promoter constructs is sufficient to confer cell cycle-dependent regulation and that additional factors beyond pRB/E2F are also involved, as also concluded previously based on studies of E7 mutations in raft cultures (39).

Only when the full-length gene was used was the native cell cycle-dependent expression pattern largely restored, especially the induction by E7 (Fig. 6C). Thus, additional important regulatory sequences must reside within the gene outside the promoter and the first intron. A cryptic promoter was previously identified in intron 4 of the gene (86); its presence seems to be required for the proper regulation of PCNA mRNA in G0 cells (8,87). In addition, an antisense transcript, 500 to 600 bases in length with an unknown function, was reported to be constitutively synthesized from a promoter located within the first intron (11). Post-
transcriptional regulation was proposed in each of these cases, although our in situ analyses revealed no such evidence in postmitotic cells (Fig. 1). However, despite the induction of myc-hPCNA transcription by E7 in post-mitotic cells, as does the endogenous hPCNA, we note that transcription of myc-hPCNA is not entirely shut off in the post-mitotic cells, as does the endogenous gene in the absence of E7 (compare Fig 1A and 6C). This leakiness might suggest that sequences outside the cloned gene examined in this study could be additionally required in fine-tuning the cell cycle-dependent regulation of the hPCNA gene. A well known example of transcriptional control from a distance is the β-globin genes for which the locus control region many kilobase pairs upstream of the family of genes determines the timing and levels of their expression (88).

In summary, our findings highlight how the use of a culture system that closely resembles native tissues can reveal unanticipated complexities in the regulation of cellular gene expression. The requirement for certain cell-cycle related transcription factors might not be evident in actively dividing cells in submerged cultures. In the case of the human PCNA gene, at least part of the cell cycle-dependent signals appears to reside outside the extensively investigated promoter region. Intron 1, which contains two E2F consensus sites, and additional sequences within or even beyond the cloned gene are also important.

Acknowledgments

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Figure Legends

Figure 1. Expression of PCNA in raft cultures. A. Dark field images of in situ hybridization with sense-strand (left panels) or antisense-strand (right panels) $^{35}$S-labeled PCNA RNA probes on 4 µm sections of raft cultures transduced with vector-only virus (pLC) or HPV-18 URR E7-containing virus (E7). Arrows point at the stratum basale. B. Total protein extracts from COS-7 cells transfected with pMTX-based expression plasmids for wild type HPV-18 E7, empty vector, or HPV-18 E7 H2P were immunoblotted with an HPV18-E7 polyclonal antibody. Fifty micrograms of total cell extracts in RIPA buffer were loaded per lane. C. 250 µg of total extracts from transfected COS-7 cells were precipitated with GST-Rb pocket domain immobilized onto Sepharose beads, and immunoblotted with E7 polyclonal antibody. The lanes labeled ‘input’ were 20 µg of total cell extract as a control. D. Immunohistochemistry with antibodies to BrdU and PCNA to reveal DNA synthesis and PCNA protein, respectively. Raft cultures were transduced with the vector-only virus (pLC), a retrovirus containing HPV-18 URR-E7 (E7), or a E7 point mutation, H2P. The positive signal is red and nuclear. The histology of the tissues was revealed by counterstaining with hematoxylin.

Figure 2. Schematics of the reporter constructs containing the $h$PCNA promoter or $h$PCNA gene. A. The 1.2-kb promoter (hPCNAp) (-1265 to +61) fused to either the LacZ or the chloramphenicol acetyl transferase (CAT) gene. B. The extended promoter (hPCNAep) (-1265 to +1173) spanning the conventional promoter, first exon, the first intron, and 85 bases of the second exon fused in frame with the LacZ gene. The two E2F binding sites located in the first intron are indicated. Small arrows indicate the approximate positions of primers used in
genomic DNA PCR to detect the presence or absence of intron 1 in proviruses in transduced keratinocytes. C. An SIN lentiviral vector containing myc-\(hPCNA\) in the opposite orientation. The myc-tagged full-length \(hPCNA\) gene (-1265 to +5198) spans the conventional promoter, all exons and introns, and the 3’ untranslated region, including the poly A conding sequence. The myc tag is inserted in exon 1 near the amino terminus of the protein. \(hPCNA\) exons are shown as boxes with roman numerals. Sequence elements in the vector include RRE: rev recognition element; CTS: central termination sequence; WPRE: woodchuck hepatitis virus postregulatory element; PPT: central polypurine tract. The representations are not to scale.

**Figure 3.** HPV-18 E7 and adenovirus E1A proteins repress the \(hPCNA\) promoter in submerged cell cultures. **A.** CAT reporter assays in CHO cells. Ten \(\mu\)g of the plasmid containing the \(hPCNA\) promoter driving the reporter gene was co-transfected into CHO cells with 2 \(\mu\)g of pMTX or pMTX-18E7. **B.** CAT reporter assays in L cells for hPCNAp and the promoter of the human DNA polymerase \(\alpha\) p180 subunit. Ten \(\mu\)g of reporter plasmid were separately co-transfected into cells with 2 \(\mu\)g of pMTX or MTX-based expression vectors of HPV-18 E7, E7 dDLLC, E7 H2P, or adenovirus E1A. Relative CAT activities were determined 48 hours post transfection. The experiments were performed in triplicates, and error bars represent standard deviations.

**Figure 4.** The transduced \(hPCNA\) promoter or the extended promoter are not regulated in a cell cycle-dependent manner in raft cultures. **A.** Recombinant retroviruses expressing the \(LacZ\) reporter gene from the \(hPCNA\) promoter (hPCNAp), the keratin 14 promoter (K14), the involucrin promoter (inv), or a promoterless retrovirus (pLN-LacZ) were used to infect PHKs and raft cultures were prepared. The cultures were stained with X-gal prior to embedding in
paraffin. Four-μm sections were counterstained with eosin. B. PHKs infected with a retrovirus expressing the *LacZ* reporter gene from the hPCNAp, or a promoterless retrovirus (pLN-LacZ) were grown in submerged cultures, fixed, stained with X-gal, and lightly counterstained with hematoxylin. C. PHKs infected with pLN-hPCNAp-LacZ from part A were super-infected with an empty retrovirus (pBabe) or a retrovirus containing HPV-18 URR-E7 (pBabe-URR-E7). The cultures were stained with X-gal prior to paraffin embedding. Immunohistochemistry for hPCNA was performed on 4-μm sections. The signal is red and nuclear. Arrows point at the basal stratum. D. The extended *hPCNA* promoter containing the first intron is not sufficient to confer a cell cycle-dependent regulation. Raft cultures transduced with lentivirus containing hPCNAep:LacZ were stained with X-gal, and sections were counterstained with eosin. Arrows point at the basal stratum. E. Genomic DNA PCR of hPCNAep:LacZ lentivirus-transduced PHKs. Primers flank the first intron of the reporter construct as shown by arrows in Fig. 2B. The amplification products were analyzed by agarose gel electrophoresis and revealed by ethidium bromide staining. Both intron-containing (a band of 1250-bp) and intronless (a band of 542-bp) proviruses are present (marked with arrows), but only in transduced cells. A nonspecific band (marked with ‘*’) was observed in both the uninfected control and transduced PHKs. U: uninfected PHKs; I: lentivirus-infected PHKs.

**Figure 5.** hPCNAep transactivation by HPV-18 E7 and E1A is mediated by E2F sites in the first intron and requires E7 CR2. A. Activation of hPCNAep in CHO cells by HPV-18 E7, and adenovirus E1A. 5 μg of a plasmid containing the hPCNAep:LacZ construct depicted in Fig. 2B was electroporated into CHO cells together with 0.5 μg, 1 μg, 5 μg or 10 μg of pcDNA3.1-E7, or 2 μg of pMTX-E1A, as well as 1 μg of a luciferase expressing vector as a control for transfection
efficiency. 48 hours post electroporation, cells were harvested and normalized βgal activities are shown. **B.** Similar to A, but 5 µg of pcDNA3.1-based expression vectors of HPV-18 E7, E7 H2P or E7 dDLLC were transfected. **C.** Similar to A, except the two E2F sites in the first intron of the hPCNAep were mutated in the reporter plasmid (E2F mt). 1 µg, 5 µg or 10 µg of pcDNA3.1-E7 or 2 µg of pMTX-E1A were cotransfected. Cotransfection with the wild type hPCNAep:LacZ alone was used as a reference. The experiments were performed in triplicates, and error bars represent standard deviations.

**Figure 6.** A myc-tagged hPCNA gene exhibits the cell cycle-dependent regulation. **A.** Expression of the myc:hPCNA fusion protein in 293 cells. Increasing amounts of pBS-myc-hPCNA were electroporated into 293 cells. Crude protein extracts were analyzed by SDS-10% PAGE and western blotting. The blots were probed with antibodies specific for either hPCNA (top panel) or the myc epitope (bottom panel). **B.** Expression of myc:hPCNA fusion protein in PHK rafts cultures. Increasing amounts of an SIN lentivirus carrying the myc-hPCNA construct were used to infect PHKs. An SIN lentivirus carrying the eGFP gene under the control of the CMV IE promoter was used as a control. Without selection, the PHKs were used to develop raft cultures and crude protein extracts were then analyzed as described in A. The top panel shows the detection of the myc:hPCNA fusion protein using a myc-epitope antibody, while the bottom panel shows the detection of the endogenous hPCNA as a control of loading. **C.** Expression of myc-hPCNA RNA by in situ hybridization. PHKs infected either with an SIN lentivirus containing CMV-eGFP or a virus containing myc-hPCNA as described in part B were superinfected with control vector MLV (pLC) (left panels) or MLV carrying the HPV-18 URR-E7 (right panels). After selection, the cells were used to develop raft cultures. Four-µm sections
were probed with a myc-specific biotinylated DNA probe. The signals were developed using deposition of tyramide conjugated with fluorescein (green). The slides were mounted in the presence of DAPI (blue) to reveal nuclei. GFP fluorescence was not detected due to inactivation by the high temperature paraffin embedding. Arrows point at the basal stratum. D. Percentage of cells positive for myc-hPCNA RNA in the basal or suprabasal compartments of raft cultures from PHKs infected with myc-hPCNA lentivirus followed by superinfection with the control (pLC) or E7 retrovirus. Between 500 and 700 cells from cultures shown in the bottom panels of part C were visually assessed for the presence of myc-hPCNA RNA. The averages and standard deviations of the percentage of positive cells from 6 different regions of the rafts are shown.
Noya et al., Figure 1
Noya et al. Figure 2
Noya et al. Figure 3
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Noya et al. Figure 5
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The promoter of the human proliferating cell nuclear antigen gene is not sufficient for cell cycle-dependent regulation in organotypic cultures of keratinocytes
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