VIROLOGY 238, 40–52 (1997)
ARTICLE NO. VY978768

Characterization of Events during the Late Stages of HPV16 Infection *in Vivo* Using High-Affinity Synthetic Fabs to E4

John Doorbar,*,†,[1] Charmaine Foo,‡ Nick Coleman,‡ Liz Medcalf,‡ Oliver Hartley,§ Terence Prospero,§ Sawsan Napthine,‡ Jane Sterling,‡ Greg Winter,§ and Heather Griffin§

**National Institute for Medical Research, The Ridgeway, Mill Hill, London; ‡Department of Pathology, University of Cambridge, Cambridge; †Institute of Virology, University of Glasgow, Glasgow; and §MRC Centre for Protein Engineering, Hills Road, Cambridge, United Kingdom*

Received June 19, 1997; returned to author for revision July 7, 1997; accepted August 7, 1997

HPV late gene expression is initiated as an infected basal cell migrates through the differentiating layers of the epidermis, resulting in the onset of vegetative viral DNA replication and the expression of viral late proteins. We have used a large synthetic immunoglobulin library displayed on phage (diversity 6.5×10^{10} phage) to isolate three Fabs (TVG405, 406, and 407) which recognize distinct epitopes on the E4 late protein of HPV16. A C-terminal monoclonal (TVG404) was generated by hybridoma technology, and N-terminal polyclonal antiserum was prepared by peptide immunization (α N-term). The most potent antibody (TVG405) had an affinity for E4 of approximately 1.0 n*M*. All antibodies recognized the protein in paraffin-embedded archival material, allowing us to map events in the late stages of virus infection. Expression of E4 *in vivo* does not coincide with synthesis of the major virus coat protein L1, but precedes it by 1 or 2 cell layers in premalignant lesions caused by HPV16 and by up to 20 cell layers in HPV63-induced warts. In higher grade lesions associated with HPV16, E4 is produced in the absence of L1. By contrast, vegetative viral DNA replication and E4 expression correlate exactly and in some lesions begin as the infected epithelial cell leaves the basal layer. Differentiation markers such as filaggrin, loricrin, and certain keratins are not detectable in E4-positive cells, and nuclear degeneration is delayed. HPV16 E4 has a filamentous distribution in the lower epithelial layers, but associates with solitary perinuclear structures in more differentiated cells. Antibodies to the N-terminus of the protein stained these structures poorly. Our findings are compatible with a role for the HPV16 E4 protein in vegetative DNA replication or in modifying the phenotype of the infected cell to favor virus synthesis or virus release. The Fabs will be of value in the evaluation of model systems for mimicking HPV infection *in vitro*.

Key Words: HPV E4; late gene expression; vegetative viral DNA replication; epithelial differentiation; phage display; antibodies.

INTRODUCTION

Papillomaviruses (PVs) cause epithelial tumors in humans which vary in severity depending on the site of infection and the HPV type involved (Laimins, 1993; de Villiers, 1994). Low-risk types such as HPV 1 or HPV63 (Egawa *et al.,* 1993a,b) cause benign cutaneous warts which progress to malignancy only rarely, while high-risk viruses such as HPV 16 and HPV31 cause flat warts at mucosal sites and are associated with high-grade cervical intraepithelial neoplasia (CIN) and cancer (Schneider, 1994). Formation of an HPV-induced tumor is thought to require infection of an epithelial basal cell and the expression of viral early proteins in order to stimulate cell proliferation. The late stages of the virus life cycle, which ultimately lead to the production of infectious virions, are initiated only as the infected cell migrates through the upper differentiated layers of the epidermis. Viral and cellular events which influence HPV late gene expression are only poorly understood because until recently there has been no convenient system for mimicking productive infection *in vitro* (Laimins, 1993).

Studies on naturally occurring warts have revealed the virus to encode three late proteins—L1 and L2, which are virion coat proteins (Doorbar and Gallimore, 1987), and E1 ∧ E4, a nonstructural late protein of unknown function (Doorbar *et al.,* 1986). The abundance of E1 ∧ E4 in HPV1-induced warts (Breitburd *et al.,* 1987; Doorbar *et al.,* 1986) and the availability of well-characterized antibodies (Doorbar *et al.,* 1988) (Mabs) have allowed the protein to be extensively characterized *in vivo.* The HPV1 E1 ∧ E4 protein is first expressed in cells of the lower spinous layer and assembles into distinctive cytoplasmic and nuclear inclusions. During terminal differentiation it is posttranscriptionally modified by phosphorylation (Grand *et al.,* 1989) and by removal of sequences from the N-terminus (Doorbar *et al.,* 1988; Roberts *et al.,* 1994). By contrast, the E1 ∧ E4 proteins of high-risk viruses are poorly characterized, largely because many HPV16-induced lesions contain only small numbers of productively infected cells, which contain relatively low levels of E4 (Doorbar, 1996; Doorbar and Myers, 1996). A single Mab to HPV16 E1 ∧ E4 has been used to localize the protein to the cytoplasm (TVG 402) but has been reported not to work well on paraffin-embedded archival material (Doorbar *et al.,* 1992). Polyclonal antibody studies have yielded conflicting results.

[1] To whom correspondence and reprint requests should be addressed. Fax: +44 (0) 181 906 4477. E-mail: jdoorba@nimr.mrc.ac.uk.

0042-6822/97 $25.00

One study has supported the above findings (Crum *et al.,* 1990), while another has indicated that the protein is localized to the nucleus (Palefsky *et al.,* 1991), suggesting that different forms of the protein may have different distributions.

Here we have generated five new epitope-mapped antibodies to the HPV16 E4 protein using a combination of phage display technology, hybridoma production, and immunization of mice using synthetic peptides. Using these reagents we have characterized the E4 protein of HPV16 and its associated structures *in vivo* and have correlated its expression with events in the late stages of papillomavirus infection. These results show that expression of E4 coincides almost exactly with the onset of vegetative viral DNA replication but not with virus synthesis and that cells expressing E4 lack normal markers of terminal differentiation.

MATERIALS AND METHODS

Production of monoclonal and polyclonal antibodies by immunization

Monoclonal antibody TVG404 which recognized an epitope at the very C-terminus of E4 was prepared by standard hybridoma technology as described previously (Doorbar *et al.,* 1992). Antibodies to the N-terminus of the protein were raised against the synthetic peptide MADPAAATKYPLC after conjugation to thyroglobulin or keyhole limpet hemocyanin through its C-terminal cystein residue. Conjugation was carried out using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as described by Green *et al.* (1982). Polyclonal antibodies (to HPV16 and HPV63 E4 proteins) were prepared by immunization of rabbits with maltose-binding protein E4 fusion protein (MBP-E4). Antibody titers were monitored in ELISA using purified glutathione *S*-transferase E4 fusion protein (GST-E4).

Selection of synthetic Fabs by *in vitro* selection

Fabs were isolated from a synthetic antibody displayed on fd bacteriophage (Griffiths *et al.,* 1994) as described below. Immunotubes (Life Technologies, Paisley, UK) were coated overnight at 4° with either MBP-E4 or GST-E4 at a concentration of 0.1 μg/ml. These were subsequently blocked at 37° for 1 hr in PBS/2% marvel prior to incubation in the presence of 10^{11} phage on a blood tube rotator (37°). Unbound phage were poured off, and tubes were washed 10$\times$ with PBS/0.1% Tween 20. Binders were eluted with 100 m*M* triethylamine, pH 11.0 (1 ml), and immediately neutralized with 1 *M* Tris (pH 8.0) before being reintroduced into *Escherichia coli* TG1 cells. The enriched library was grown and the selection procedure repeated three more times. Phage displaying antibodies with affinity for E4 were identified by ELISA (against GST-E4, MBP-E4, GST, and MBP), and activity was confirmed by phage Western blotting. Immunoglobulin genes were transferred from the isolated phage into the bacterial expression vector pUC119.His.myc (Griffiths *et al.,* 1994), and soluble Fabs were purified from the periplasmic space of induced bacteria by nickel-NTA chromatography (Qiagen, Crawley, UK). Antibody titers were established by ELISA.

Epitope mapping of antibodies using overlapping peptides

The HPV16 E4 protein was synthesized as a series of 85 overlapping octomers (single amino acid overlap) by solid-phase fmoc chemistry using the SPOTS epitope mapping system (Genosys Biotechnologies, Cambridge, UK). Accuracy of synthesis was confirmed using the HPV16 E1 $\wedge$ E4 monoclonal TVG402 which binds the major antigenic site of the protein (Doorbar *et al.,* 1992). Filters were regenerated as described by the manufacturers and antibody binding was visualized by ECL (Amersham, Little Chalfont, UK). Polyclonal serum was used at 1/250 dilution, purified Fabs were used at approximately 1 μg/ml, and hybridoma supernatant was used at 1/10 dilution.

Measurement of antibody affinities by surface plasmon resonance

Affinities of synthetic (TVG405, TVG406, and TVG407), and hybridoma-derived Fabs (TVG402) were analyzed by surface plasmon resonance using a Biacore 2000 (Pharmacia Biosensor, St. Albans, UK) as described by the manufacturer. MBP-E4 aggregates were dissociated under reducing conditions in 0.5% SDS, 1 m*M* β-mercaptoethanol, 50 m*M* $Na_2CO_3/NaHCO_3$ (pH 8.5) and biotinylated using NHS-LC–biotin (Sigma, St. Louis, MO; 25 mg/ml in DMSO) at a biotin:protein molar ratio of 6:1 (Johnson *et al.,* 1991). Monomeric MBP-E4 was recovered by FPLC chromatography using a Superdex S200 HR10/30 column run in 6 *M* urea/1 m*M* β-mercaptoethanol/PBS/0.2 m*M* EDTA (pH 7.2), before being bound to a streptavidin-coated sensor chip and "refolded *in vitro*" in PBS/0.2 m*M* EDTA/0.1 mg/ml protease-free BSA (Sigma). Fabs were isolated from purified TVG402 using an Immunopure Fab kit (Pierce, Rockford, IL), and monomeric preparations were obtained by FPLC gel chromatography (Superdex S200 HR10/30 column run in PBS/0.2 m*M* EDTA (pH 7.2)). Sensor chip surfaces were regenerated using 6 *M* urea column buffer (described above). On and off rates were derived by nonlinear curve fitting using the proprietary BIAanalysis software.

Detection of proteins by immunofluorescence

Immunofluorescence was carried out on 5-μm sections of formalin-fixed paraffin-embedded tissue mounted on Apes-coated microscope slides (Addenbrookes Hospital, Cambridge, UK). Eight CIN1 and five CIN 2 lesions associated with HPV16 were examined. Three HPV1-associated lesions and two associated with HPV63

were investigated (one kindly provided by K. Egawa, Kumomoto Medical School, Japan). Antibodies were used at the concentrations described for epitope mapping (outlined above) and were detected using anti-mouse or anti-rabbit secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or Texas red (TR). Nuclei were counterstained with 0.001% 4′,6′-diamidino-2-phenylindole (DAPI). Synthetic Fabs were detected using mouse monoclonal 9E10, which recognizes the myc tag (present on the synthetic Fabs) (Boehringer Mannheim, Lewes, UK), reagents directed against the His tag (e.g., Ni-NTA peroxidase conjugate; Qiagen, Crawley, UK), or secondary antibodies to human light chains (e.g., anti-κ/λ light chain conjugates, The Binding Site, Birmingham, UK). For immunofluorescence studies on human tissue, monoclonal 9E10 was used as the second step followed by immunofluorescent detection with anti-mouse conjugate.

For double-staining, synthetic Fabs were purified by Ni-NTA affinity chromatography and directly labeled using fluorescein-EX succinimidyl ester or Texas red-X succinimidyl ester (Molecular Probes, Eugene, OR). TVG402 was purified from hybridoma supernatant by Protein A affinity chromatography and labeled in the same way. Antibodies to specific keratins (LHK1/LL017, keratin 1; LH2, keratin 10; 6B10, keratin 4; 2D7, keratin 13) and involucrin (Hudson *et al.,* 1992) were generously provided by Irene Leigh (Dept. Dermatology, Queen Mary and Westfield College, London). Filaggrin antibodies were obtained commercially (Biogenesis, Bournemouth, UK). L1 staining was carried out either using the Camvir 1 monoclonal which recognizes HPV16 L1 (McClean *et al.,* 1990) or using cross-reactive anti-BPV1 polyclonal serum (Dako Ltd., High Wycombe, USA). For double stains, the antibody requiring the secondary conjugate was used first. Fluorescent detection was completed by adding the secondary conjugate before the directly conjugated anti-E4 Fab was added. Immunofluorescence staining was enhanced by microwave treatment and was used in some instances. Sections were dewaxed as normal and microwaved on full power (750-W oven) in 500 ml of 0.01 *M* citrate buffer (pH 5.0) for 15 min. After cooling for 20–30 min sections were washed in PBS and blocked in 10% FCS prior to staining.

Detection of vegetative viral DNA replication by *in situ* hybridization

DNA *in situ* hybridization was carried out using HPV16 DNA which had been separated from the pBR322 cloning vector by *Bam*HI digestion and gel electrophoresis and purified using GeneClean (Anachem, Luton, UK). HPV18, HPV11, and HPV6 DNAs were released from their parental vector by digestion with *Eco*RI, *Bam*HI, and *Eco*RI, respectively, and used for initial typing and as controls. DNA was labeled by the random primer method using digoxigenin-labeled deoxyuridine-triphosphate. Hybridization was carried out according to the manufacturers' guidelines and detected using an anti-digoxigenin alkaline phosphatase conjugate using bromochloroindolyl phosphate/nitro blue tetrazolium as described by the manufacturers (Boehringer Mannheim). E4 staining was carried out on the developed slides using a mixture of anti-E4 reagents (TVG402, 405, 406 for HPV16 and 4.37 and 9.95 for HPV1) followed by the appropriate FITC conjugate.

RESULTS

Generation of epitope defined monoclonal and polyclonal antibodies by immunization *in vivo*

Although Mabs against HPV16 E1 ∧ E4 have been described previously ((TVG401, 402, 403) (Doorbar *et al.,* 1992)), these reagents recognize a single overlapping epitope at the major antigenic site of E4 and have been reported not to detect the protein in archival tissue biopsies (Doorbar *et al.,* 1992). Attempts to generate further Mabs by standard hybridoma technology resulted in the isolation of TVG 404, an IgM which recognized an epitope at the very C-terminus of the protein. To complement this reagent, polyclonal antiserum to the N-terminus of the protein was raised against an N-terminal synthetic peptide (α-E4 N term). Antibody specificities were confirmed by epitope mapping (see Materials and Methods), and the results are summarized in Fig. 1. Our difficulties in isolating high-affinity Mabs to epitopes outside the major antigenic region of E4 led us to use a synthetic immunoglobulin repertoire displayed on phage. Such libraries are not influenced by clonal deletion and anergy that result in self-tolerance *in vivo* and contain binding specificities not present in the natural B-cell repertoire.

In vitro generation of epitope mapped antibodies using a synthetic immunoglobulin repertoire displayed on phage

High-affinity antibodies (<10 n*M*) can be isolated directly from synthetic immunoglobulin repertoires of sufficiently large size and diversity (Griffiths *et al.,* 1994). Phage selections were carried out alternately against GST 16 E1 ∧ E4 and MBP 16 E1 ∧ E4 in order to prevent isolation of antibodies to MBP or GST protein, using a repertoire of 6.5×10^{10} Fabs (Griffiths *et al.,* 1994). Although MBP 16 E4 was expressed at higher levels (>50 mg/liter of bacteria) than the GST fusion (approx. 5 mg/liter of bacteria) antibody isolation requires as little as 1 μg of antigen (Hawkins *et al.,* 1992). After four rounds of selection, individual clones were examined for their ability to bind E1 ∧ E4, unfused GST or MBP, or bovine serum albumin (BSA). Forty-seven clones (of 100) were able to bind MBP 16 E1 ∧ E4, 39 of which could also bind GST 16 E4. None of these clones interacted with BSA, GST, or MBP. *Bst*NI fingerprinting (Marks *et al.,* 1992; Nissim *et al.,* 1994) revealed three distinct Fabs among these clones, and their immunoglobulin genes

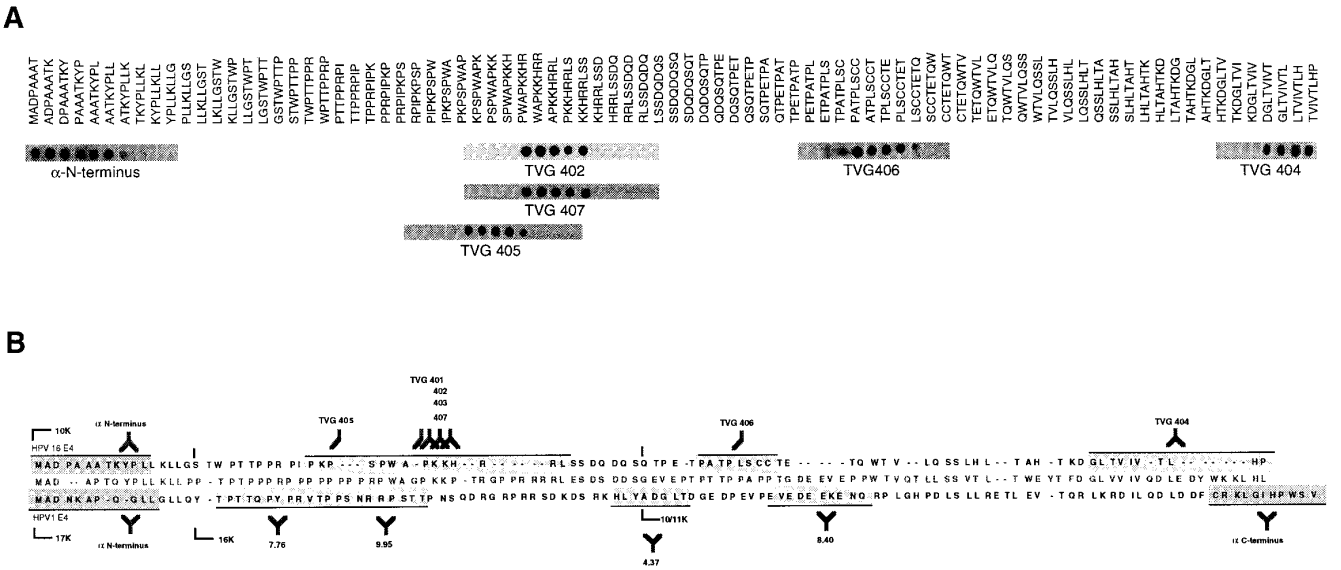

FIG. 1. Location of epitopes on the E1 ∧ E4 protein of HPV16. (A) The sequences of the 85 overlapping E4 synthetic peptides are shown at the top of the figure, and the results of the epitope mapping experiments are shown at the bottom. The dark spots represent binding of the antibody to the synthetic peptide shown above it. Only the portion of the filter containing peptides which reacted with each antibody are shown. (B) The location of epitopes on the E1 ∧ E4 amino acid sequence are summarized above the HPV16 sequence. Alignment with a consensus E4 sequence prepared by comparison of 70 putative E1 ∧ E4 sequences (Doorbar and Myers, 1996) is shown below the sequence of HPV16 E1 ∧ E4, and the sequence of the HPV1 E1 ∧ E4 protein is shown below this. The binding sites of the existing HPV1 E1 ∧ E4 Mabs (Doorbar *et al.,* 1988) are shown beneath the HPV1 sequence. The proteolytic cleavage sites that give rise to the 16K and 10/11K gene products in the E1 ∧ E4 protein of HPV1 (Doorbar *et al.,* 1988; Roberts *et al.,* 1994) are indicated below the HPV1 sequence, allowing prediction of putative sites in the E1 ∧ E4 sequence of HPV16.

were subcloned into the prokaryotic expression vector pUC119His.6myc to allow the production of soluble anti-E4 Fabs (Griffiths *et al.,* 1994). Approximately 5 mg (per liter of bacteria) of anti-E4 Fab (TVG 405, 406, and 407) could be extracted from the periplasmic space of induced bacteria and all were found to specifically detect E1 ∧ E4 by ELISA and Western blotting. Fab TVG 407 bound an epitope which was identical to that recognized by the hybridoma-derived Mab, TVG 402 (Fig. 1). The remaining synthetic Fabs recognized novel epitopes upstream (TVG 405) or downstream (TVG 407) of this major antigenic region of E4, and the results are summarized in Fig. 1.

The highest affinity antibodies to HPV16 E4 proteins were obtained by selection of the synthetic repertoire *in vitro*

Affinities of Fab fragments for MBP-E4 were measured by surface plasmon resonance using purified monomeric Fabs. Binding activities were on the order of 20% of total protein levels for the bacterially derived antibodies and 50% for Fabs derived from hybridoma culture supernatant. The affinities of TVG405 and TVG402 were calculated from on and off rates obtained by nonlinear curve fitting to sets of BIAcore binding curves (Figs. 2a and 2b). TVG405 had an association rate constant (k_{on}) of 1.8 $\times$ 10^6 $M^{-1} \cdot s^{-1}$ and an off rate (k_{off}) of 2 $\times$ 10^3 s^{-1}, indicating a molar dissociation constant (K_d) of approximately 1 n*M.* The best hybridoma-derived antibody — TVG402 — had an affinity of only 70 n*M* and had a k_{on} of 4.2 $\times$ 10^4 $M^{-1} \cdot s^{-1}$ and a k_{off} value of 3 $\times$ 10^3 s^{-1}. TVG 406 and 407 displayed rapid kinetics and were thus examined by Scatchard analysis of equilibrium binding data, as shown for TVG407 (Figs. 2c and 2d; see legend for further details). TVG407 had an affinity (K_d) of 50 n*M* after correction for biological activity, and TVG406 had an affinity (K_d) of 28 n*M* (data not shown). The amino acid sequences of the heavy and light chain CDR3 loops were established by DNA sequencing (data not shown), further confirming that the three antibodies were distinct.

E4 late gene expression precedes virus synthesis to varying extents in low- and high-grade mucosal lesions caused by HPV16 and in benign cutaneous lesions caused by HPV1 and HPV63

All the synthetic Fabs detected the HPV16 E1 ∧ E4 protein in formalin-fixed paraffin-embedded tissue, although TVG405 consistently showed the highest level of staining (Fig. 3). Epitope exposure by microwave treatment enhanced the sensitivity of E4 detection and even allowed staining using TVG402 (Doorbar *et al.,* 1992). The extent of E4 expression varied greatly between different lesions (eight HPV16-associated CIN1 biopsies were examined), ranging from expression only in rare cells scattered throughout the biopsy (Fig. 3) to widespread distribution throughout the most differentiated layers of the epidermis (Fig. 4) comparable to the distribution of E4 in cutaneous warts caused by HPV1 and HPV63 where the production of infectious virions is also high (Fig. 4). In low-grade cervical intraepithelial neoplasia (CIN 1) caused by HPV16, E4 and L1 levels were also found to correlate

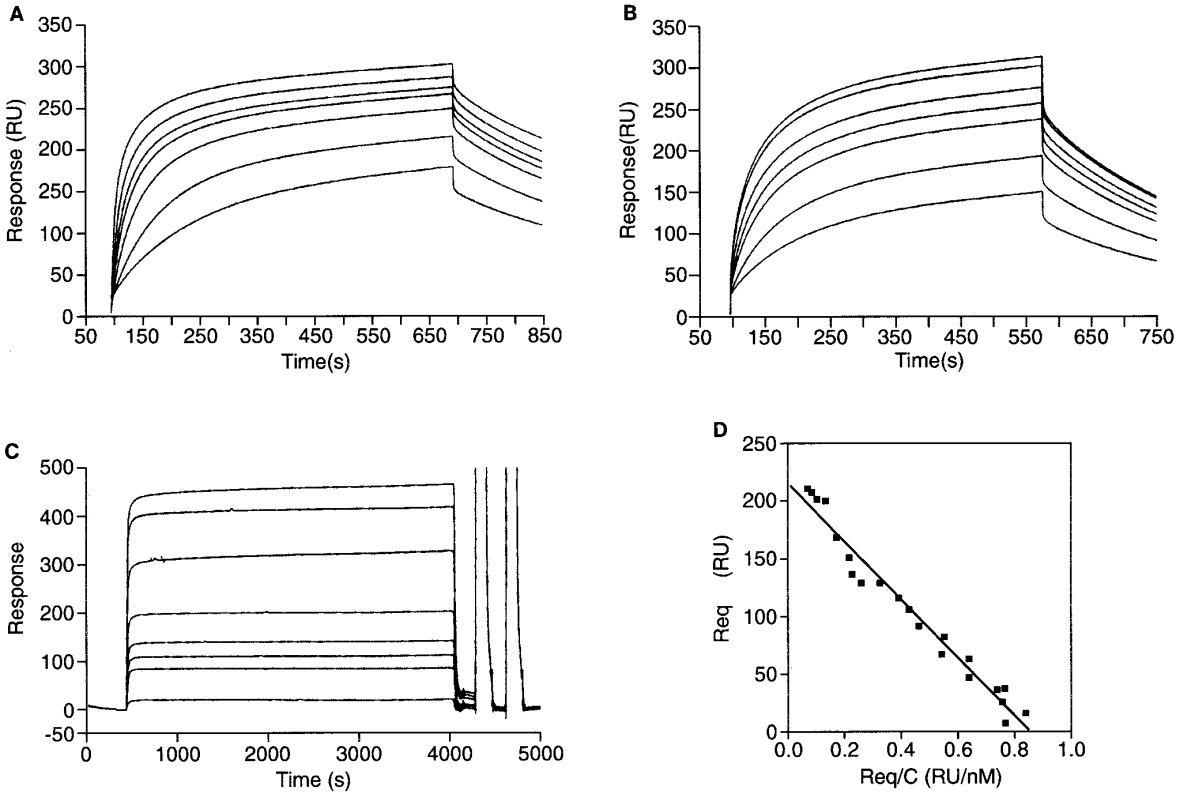

FIG. 2. Measurement of affinities by surface plasmon resonance. (A) Overlay of binding curves (sensograms) obtained after passing Fab TVG405 over a biacore chip coated with MBP–E4 fusion protein as described in the text. Fab concentrations ranged from 10 n*M* (lowest curve) to 300 n*M* (upper curve) through five intermediate dilutions. The extent of binding is indicated in resonance units on the *X* axis, against time in seconds on the *Y* axis. Purified Fab was injected at around 100 sec and washing initiated at 700 sec. The affinity (K_d) of TVG405 was calculated as between 0.3 and 1.25 n*M* by analysis of the association and dissociation curves using BIAevaluation software (Pharmacia, UK). (B) Overlay of binding curves (as described above) for the hybridoma-derived Fab TVG402 over a concentration range 100 n*M* to 1 μM. The K_d was estimated as 70 n*M*. (C) Equilibrium binding curve of Fab TVG407, which displayed rapid kinetics. (D) Scatchard analysis of the data presented in C using BIAevaluation software. Equilibrium values were corrected for bulk refractive index changes by subtracting values from a surface blocked with biotin, from the values shown in C. In the plot shown the slope is $-K_d$ and the *Y*-axis intercept is R_{max}, i.e., the binding level at saturation with Fab. The uncorrected K_d values for TVG407 and TVG406 were 250 and 140 n*M*, which, when the activity of the Fab preparation was considered, indicated actual affinities of 50 and 28 n*M*.

closely, although expression of the two proteins was not coincident (as previously suggested by Brown *et al.* (1994)). E4 expression preceded the synthesis of the major capsid protein by several cell layers (as revealed by double-staining, see Fig. 4) and in high-grade cervical lesions (CIN 2/CIN 3), E4 was often abundant, even though the expression of L1 was no longer supported (Fig. 4). This time delay between the commencement of E4 synthesis and the assembly of infectious virions was most apparent in HPV63, where E4 expression coincided with migration of an infected basal cell into the parabasal layers, while expression of L1 was restricted to a narrow strip of cells in the upper granular layer.

Onset of vegetative viral DNA replication and expression of E4 coincide exactly in mucosal tumors induced by HPV16, as well as in cutaneous lesions caused by HPVs 1 and 63

Vegetative viral DNA replication was found to begin in cells of the midspinous layer and to correlate exactly with the onset of E4 expression (Fig. 5). In HPV1-induced warts, vegetative viral DNA replication and E4 synthesis commence much earlier and are evident immediately after the infected basal cell migrates into the superficial layers (Fig. 5). Only a proportion of the differentiating cells were permissive for vegetative viral DNA replication, and only in these cells was E4 detectable. Neighboring cells showed neither late gene expression nor vegetative viral DNA replication, suggesting that onset of the two events is closely linked. Although the sensitivity of DNA and E4 detection was not established, these "normal" cells are likely to be either nonpermissive for viral replication or uninfected. This precise correlation between E4 expression and onset of vegetative viral DNA replication was also seen in cutaneous warts caused by HPV63 and 65 and in common warts caused by HPV2 (data not shown).

Cells undergoing late gene expression show an abnormal pattern of terminal differentiation compared to nonpermissive or uninfected cells

Cells supporting the late stages of HPV infection could thus be identified by immunostaining with Fab TVG405

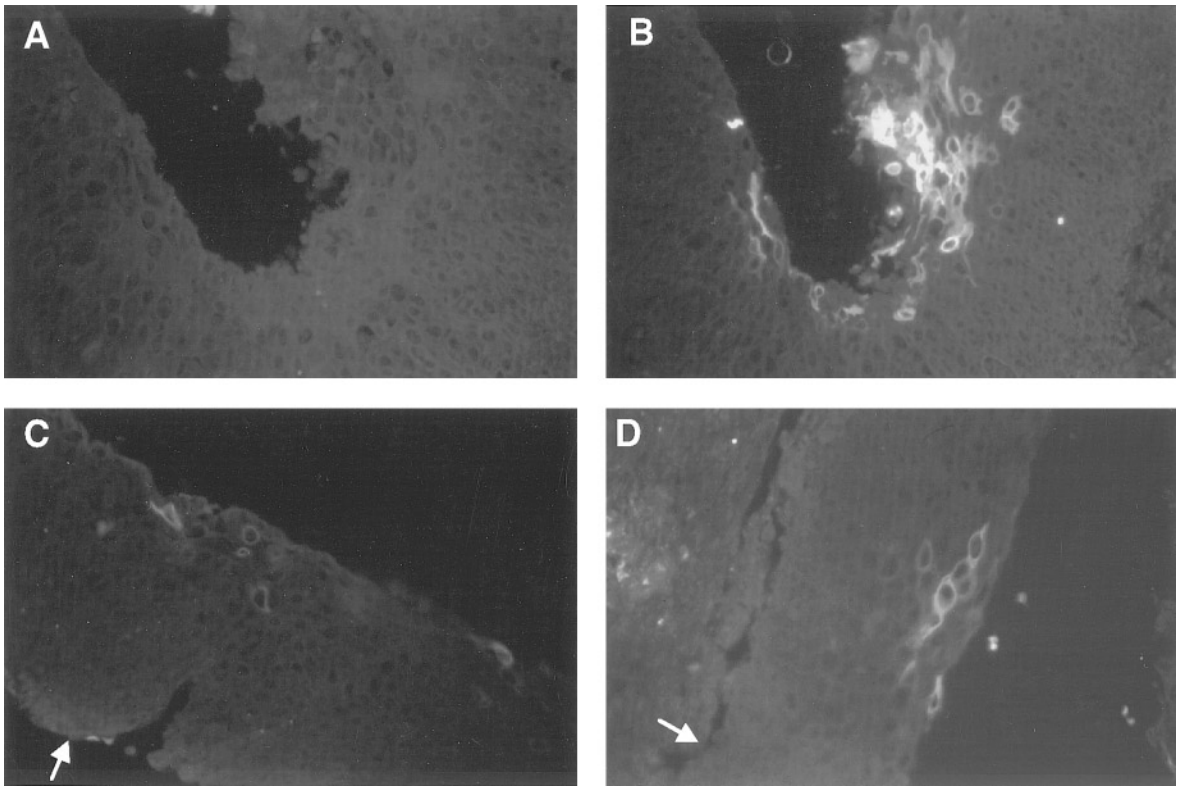

FIG. 3. Use of synthetic Fabs to localize HPV16 E4 protein *in vivo.* Immunostaining of a low-grade HPV16 CIN I with Fab NIP-C11 (Griffiths *et al.,* 1994), which has no reactivity toward HPV16 E4 (A), and the E4-specific Fab TVG405 which is described here (B, C, D). Fabs were detected using 9E10 as secondary antibody followed by anti-mouse FITC conjugate. E4 is detectable in the upper layers of the epidermis but at greatly varying levels between different lesions with often only a few positive cells being apparent (C, D). The position of the basal layer is arrowed in C and D. Magnification, 200×.

(for HPV16), Mab 4.37 (for HPV1), or polyclonal antisera to E4 (HPV63). In warts caused by HPV1, E4-positive cells lacked detectable levels of filaggrin or involucrin (Fig. 6i). Nonpermissive (or uninfected) cells in the same lesion, which showed neither E4 expression nor vegetative viral DNA replication, expressed filaggrin and loricrin at levels indistinguishable from those in the surrounding epidermis. Although we could not easily detect filaggrin in CIN biopsies using our available antibodies, correlation of E4 synthesis with the differentiation-specific keratins K4 and K13 revealed an identical pattern of inhibition. The intensity of K4 and K13 staining was always lower in E4-positive cells than in neighboring cells that were not expressing E4 (Fig. 6ii). K5 and 14, which are present in the basal and lower parabasal cells, were unaffected (data not shown). This interference with the detection or expression of differentiation-specific keratins (K1 and K10 in cutaneous skin) was also apparent in cutaneous warts caused by HPV1 (Fig. 6ii), but interestingly was not evident in warts caused by HPV63 (Fig. 6ii). The E4 protein of HPV63 is most closely related to that of HPV1.

The intracellular distribution of the HPV16 E4 proteins is distinct from the distribution of E4 in cutaneous lesions caused by HPV1 and HPV63

The E1 ∧ E4 protein of HPV1 is predominantly cytoplasmic and assembles into inclusions that coalesce and increase in size as the cell migrates toward the surface of the skin. The E1 ∧ E4 protein of HPV63 was found to have a fibrous and granular distribution. By contrast, HPV16 E4 had a filamentous and perinuclear distribution in cells of the lower epidermal layers (Fig. 7) and assembled into prominent structures only in the more differentiated cell layers. These "inclusions" were always found singly per cell (cf. multiple inclusions found in most cutaneous lesions), were located adjacent to the nucleus, and were nearly always detected on the side of the nucleus closest to the surface of the epidermis. Although similar in appearance to the E4/intermediate filament bundles which form after expression of the HPV16 E1 ∧ E4 protein in epithelial cells *in vitro,* we have not detected the presence of keratins in these structures *in vivo.* Antibodies to the very N-terminus of HPV16 E1 ∧ E4 stained the structures much less readily than antibodies to C-terminal epitopes (TVG 404, TVG405, TVG406), suggesting that the N-terminal region may be either hidden or lost. Confocal imaging revealed that the N-terminal antibodies localize primarily at the edge of the E4 structures while anti-C-terminal staining was strongest in the center (data not shown). When compared to the distribution seen with TVG405 and TVG406, the anti-N-terminal reagent revealed HPV16 E1 ∧ E4 to have a more diffuse distribution in the cell (Fig. 8). No significant difference

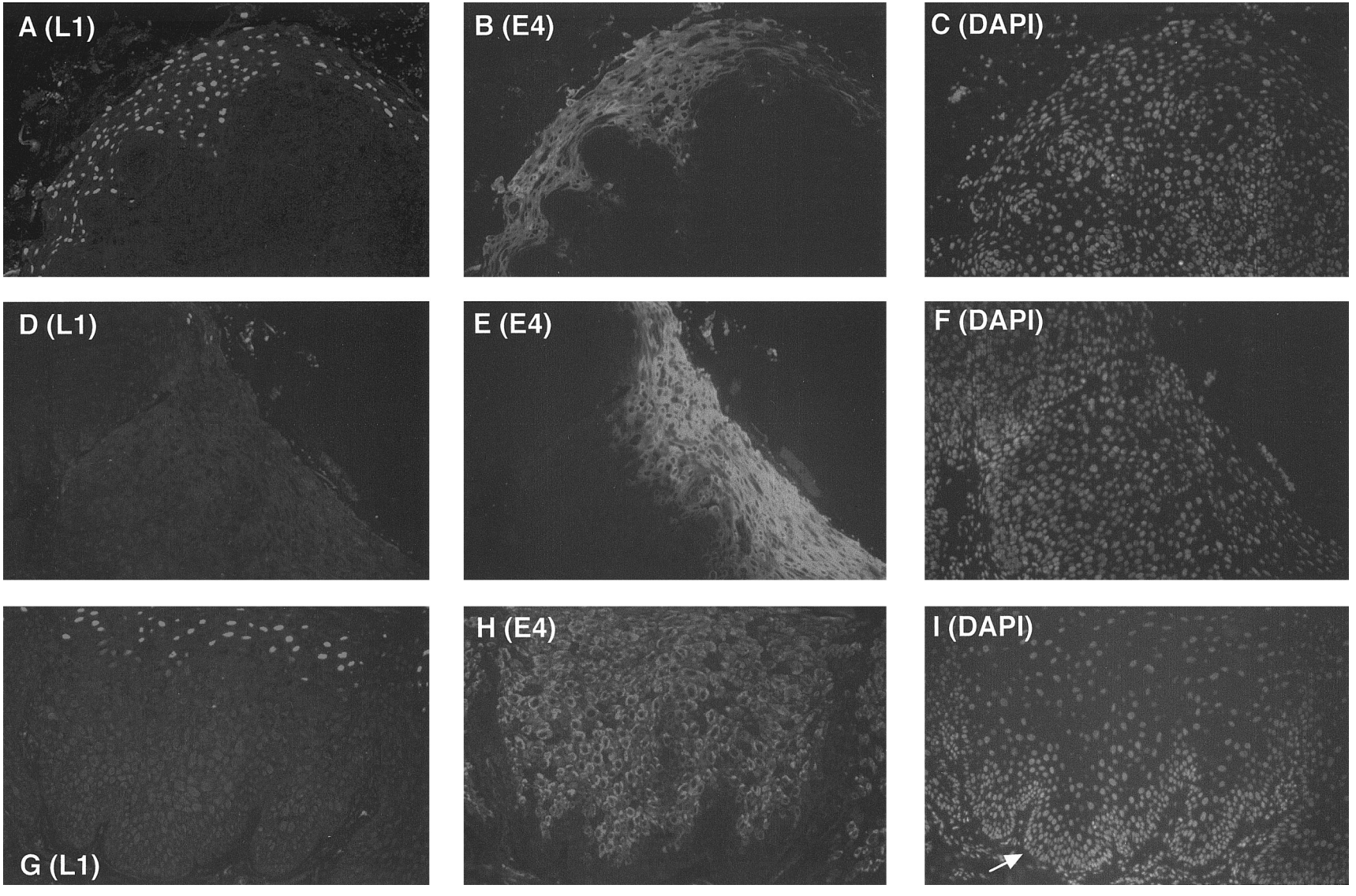

FIG. 4. Synthesis of E4 is not directly linked to the expression of capsid proteins in high- and low-grade HPV16 lesions and benign warts. Triple staining using anti-L1 antisera (A, D, G), HPV16 E4 Fab TVG405 (B, E) and polyclonal antisera to HPV63 E4 (H), and DAPI (C, F, I). A, B, and C represent a low-grade HPV16-induced lesion (CIN I). D, E, and F represent a high-grade HPV16-induced lesion (CIN II/III). G, H, and I represent a section through a verruca caused by HPV63. In all cases E4 expression precedes L1 expression although by only a few cell layers in CIN I (A, B). In the CIN II/III we assume that terminal differentiation is insufficient to support synthesis of virion structural proteins (D) although E4 expression is abundant (E). The contrast between the onset of E4 expression and the detection of virus structural proteins is most apparent in cutaneous verrucas caused by HPV63 (G, H). The basal layer is indicated by an arrow on the DAPI-stained images. Magnification, 100×.

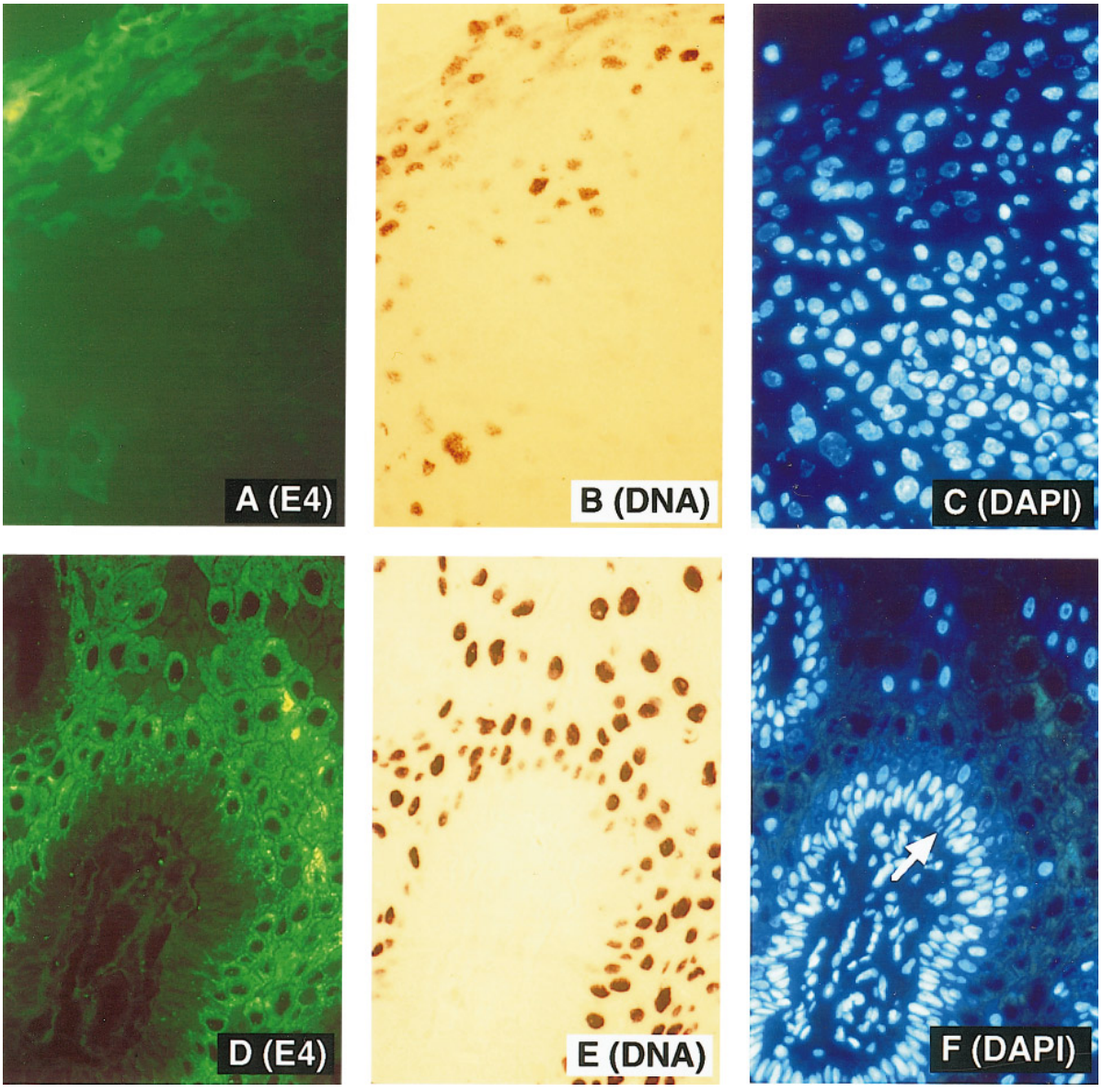

FIG. 5. Onset of vegetative viral DNA replication coincides with E4 expression in low-grade HPV16 lesions and in benign cutaneous warts. Triple staining using the HPV16 E4 antibodies TVG402, 405, and 406 (A) and HPV1 E4 antibodies 4.37 and 9.95 (D), biotinylated DNA probe (B, HPV16; E, HPV1), or DAPI (C and F). A, B, and C represent a section through a HPV16-induced CIN I, and D, E, and F represent a section through a HPV1-induced verucca. In the HPV16 CIN I, vegetative viral DNA replication and E4 synthesis correlate in the middle to upper layers of the epidermis (A, B). In cutaneous lesions the two events are initiated as soon as the infected cell leaves the basal layer (D, E). Basal cells are illustrated in the DAPI-counterstained image (F). Magnification, 200×.

was apparent among the staining patterns of TVG405, 406, 407, and the C-terminal antibody.

DISCUSSION

Papillomavirus late gene expression is initiated as the infected cell migrates through the differentiating layers of the epidermis. Here we have correlated events which lead to the production of HPV16 virions in premalignant cervical lesions and have compared these events with the process of virus production in benign cutaneous warts caused by HPV1 and 63. Three clear conclusions can be drawn from this work. First, E4 expression and the onset of vegetative viral DNA replication coincide almost exactly, not only in HPV16 and HPV1/HPV 63 lesions, but also in common warts caused by HPV2 and HPV57. Second, E4 expression and the production of virion structural proteins are not coincident. E4 expression precedes L1 synthesis, often by many cell layers, and in HPV16-associated CIN 2/3 lesions, E4 expression is abundant even though the infected cells are nonpermissive for virus production. And third, it is apparent that terminal differentiation is abnormal in cells supporting HPV16 and HPV1 late gene expression, suggesting that virion synthesis is not passively regulated during keratinocyte differentiation. In addition, the generation of

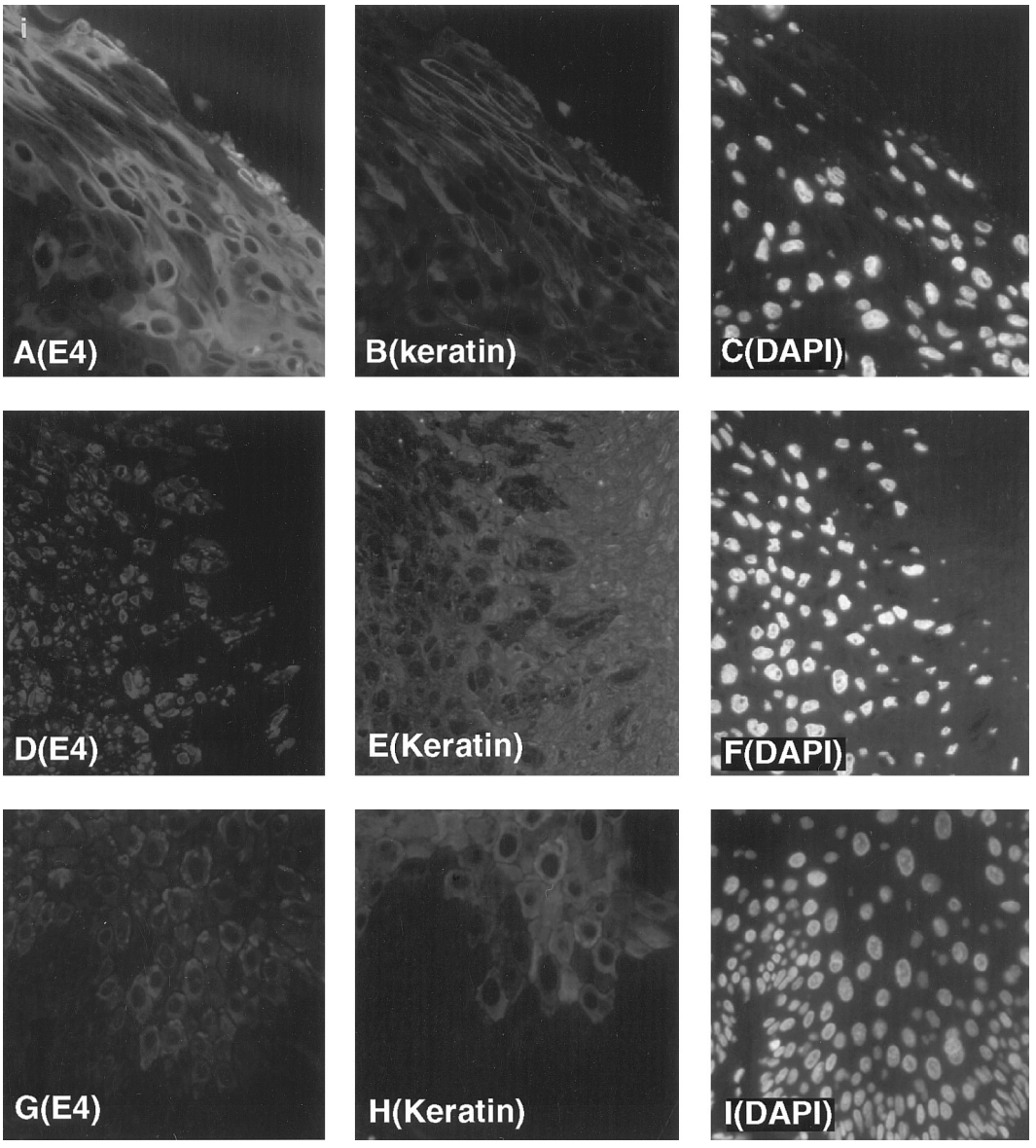

FIG. 6. Productive infection interferes with normal epithelial terminal differentiation in low-grade HPV16 lesions and in benign cutaneous warts. (i) Keratin expression. Triple staining using the HPV16 E4 Fabs TVG405/TVG406 (A), HPV 1 E4 monoclonals 4.37/9.95 (D), and HPV63 E4 polyclonal antibodies (B), in conjunction with antibodies to the differentiation-specific mucosal keratins 4 and 13 (B) or cutaneous keratins 1 and 10 (E, H). C, F, and I show the DAPI counterstain. A, B, and C represent a section through a HPV16-induced CIN I. D, E, and F show a section through the edge of a HPV1-induced verucca, while G, H, and I show a section through a HPV63-induced wart. In HPV16 and HPV1-induced lesions, differentiation-specific keratins are less apparent in E4-positive cells than in neighboring cells (A, B, D, E) although this was not the case with HPV63 (G, H). Nuclear degeneration (visualized by DAPI counterstaining) is retarded in E4-expressing cells (A, C, D, F). Magnification, 200×. (ii) Filaggrin expression. Triple staining, as described above, except that B and E show filaggrin staining. E4 staining is shown in A and D, and DAPI counterstaining is shown in C and F. A, B, and C represent the edge of a HPV63-induced wart where normal skin (left-hand side of the panel) meets the benign tumor (right-hand side of panel). D, E, and F show the granular layer of a HPV1-induced wart. E4-positive cells do not express detectable levels of the differentiation-specific marker filaggrin and show marked nuclear preservation when compared to neighboring uninfected or nonpermissive cells. Magnification, 200×.

high-affinity Fabs has allowed us to characterize HPV16 E4 and its associated structures *in vivo.*

E4 expression and the onset of vegetative viral DNA replication are among the earliest events that are detectable in the late stage of the HPV life cycle. It has been suggested that E4 may influence episomal copy number by sequestering cellular factors which inhibit vegetative viral DNA replication in cells of the basal layer (Breitburd *et al.,* 1987; Rogel-Gaillard *et al.,* 1992). Interestingly, vegetative viral DNA replication can be initiated in BPV-transformed fibroblasts by serum starvation, and in these cells high-level expression of E4 is apparent (Jareborg and Burnett, 1991). While these findings and ours are compatible with the suggested role for E4 in vegetative viral DNA replication, the converse may be true. In SV40 infection, cellular factors (IBPs) which repress the viral late pro-

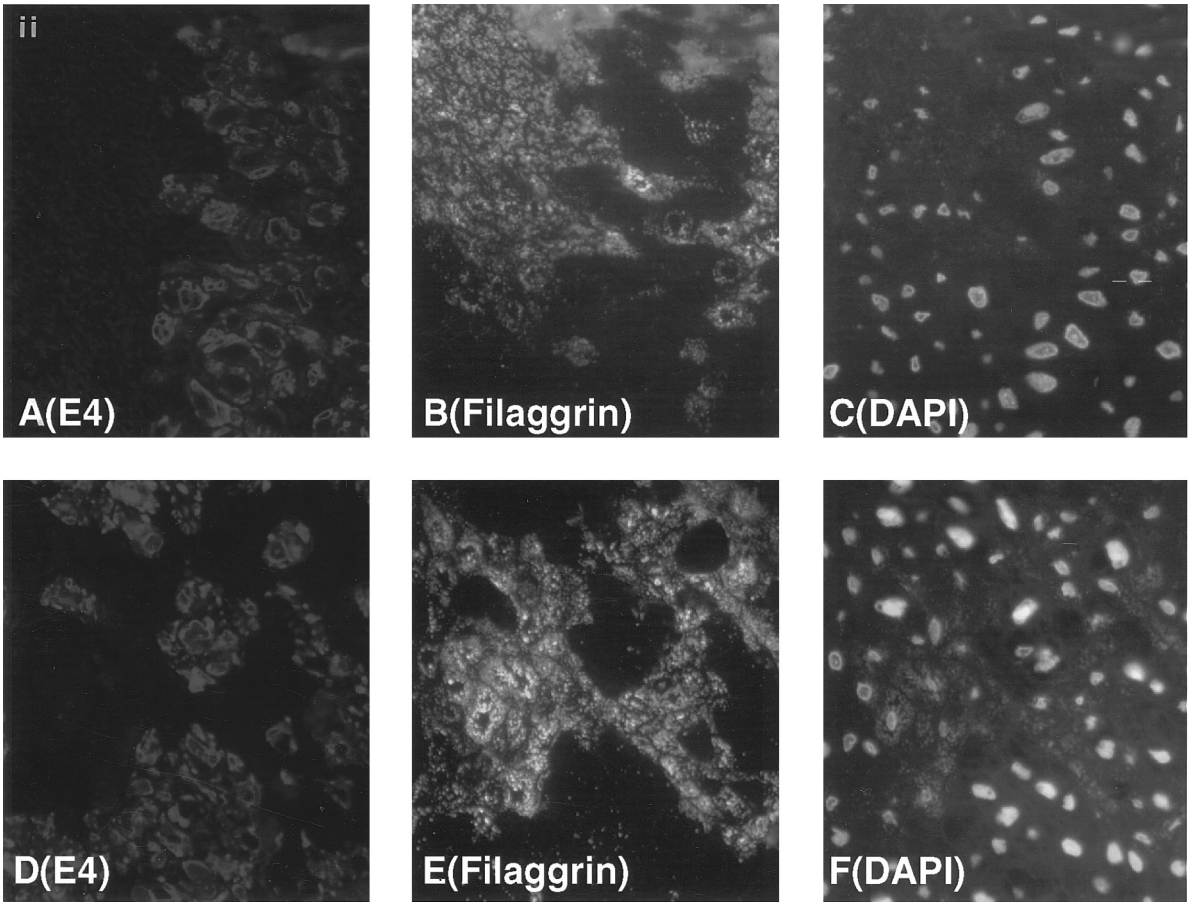

FIG. 6 — *Continued*

moter (Wiley *et al.,* 1993) are titrated out during vegetative viral DNA replication leading promoter activation and synthesis of late proteins, and it has recently been suggested that vegetative viral DNA replication may be a prerequisite for late gene expression in papillomaviruses (Frattini *et al.,* 1996). Although the molecular events which influence vegetative viral replication appear closely linked to those that regulate E4 expression, additional work will be needed to establish whether E4 stimulates DNA replication or vice versa.

The close correlation between E4 expression and the onset of DNA replication was in contrast to the variation observed between E4 expression and the production of virion coat proteins. Expression of E4 and L1 has previously been shown to occur in similar areas in lesions induced by HPV1, 2, 4, and 16 (Crum *et al.,* 1990; Doorbar *et al.,* 1989), and the two proteins have been reported to colocalize exactly in HPV11-infected foreskin implants grown in athymic mice (Brown *et al.,* 1994). In general, these earlier studies were carried out on serial tissue sections using polyclonal antisera, making it difficult to draw conclusions about E4 and L1 expression in individual cells. The double-staining approach used here has revealed that E4 expression and L1 expression are not coincident in low-grade HPV 16-induced lesions but rather that E4 expression precedes that of L1 as suggested by the work of Crum *et al.* (1990). In the most extreme case (a cutaneous wart caused by HPV63) at least 20 cell layers separated the two events. This argues against the assumption that E4 expression is regulated by the same factors that control expression of L1 (Brown *et al.,* 1994). Phorbol esters, which are potent activators of protein kinase C (PKC), have been shown to stimulate L1 synthesis in HPV31-containing cell lines grown in raft culture (Meyers *et al.,* 1992). E4 expression is unaffected by such activators of PKC, strengthening the conclusion that its regulation is distinct from that which controls capsid synthesis (Pray and Laimins, 1995).

The absence of filaggrin and involucrin in productively infected cells in warts induced by HPV1 and 63 and the down-regulation of differentiation-specific keratins in lesions caused by HPV16 and HPV1 suggest that HPVs do not depend on normal differentiation for completion of their life cycle (see also (Rogel-Gaillard *et al.,* 1993)). It has previously been suggested that E4 may associate with "death proteins," in order to inhibit differentiation and to allow virus production in cells that would otherwise be dying (Rogel-Gaillard *et al.,* 1992, 1993). Although our findings are compatible with such a hypothesis, recent evidence suggests that other viral proteins may be involved. Transfection of keratinocytes with E6-expressing plasmids leads to inhibition of calcium and serum-in-

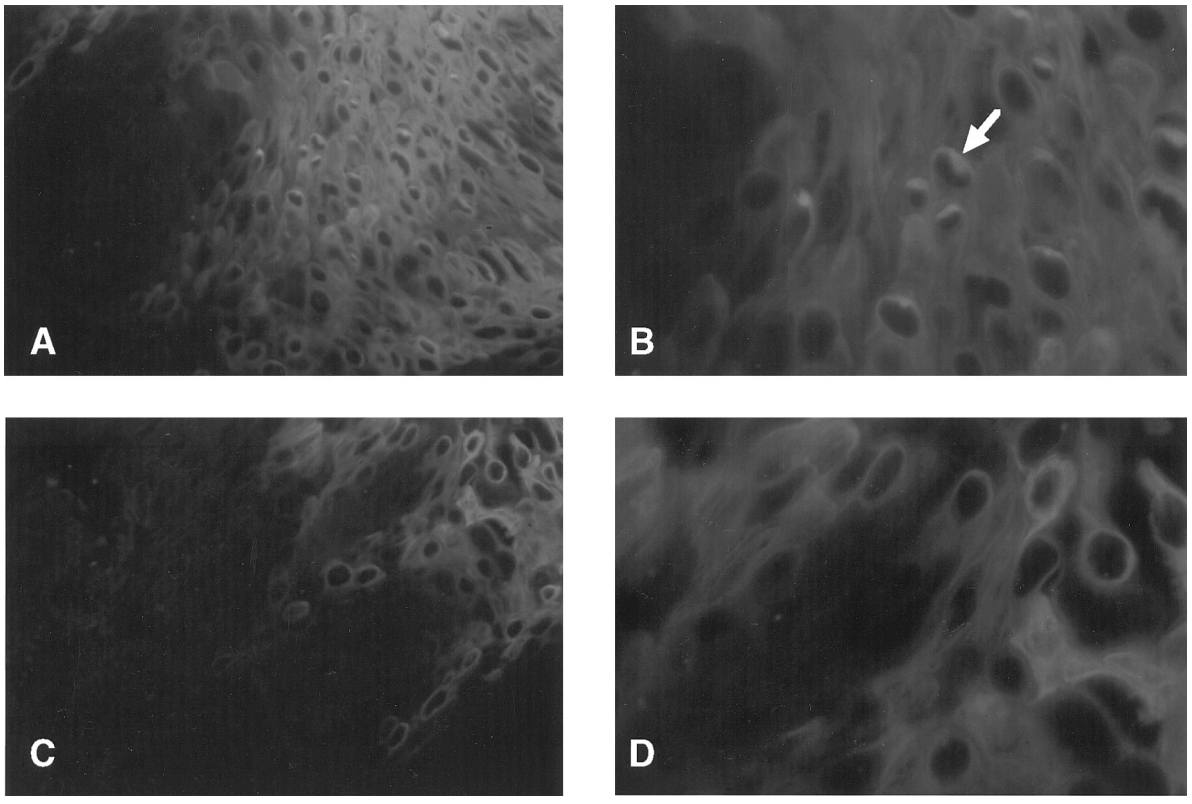

FIG. 7. Association of the HPV16 E4 proteins with perinuclear bundles and filamentous structures *in vivo.* Detection of HPV16 E4 proteins in the upper layers (A, B) and lower layers (C, D) of a HPV16 CIN I using a mixture of Fabs TVG405 and TVG406. In the upper layers E4 is diffuse throughout the cytoplasm but with a prominent perinuclear pattern. Concentration of E4 into perinuclear bundles (arrowed in B) is apparent in the these cells. In the lower layers, E4 has a predominantly perinuclear and filamentous appearance (C, D), but is not concentrated into perinuclear bundles. Magnification (A, C) 200×; (B, D) 400×.

duced terminal differentiation (Sherman and Schlegel, 1996; Stoppler *et al.,* 1996). This may be manifest *in vivo* as the loss of differentiation-specific antigens in cells expressing viral late proteins as observed here.

In the lower layers of naturally occurring lesions, the E4 protein of HPV16 was found to have a diffuse and filamentous appearance, while in higher layers, the protein had a perinuclear distribution and was concentrated on the side of the nucleus closest to the epithelial surface. Although this distribution is reminiscent of the collapsed E4/keratin bundles seen following expression of mucosal HPV E4 proteins *in vitro* (Doorbar *et al.,* 1991, 1996a; Pray and Laimins, 1995; Roberts *et al.,* 1993), these structures did not stain with anti-keratin antibodies

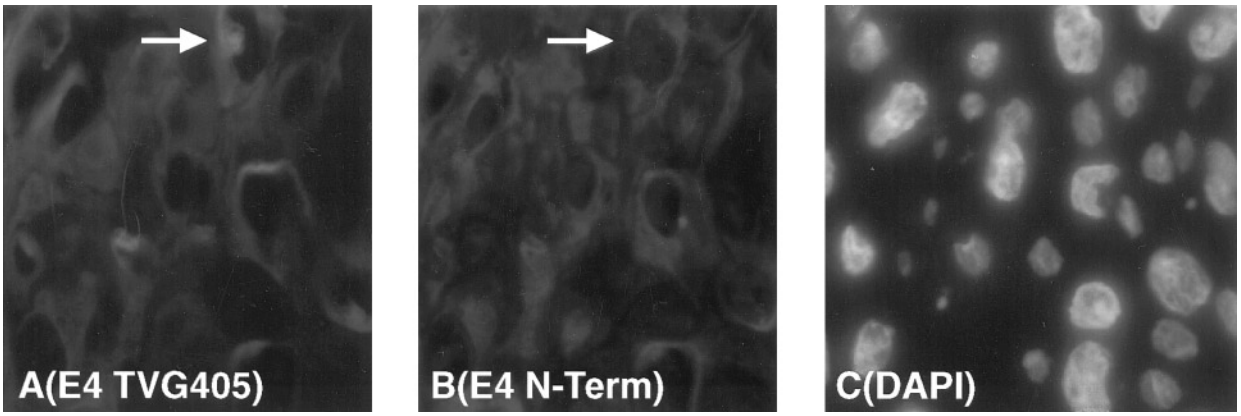

FIG. 8. Evidence for processing of the HPV16 E4 proteins *in vivo.* Triple staining in the upper layers of a HPV16 CIN using HPV16E4 Fab TVG406 which recognizes an epitope in the C-terminal half of the E4 protein (A), an antibody to the N-terminal 12 amino acids of the HPV16 E1 ∧ E4 protein (B), and DAPI (C). TVG402, 403, 404, 405, and 407 produced staining patterns that were not significantly different from that of TVG 406. Anti-N-terminal antibodies did not effectively stain the perinuclear bundles (B) which were evident with TVG406 (arrowed in A), suggesting that as in HPV1, different forms of the protein have different intracellular locations. Magnification, 400×.

and may be similar to the prominent cytoplasmic E4 inclusions found in HPV1 warts — which stain with keratin antibodies only at their periphery (Rogel-Gaillard *et al.,* 1993). The *in vivo* significance of E4s association with keratins is uncertain, although the filamentous staining pattern and preliminary double-staining experiments using anti-keratin antibodies (unpublished results) suggest that some degree of interaction may occur in the lower layers. Studies on the E4 protein of HPV 1 have also shown that only a fraction of the protein is filament-associated in naturally occurring lesions *in vivo* and that most exists as soluble cytoplasmic complexes (Doorbar *et al.,* 1996).

Antibodies to epitopes away from the N-terminus of the protein revealed the perinuclear structures more clearly than antibodies to the very N-terminus of the protein, which showed a diffuse staining pattern. At least 50% of the E4 protein extractable from HPV1-induced tumors is processed by cleavage at the N-terminus (Breitburd *et al.,* 1987; Doorbar *et al.,* 1986, 1988, 1996a), and it is likely that similar processing may also occur in HPV16 E4. The leucine cluster, which is present toward the N-termini of most E4 proteins (Doorbar and Myers, 1996), is important for the localization of E4 to intermediate filaments *in vitro* (Roberts *et al.,* 1994) and its loss may influence E4 location *in vivo.* N-terminal antibodies stained the very edge of these aggregates while C-terminal antibodies stained the center.

The direct isolation of Fabs with nanomolar affinities demonstrates the advantage of using large immunoglobulin repertoires for the generation of high-affinity reagents. Fab TVG405 compares well with antibodies produced from either animal or bacterial systems and is the best Fab yet reported from the lox-p library (Griffiths *et al.,* 1994). All of the Fab fragments had high k_{on} values, however, suggesting that an isolation strategy biased toward selection by the off rate may generate even higher affinity reagents. Despite the potential of this approach, few clinically useful antibodies have yet been generated by phage display. Pope *et al.* (1996) isolated an estradiol binder with affinity similar to that of TVG405, which may be useful in determining circulating steroid levels in human patients. The correlation between vegetative viral DNA replication and E4 expression, and the ease with which E4 can be detected in the superficial epithelial layers suggests a role for E4 Fabs in routine cervical smear testing. Current DNA-based techniques such as hybrid capture (Digene, USA) are expensive, time-consuming, and not ideally suited to large-scale screening. As the infecting virus type is an important indicator of disease prognosis, antibody-based detection would provide a cheaper and more rapid approach.

Our work has provided insight into the pattern of events which lead to the formation of HPV16 virions *in vivo.* Functional analysis of E4 will require further *in vitro* analysis and will be facilitated by recent developments in raft culture (Frattini *et al.,* 1996; Meyers *et al.,* 1992). The reagents and observations described here will be of value in these studies and should provide a standard to which the *in vitro* analysis can be compared.

ACKNOWLEDGMENTS

We thank Professor Tony Minson for his support during the course of this work which was carried out largely in the Division of Virology at Cambridge. The project was funded by the Medical Research Council and by the Royal Society. J.D. is a Royal Society University Research Fellow.

REFERENCES

Beyer-Finkler, E., Stoler, M. H., Giradi, F., and Pfister, H. (1990). Cell-differentiation-related gene expression of human papillomavirus 33. *Med. Microbiol. Immunol.* 179, 185–192.

Breitburd, F., Croissant, O., and Orth, G. (1987). Expression of human papillomavirus type-1 E4 gene products in warts. *In* "Cancer Cells 5" (B. M. Steinberg, J. L. Brandsma, and L. B. Taichman, Eds.), pp. 115–122. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Brown, D. R., Fan, L., Jones, J., and Bryan, J. (1994). Colocalization of human papillomavirus type 11 E1 ∧ E4 and L1 proteins in human foreskin implants grown in athymic mice. *Virology* 201, 46–54.

Chow, L. T., and Broker, T. R. (1994). Papillomavirus DNA replication. *Intervirology* 37, 150–158.

Croissant, O., Breitburd, F., and Orth, G. (1985). Specificity of the cytopathic effect of cutaneous human papillomaviruses. *Clin. Dermatol.* 3(4), 43–55.

Crum, C. P., Barber, S., Symbula, M., Snyder, K., Saleh, A. M., and Roche, J. K. (1990). Coexpression of the human papillomavirus type 16 E4 and L1 open reading frames in early cervical neoplasia. *Virology* 178, 238–246.

de Villers, E. M. (1994). Human pathogenic papillomavirus types: An update. *In* "Human Pathogenic Papillomaviruses" (H. Zur Hausen, Ed.), pp. 1–12. Springer-Verlag, Heidelberg.

Doorbar, J. (1996). The E4 proteins and their role in the viral life cycle. *In* "Papillomavirus Reviews: Current Research on Papillomaviruses" (C. Lacey, Ed.), pp. 31–38. Leeds Medical Information, Leeds Univ. Press, Leeds.

Doorbar, J., Campbell, D., Grand, R. J. A., and Gallimore, P. H. (1986). Identification of the human papillomavirus-1a E4 gene products. *EMBO J.* 5(2), 355–362.

Doorbar, J., Coneron, I., and Gallimore, P. H. (1989). Sequence divergence yet conserved physical characteristics among the E4 proteins of cutaneous human papillomaviruses. *Virology* 172, 51–62.

Doorbar, J., Ely, S., Coleman, N., Hibma, M., Davies, D. H., and Crawford, L. (1992). Epitope mapped monoclonal antibodies against the HPV16 E1 ∧ E4 protein. *Virology* 187, 353–359.

Doorbar, J., Ely, S., Sterling, J., McLean, C., and Crawford, L. (1991). Specific interaction between HPV16 E1 ∧ E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* 352, 824–827.

Doorbar, J., Evans, H. S., Coneron, I., Crawford, L. V., and Gallimore, P. H. (1988). Analysis of HPV1 E4 gene expression using epitope-defined antibodies. *EMBO J.* 7(3), 825–833.

Doorbar, J., and Gallimore, P. H. (1987). Identification of proteins encoded by the L1 and L2 open reading frames of human papillomavirus type 1a. *J. Virol.* 61, 2793–2799.

Doorbar, J., Medcalf, M., and Napthine, S. (1996). Analysis of HPV1 E4 complexes and their association with keratins *in vivo. Virology* 218, 114–126.

Doorbar, J., and Myers, G. (1996). The E4 protein. *In* "Human Papillomaviruses 1996" (G. Myers, H. Delius, J. Icenogel, H.-U. Bernard, C. Baker, A. Halpern, and C. Wheeler, Eds.), Vol. III, pp. 58–80. Los Alamos National Laboratory, Los Alamos, NM.

Egawa, K. (1994). New types of human papillomaviruses and intracytoplasmic inclusion bodies: A classification of inclusion warts according to clinical features, histology and associated HPV types. *Br. J. Dermatol.* 130, 158–166.

Egawa, K., Delius, H., Matsukura, T., Kawashima, M., and Villiers de, E.-M. (1993a). Two novel types of human papillomavirus, HPV63 and HPV65: Comparison of their clinical and histological features and DNA sequences to other HPV types. *Virology* 194, 51–62.

Egawa, K., Inaba, Y., Yoshimura, K., and Ono, T. (1993b). Varied clinical morphology of HPV-1-induced warts, depending on anatomical factors. *Br. J. Dermatol.* 128, 271–276.

Frattini, M. G., Lim, H. B., and Laimins, L. A. (1996). In-vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late gene expression. *Proc. Natl. Acad. Sci. USA* 93(7), 3062–3067.

Grand, R. J. A., Doorbar, J., Smith, K. J., Coneron, I., and Gallimore, P. H. (1989). Phosphorylation of the human papillomavirus type 1 E4 proteins *in vivo* and *in vitro. Virology* 170, 201–213.

Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T. M., Sutcliffe, J. G., and Lerner, R. A. (1982). Immunogenic structure of the influenza virus hemagglutinin. *Cell* 28, 477–487.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H. R., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaccolo, M., Gherardi, E., and Winter, G. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* 13, 3245–3260.

Hawkins, R. E., Russell, S. J., and Winter, G. (1992). Selection of phage antibodies by binding affinity. *J. Mol. Biol.* 226, 889–896.

Hudson, D. L., Weiland, K. L., Dooley, T. P., Simon, M., and Watt, F. M. (1992). Characterization of eight monoclonal antibodies to involucrin. *Hybridoma* 11(3), 367–378.

Jareborg, N., and Burnett, S. (1991). Immunofluorescent detection of bovine papillomavirus E4 antigen in the cytoplasm of cells permissive in vitro for viral DNA amplification. *J. Gen. Virol.* 72, 2269–2274.

Johnson, B., Lofas, S., and Lindquist, G. (1991). Imobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* 198, 268–277.

Laimins, L. A. (1993). The biology of human papillomaviruses: From warts to cancer. *Infectious Agents Dis.* 2, 74–86.

Lambert, P. F. (1991). Papillomavirus DNA replication. *J. Virol.* 65, 3417–3420.

Low, N. M., Holliger, P., and Winter, G. (1996). Mimicking somatic hypermutation — Affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. *J. Mol. Biol.* 260(3), 359–368.

Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T., Bye, J. M., and Winter, G. (1992). Bypassing immunization — Building high affinity human antibodies by chain shuffling. *Biotechnology* 10(7), 779–783.

McClean, C. S., Churcher, M. J., Meinke, J., Smith, G. L., Higgins, G., Stanley, M. A., and Minson, M. C. (1990). Production and characterisation of a monoclonal antibody to human papillomavirus type 16 using a recombinant vaccinia virus. *J. Clin. Pathol.* 43, 488–492.

Meyers, C., Frattini, M. G., Hudson, J. B., and Laimonis, L. A. (1992). Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science* 257, 971–973.

Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D., and Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *EMBO J.* 13, 692–698.

Palefsky, J. M., Winkler, B., Rabanus, J.-P., Clark, C., Chan, S., Nizet, V., and Schoolnik, G. K. (1991). Characterisation of in vivo expression of the human papillomavirus type 16 E4 protein in cervical biopsy tissues. *J. Clin. Invest.* 87, 2132–2141.

Pope, A., Pritchard, K., Williams, A., Roberts, A., Hackett, J. R., Mandecki, W., and Johnson, K. S. (1996). In-vitro selection of a high-affinity antibody to estradiol using a phage display human-antibody library. *Immunotechnology* 2(3), 209–217.

Pray, T. R., and Laimins, L. A. (1995). Differentiation-dependent expression of E1 ∧ E4 proteins in cell lines maintaining episomes of human papillomavirus type 31b. *Virology* 206, 679–685.

Roberts, J. M., and Weintraub, H. (1986). Negative control of DNA replication in composite SV40-bovine papillomavirus plasmids. *Cell* 46, 741–752.

Roberts, S., Ashmole, I., Gibson, L., Rookes, S. M., Barton, G. J., and Gallimore, P. H. (1994). Mutational analysis of human papillomavirus E4 proteins: Identification of structural features important in the formation of cytoplasmic E4/cytokeratin networks in epithelial cells. *J. Virol.* 68(10), 6432–6455.

Roberts, S., Ashmole, I., Johnson, G. D., Kreider, J. W., and Gallimore, P. H. (1993). Cutaneous and mucosal human papillomavirus E4 proteins form intermediate filament-like structures in epithelial cells. *Virology* 197, 176–187.

Rogel-Gaillard, C., Breitburd, F., and Orth, G. (1992). Human papillomavirus type 1 E4 proteins differing by their N-terminal ends have distinct cellular locations when transiently expressed in vitro. *J. Virol.* 66(2), 816–823.

Rogel-Gaillard, C., Pehau-Arnaudet, G., Breitburd, F., and Orth, G. (1993). Cytopathic effect in human papillomavirus type 1-induced inclusion warts: *In vitro* analysis of the contribution of two forms of the viral E4 protein. *J. Invest. Dermatol.* 101, 843–851.

Schier, R., McCall, A., Adams, G. P., Marshall, K. W., Merritt, H., and Yim, M. (1996). Isolation of picomolar affinity anti-c-erbb-2 single chain FV by molecular evolution of the complimentary-determining regions in the centre of the antibody-binding site. *J. Mol. Biol.* 263(4), 551–567.

Schneider, A. (1994). Natural history of genital papillomavirus infections. *Intervirology* 37(3–4), 201–214.

Sherman, L., and Schlegel, R. (1996). Serum-induced and calcium-induced differentiation of human keratinocytes is inhibited by the E6 protein of human papillomaviruses. *J. Virol.* 70, 3269–3279.

Stoler, M. H., Whitbeck, S. M., Wolinsky, S. M., Broker, T. R., Chow, L. T., Howett, M. K., and Kreider, J. W. (1990). Infectious cycle of human papillomavirus type 11 in human foreskin xenografts in nude mice. *J. Virol.* 64, 3310–3318.

Stoppler, M. C., Ching, K., Stoppler, H., Clancey, K., Schlegel, R., and Icenogle, J. (1996). Natural variants of the human papillomavirus type 16 E6 protein differ in their abilities to alter keratinocyte differentiation and to induce p53 degradation. *J. Virol.* 70, 6987–6993.

Wiley, S. R., Kraus, R. J., Zuo, F. G., Murray, E. E., Loritz, K., and Mertz, J. E. (1993). SV40 early to late switch involves titration of cellular transcription repressors. *Genes Dev.* 7, 2206–2219.

Wright, T. C., Kurman, R. J., and Ferenczy, A. (1994). Precancerous lesions of the cervix. *In* "Blaustein's Pathology of the Female Genital Tract" (R. J. Kurman, Ed.), pp. 229–277. Springer-Verlag, New York.

Zheng, Z.-M., He, P., and Baker, C. C. (1996). Selection of the bovine papillomavirus type 1 nucleotide 3225 3′ splice site is regulated through an exonic splicing enhancer. *J. Virol.* 70, 4691–4699.