was subcloned as a Hind III–Eco RI fragment into M13mp18 and M13mp19 and sequenced. The DNA sequencing result showed that the poly(A) tail of TK cDNA clone consists of 11 A's.
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Biosynthesis of Human Papillomavirus from a Continuous Cell Line Upon Epithelial Differentiation

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The study of the human pathogen papillomaviruses (HPVs) has been hampered by the inability to propagate the virus in tissue culture. The addition of 12-*O*-tetradecanoyl phorbol-13-acetate to the media of organotypic (raft) cultures increased expression of physiological markers of keratinocyte differentiation and concomitantly induced production of virions. Capsid production was detected in differentiated suprabasal cells. Virions approximately 54 nanometers in size were observed by electron microscopy in raft tissue cross sections in the suprabasal layers. Virions purified through isopycnic gradients were found to contain type 31b DNA and exhibited an icosahedral shape similar to that of papillomaviruses found in clinical samples.

Human papillomaviruses (HPVs) are important human pathogens associated with a variety of neoplasias. HPV types 16, 18, 31, 33, 35, and 51 have been associated with malignant lesions of the anogenital area, and types 6 and 11 are found in benign genital lesions (*1*, *2*). Study of the complete viral life cycle has been prevented by the lack of a cell culture system that will permit vegetative viral replication. Researchers have propagated HPVs in rodents by grafting infected tissue either under the renal capsule (*3*) or under the flank skin (*4*) of a nude mouse, but no reproducible permissive in vitro system has yet been described. This is probably a result of the evolution of a viral life cycle that is tightly coupled to the differentiation program of keratinocytes in which virion production is limited to differentiating suprabasal cells (*5*).

Organotypic (raft) cultures recreate important features, both morphological and physiological, of epithelial differentiation in vitro by raising the cells to an air-liquid interface. This has been accomplished by the recombination of epidermal cells with a collagen matrix maintained on rigid support (*6*). However, raft cultures have continued to be deficient in the most important aspect of papillomavirus research, the ability to propagate virions. One stage of productive infection, the differentiation-specific amplification of episomal viral DNA in the upper layers of the epithelium, has been achieved in raft cultures (*7*). This was done with a cell line (CIN-612) derived from a cervical intraepithelial neoplasia type 1 (CIN 1) lesion that maintains episomal copies of HPV type 31b DNA. No evidence of virion production was detected with this cell line. Although important for replication studies, the ultimate goal of complete vegetative viral replication in vitro was still lacking.

One explanation for this inability to propagate HPVs in culture was the failure of the raft system to faithfully duplicate all aspects of the differentiation program of epithelial cells to which viral production is closely linked (*8*, *9*). In normal keratinocytes, keratin 10 is expressed throughout the suprabasal layers of the differentiating epithelium (*9*, *10*), and filaggrin is generally restricted to the stratum granulosum of the differentiating epithelium (*11*). In raft cultures of CIN-612 cells, keratin 10 (Fig. 1C) was only weakly present in the intermediate layers, and filaggrin was only weakly expressed in the stratum corneum and occasionally in the upper part of the stratum granulosum (Fig. 1E). When 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) was added to the raft culture media of invasive cervical carcinoma cell lines (*12*), morphological differentiation was induced, as shown by cross sections of raft cultures (*13*). In TPA-treated CIN-612 rafts, keratin 10 was ex-

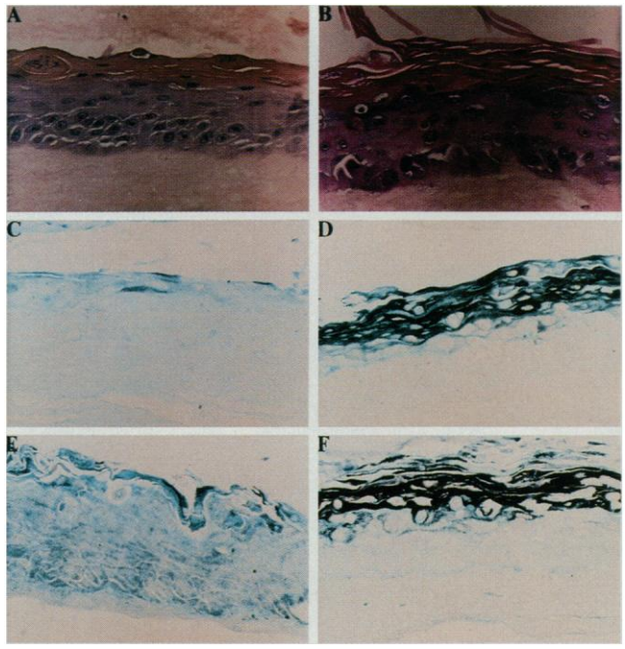

Fig. 1. Effect of TPA treatment on the expression of epithelial differentiation-specific markers in CIN 1–derived CIN-612 raft cultures. We examined the expression of keratin 10 and filaggrin by immunostaining thin sections of paraformaldehyde-fixed, paraffin-embedded raft tissue with the VECTASTAIN Elite ABC Kit (Vector Laboratories). (**A** and **B**) CIN-612 raft cultures with haematoxylin and eosin staining. (**C** and **D**) CIN-612 raft cultures immunostained with a keratin 10–specific monoclonal antibody (Accurate Chemical & Scientific Corp.) as the primary antibody. (**E** and **F**) Immunostaining of CIN-612 raft cultures with a filaggrin-specific monoclonal antibody (Biomedical Technologies Inc.) as the primary antibody. In (A), (C), and (E), CIN-612 raft cultures were grown as described (*24*) without TPA in the media. In (B), (D), and (F), CIN-612 raft cultures were treated with 16 nM of TPA for 16 to 24 hours every fourth day for 16 days.

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pressed throughout most of the suprabasal layers, including the stratum corneum (Fig. 1D), and a strong expression of filaggrin was observed throughout the stratum granulosum and into the stratum corneum (Fig. 1F).

In vivo expression of viral late genes can be detected only in the highly differentiated suprabasal cells of infected tissue (*14*). Using antiserum to the HPV 16 L1 major capsid protein (*7*), which cross-reacts with the L1 major capsid protein of various HPV types (*7*), we screened for the production of the L1 major capsid protein in CIN-612 rafts grown with or without TPA treatment (Fig. 2). Untreated CIN-612 raft cultures showed no specific nuclear staining with the L1 antiserum (Fig. 2A), whereas specific nuclear staining was seen in the stratum granulosum and in the stratum corneum of TPA-treated CIN-612 raft cultures (Fig. 2B). Normal rabbit serum was used as a control and showed no evidence of staining (*15*). The specific nuclear staining observed in the TPA-treated CIN-612 raft cultures (Fig. 2B) is similar to the staining patterns seen in biopsy tissue of HPV-associated CIN 1 (Fig. 2C). These results show that major late capsid protein production occurs concomitantly with the induction of keratin 10 and filaggrin synthesis in TPA-treated raft cultures. Similar results have been obtained in six separate experiments with CIN-612 cells at different passages and with different clonal cell lines.

Electron microscopy of cross sections of raft culture tissue revealed that nuclei in the upper portion of the stratum granulosum and the stratum corneum contained virion particles that were approximately 54 nm in size (Fig. 3). Virions were commonly observed in bilobulated koilocytic and dyskeratotic nuclei in which the nuclear chromatin had condensed at the nuclear envelope (Fig. 3A). At higher magnification (Fig. 3B), the particles could be seen superimposed on the heterochromatin, as is often seen in vivo, but these particles are not seen in the nuclei of untreated CIN-612 raft cultures. Virions approximately 54 nm in diameter were also prevalent in the stratum corneum of raft cultures (*15*). These observations are similar to those made for virions observed in clinical biopsy material from low-grade lesions (*16*), and similar results have been obtained with material from five separate experiments with different passages of CIN-612 cells.

Because infectious assays have not yet been developed for HPVs, we sought to de-

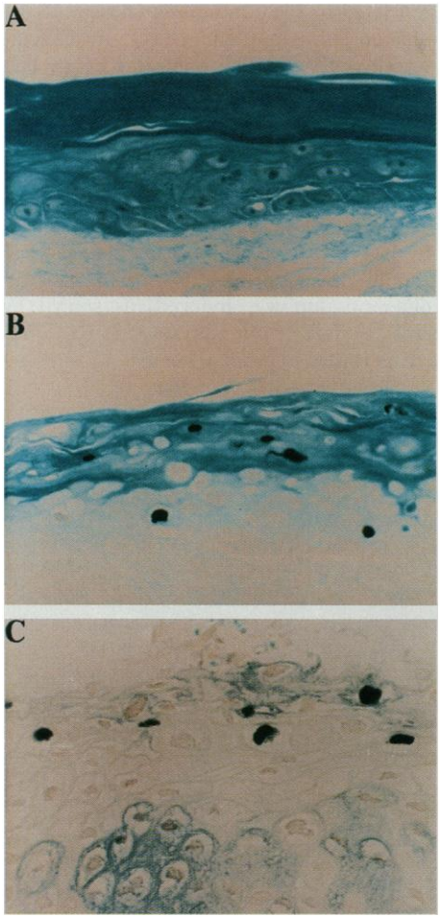

Fig. 2. Effect of TPA treatment (*11*) on the expression of the papillomavirus major late capsid protein, L1, in CIN 1–derived CIN-612 raft cultures. (**A**) No TPA. (**B**) After treatment with 16 nM TPA. (**C**) Tissue section from a clinical biopsy of a CIN 1 lesion. We examined CIN-612 raft cross sections by immunostaining with the VECTASTAIN Elite ABC Kit after treatment with antisera against L1.

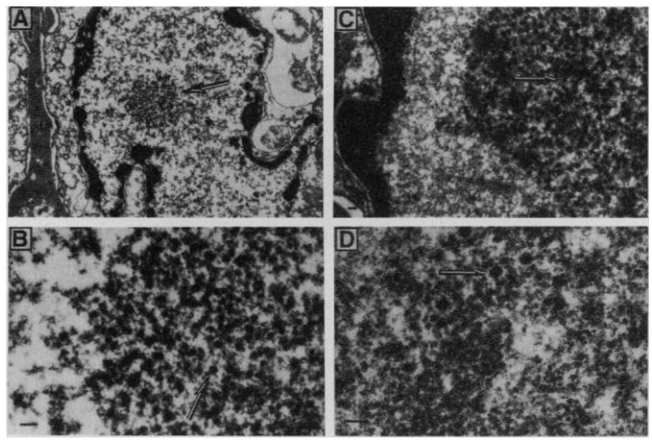

Fig. 3. Examination of TPA-treated CIN-612 raft cultures by electron microscopy for the presence of papillomavirus. Raft tissue cross sections were fixed with glutaraldehyde and stained with uranyl acetate. (**A**) A representative nucleus with patches of virion-containing material (arrow points to area containing numerous viral particles). (**B**) Enlargement of the same nucleus (arrow points to a representative viral particle). (**C**) A second example of a nucleus containing virions (arrow points to a representative viral particle). (**D**) Several viral particles approximately 54 nm in diameter within a third nucleus (arrow points to a representative viral particle. Bars in (B), (C), and (D) equal 100 nm.

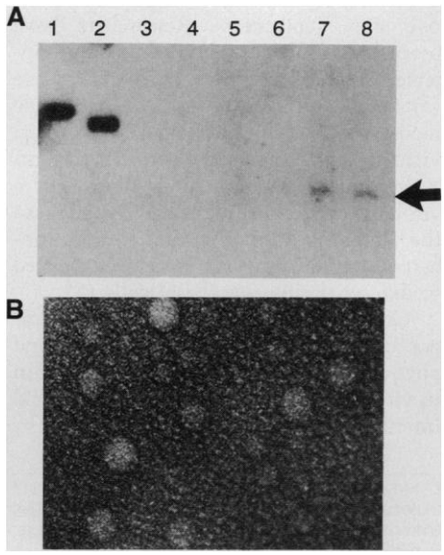

Fig. 4. Virions were isolated from eight TPA-treated raft cultures after a series of low- and high-speed centrifugations (*15*). (**A**) Southern blot. Lane 1, 15 million viral genome copies linearized (8 kb) from the pBR322 vector sequences; lane 2, 15 million viral genome copies supercoiled with pBR322 vector sequences; and lanes 3 through 8, series of fractions, with lane 3 representing the bottom of the gradient and viral DNA appearing in the middle of the gradient. Arrow indicates Form I (supercoiled) viral DNA. (**B**) Electron micrograph of numerous virions approximately 50 to 54 nm in diameter. Final purification was achieved by cesium chloride isopycnic centrifugation for 24 hours at 135,000*g*. Fractions from the bottom of the gradient through fractions determined positive for HPV 31b DNA by dot blot hybridization were dialyzed (*15*) and examined by Southern blot hybridization. One-tenth of the fractions were incubated at 50°C for 2 hours in the presence of 25 mM EDTA, 0.5% SDS, proteinase K (100 μg/ml), and carrier DNA. The samples were then extracted with phenol-chloroform, precipitated with ethanol, and run on a 0.7% agarose gel. The DNA was transferred to a nylon membrane with 0.4 N NaOH and probed with HPV 31b DNA followed by high-stringency washes. Positive fractions were stained with uranyl acetate and examined by electron microscopy.

termine if the structures we observed in nuclei of CIN-612 cells were indeed virions. Dot blot hybridization was performed on fractions from an isopycnic gradient purification (*17*) of HPV 31b virions produced in raft culture, and the presence of viral DNA was confirmed by Southern (DNA) blot hybridization (Fig. 4A). Lanes 6, 7, and 8 of Fig. 4A demonstrate the presence of HPV 31b DNA at low levels. From the copy number standards, we estimate the yield of viral particles to be at least 40 million per milliliter in lanes 7 and 8 (Fig. 4A). Fractions positive for HPV 31b DNA contained viral particles (Fig. 4B). The density gradient in fractions where virions were found was between 1.3 and 1.4 g/cm^3 as determined by weight. The presence of both HPV DNA and viral particles within the same fractions suggests that these are complete HPV virions, not empty capsids.

The ability to propagate papillomavirus in vitro is valuable not only for the understanding of the virus, but also for the eventual development of anti-viral treatments and the prevention of papillomavirus-induced lesions. These studies also establish the tight link between epithelial differentiation and HPV virion production. The ability of TPA to induce an increased expression of differentiation-specific markers suggests that a signal transduction pathway of epithelial differentiation has been identified. Virion production induced by phorbol esters has been described in other systems such as Epstein-Barr virus (*18*), Pichinde virus (*19*), Rift Valley fever virus (*20*), cytomegalovirus (*21*), and human immunodeficiency virus (*22*). The induction of the complete vegetative life cycle of HPVs in vitro requires both stratification at an air-liquid interface and protein kinase C (PKC) activation. In support of this hypothesis, the induction of viral particle biosynthesis by the addition of the synthetic diacylglycerol 1,2-dioctanoyl-*sn*-glycerol was observed (*23*). We believe that PKC activation of papillomavirus production is dependent on the induction of a more complex keratinocyte differentiation program and is not just a direct effect on capsid synthesis.

In this report, we described a system whereby latent infection of keratinocytes is converted into a productive infection leading to the formation of papillomavirus virions in culture. This tissue culture system will be useful in studies of the mechanisms whereby latency is maintained and terminated, and in the synthesis and assembly of papillomavirus virions.

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Rapamycin-Induced Inhibition of the 70-Kilodalton S6 Protein Kinase

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The immunosuppressant rapamycin inhibited proliferation of the H4IIEC hepatoma cell line. Rapamycin, but not its structural analog FK506, also inhibited the basal and insulin-stimulated activity of the p70 ribosomal protein S6 kinase. By contrast, insulin stimulation of the p85 Rsk S6 kinase and mitogen-activated protein (MAP) kinase activity were unaffected by drug. Rapamycin treatment of COS cells transfected with recombinant p70 S6 kinase completely inhibited the appearance of the hyperphosphorylated form of p70 S6 kinase concomitant with the inhibition of enzyme activity toward 40*S* subunits. Thus, rapamycin inhibits a signal transduction element that is necessary for the activation of p70 S6 kinase and mitogenesis but unnecessary for activation of p85 Rsk S6 kinase or MAP kinase.

Increased phosphorylation of multiple serine residues on the 40S ribosomal protein S6 numbers among the most rapid biochemical responses exhibited by cells stimulated with insulin or mitogens in vitro (*1*). Insulin or mitogen-stimulated S6 phosphorylation is catalyzed by one or both of two families of insulin or mitogen-activated S6 Ser-Thr protein kinases—the Rsk or p85 Rsk S6 kinases (*2*), and the p70 S6 kinases (*3*, *4*). Both families of S6 kinases are themselves regulated by Ser-Thr phosphorylation, although the immediate upstream regulators of the two S6 kinase families differ, at least in part. The *Xenopus* S6 kinase II, a p85 Rsk enzyme, is activated in vitro by phosphorylation with p42 MAP kinase (*5*). The MAP kinases are the dominant (perhaps only) immediate upstream activator of the p85 Rsk enzyme in situ in response to insulin or mitogens (*6*). Although the MAP kinases and cdc2 phosphorylate recombinant p70 S6 kinase in vitro in a putative regulatory

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