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Detection of Viral DNA and E4 Protein in Basal Keratinocytes of Experimental Canine Oral Papillomavirus Lesions

Philip K. Nicholls,*,† John Doorbar,† Richard A. Moore,*, Woei Peh,† Davina M. Anderson,*, and Margaret A. Stanley.*

*Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, United Kingdom; †National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1 AA, United Kingdom

†To whom correspondence should be addressed:

Philip K. Nicholls
Division of Veterinary & Biomedical Sciences
Murdoch University
South Street
Murdoch
WA 6150
Australia

Telephone: +61 8 9360 2599
Facsimile: +61 8 9310 4144
E-mail: nicholls@numbat.murdoch.edu.au
ABSTRACT

We studied experimental canine oral papillomavirus (COPV) infection by in situ hybridization, and immunohistochemistry of weekly biopsies. After four weeks, viral DNA in rete ridges suggested a keratinocyte stem cell target. Abundant viral DNA was seen only in E4-positive cells. E4 was predominantly cytoplasmic but also nuclear, being concentrated in the nucleoli during wart formation. Infected cells spread laterally along the basal layer and into the parabasal layers, accompanied by E7 transcription and increased mitoses. Most of the lower epithelium was positive for viral DNA but, in mature warts, higher levels of E4 expression and genome amplification occurred in only sporadic superficial cells. L1 expression was late and in only a subset of E4-positive cells. During regression, viral DNA was less abundant in deep epithelial layers, suggesting down-regulation of replication prior to replacement of infected cells from beneath. Detection of viral DNA in post-regression tissue indicated latent infection.

Key Words: COPV; gene expression; life cycle; DNA replication; E4

INTRODUCTION

The involvement of human papillomaviruses (HPVs) in cancer of the cervix is well-established, with nearly all cervical carcinomas containing HPV DNA [Walboomers, 1999 #1056]. Furthermore, over 130 HPV types have been cloned or identified by PCR detection of capsid (L1) DNA [de Villiers, 1997 #227], and the association between certain HPV types and cervical cancer is strong enough for these ‘high-risk’ HPVs to have been defined as carcinogens [Anonymous, 1996 #26]. In addition to their association with human epithelial malignancies, papillomaviruses cause a variety of benign tumours of cutaneous and mucosal epithelia [Shah, 1996 #829]. Although benign, many of these warty lesions often respond poorly to a variety of treatments [Beutner, 1997 #60], and are sometimes persistent or progressive, especially in people immunosuppressed from either HIV infection or as a result of organ transplantation [Palefsky, 1998 #698]. In addition to the efforts currently being made to find immunologically-based prophylactic or therapeutic strategies, it is clear from the diseases seen in immunosuppressed individuals that there is a need for non-immunological treatments [Stanley, 1997 #876; Phelps, 1998 #728]. Such antiviral strategies require detailed knowledge of the papillomavirus life cycle. The technical demands of in vitro culture of these viruses, and their species specificity, present difficulties in examining the viral life cycle. Additionally, it
seems that organotypic keratinocyte cultures may not accurately model all aspects of intact epithelia [Mayer, 1998 #570]. Detailed chronological analysis of the papillomavirus infectious cycle is impossible in human clinical material since the time of naturally-acquired infections is difficult to establish, making the chronology of events in subsequent biopsies uncertain. Because multiple sequential biopsies are rarely justified on clinical grounds in the human patient, the experimenter is left with only 'snapshots' of the disease taken from many different patients and lacking in coherence. Although viral DNA and RNA have been examined in biopsies of human clinical material (both warts and neoplasms), similar studies of viral DNA and RNA in the early stages of infection have not been possible because the lesions become macroscopically detectable only after a period of several weeks, and there is a further delay before patients present to clinics for examination and treatment. These problems mean that although molecular biology has enabled great progress to be made in understanding some of the details of virus-cell interactions, there remain large gaps in the understanding of papillomavirus biology. To overcome these difficulties, papillomavirus infections in animals such as the rabbit or ox have been studied, and animal models such as these have been described as the "gold standard" with which in vitro models should be compared [Chow, 1997 #172].

In the CRPV-infected rabbit, analysis of biopsies at different times post-infection has provided important data on the viral life cycle [Schmitt, 1996 #800]. However, CRPV infection involves haired-skin and there are important biological differences, especially with regard to stem cell location, between haired [Cotsarelis, 1990 #200; Michel, 1996 #590] and mucosal epithelia [Lavker, 1982 #516; Hume, 1983 #401]. Since many of the important human papillomavirus infections affect the anogenital mucosa, studies of viral biology in a mucosal model are important. One such model, palatine infection by bovine papillomavirus type 4 (BPV-4) in calves, has been used to study temporal and spatial expression of viral proteins [Anderson, 1997 #19]. However, RNA in situ hybridisation was not undertaken, and viral DNA proved undetectable in basal or suprabasal keratinocytes with the technique used.

Canine oral papillomavirus (COPV), reviewed in [Nicholls, 1999 #1057], has been used for the development of vaccines both historically [Chambers, 1960 #145] and more recently [Bell, 1994 #52; Ghim, 1995 #316]. Furthermore, COPV has been used to establish the efficacy of DNA vaccines (our unpublished data). Some COPV infections have similarities to HPV-associated recurrent laryngeal
papillomatosis [Nicholls, 1999 #1058]. Since the dog is a useful animal model of mucosal papillomavirus infections, it provides an important opportunity for studying the viral life cycle in vivo, allowing early biopsies to be taken in a controlled manner at accurately defined intervals after infection. In the COPV model, we have used a chronological series of biopsies, taken from infection through to regression, to analyse the viral life cycle by means of histopathology, in situ hybridisation, and immunohistochemistry. We were able to detect viral RNA, DNA and protein during the early stages of infection within the basal and suprabasal keratinocytes of pre-clinical lesions, providing insight into the likely location of the target cell. Double-labeling of viral E4 and L1 proteins revealed both temporal and spatial differences in expression, in distinction to the findings of previous reports using material from human warts [Brown, 1994 #100; Brown, 1995 #101]. Additionally, double-labeling of viral DNA and viral E4 protein showed a strong colocalisation even at early time points, supporting the hypothesis that, in addition to its suggested role late in the viral life cycle [Doorbar, 1991 #248; Doorbar, 1996 #250], E4 may play a role in the earlier stages of infection, perhaps in viral DNA replication [Doorbar, 1997 #252]. The detection of abundant viral DNA and protein in basal keratinocytes of COPV lesions highlights the differences in viral biology between viral types, and indicates that generalizations about the biology of different papillomavirus types should be made with caution.

RESULTS

Morphological changes during wart development and regression.

Haematoxylin and eosin stained sections from tissues taken one to four weeks post-infection were morphologically similar to pre-infection controls. At week five, an increased mean mitotic rate (Fig. 1) was associated with expansion of the stratum spinosum (Fig. 2a). After further epithelial thickening, filiform papillae appeared, with koilocytes in spinosum and granulosum strata by week seven (Fig. 2b). Frozen sections revealed a prominent hyperkeratosis, predominantly orthokeratotic. In places, a focal infiltrate of inflammatory cells obscured the dermo-epidermal interface. By week eight, mature papillomas developed multiple projecting papillae rising 5-10 mm above the surrounding mucosal surface (Fig. 2c). Significant numbers of lymphocytes were present intraepithelially and around the interface of the epithelium and lamina propria, in some places obscuring the basement membrane zone. Occasional apoptotic keratinocytes were
seen in the lower layers. In one area the apex of one of the filiform papillae had undergone focal coagulative necrosis. By week nine the infiltration of inflammatory cells, mostly lymphocytes, was more marked and the papilloma had reduced in size (Fig. 2d). There was a prominent influx of neutrophils, with spongiosis and oedema. As the papillomas resolved during week ten, the epithelium reformed, although there were still patchy foci of intraepithelial and subepithelial inflammation. By week eleven, the oral mucosa appeared morphologically normal and the cycle of papilloma progression and regression was complete.

**Analysis of viral location and replication by DNA *in situ* hybridisation**

To identify sites of viral DNA replication during wart formation and regression, the chronological series of biopsies was examined by DNA *in situ* hybridisation using a digoxigenin-labeled COPV genomic probe. No DNA was detectable in pre-infection sections, or from one to three weeks post-infection (Fig. 3a). Viral DNA was first detected at four weeks (Fig. 3b), in only some of the sections examined, and was restricted to scattered foci of positive nuclei in the basal layer or immediately above it. The tight clusters of positive cells, suggested a clonal origin. In places, these early foci were found at the lower tips of the epithelial projections or rete ridges (Fig. 3c). Some sections revealed early foci in the saddle region between the rete ridges. Occasional foci of positive nuclei in a suprabasal position, apparently unconnected to the basal layer, were tracked back to the basal layer or deep rete ridges by *in situ* hybridisation of serial sections.

From week five to week six, more positive cells appeared within the basal and suprabasal layers, until nearly every cell within the basal layer and layers immediately above was positive (Fig. 3d). The foci where viral DNA was detectable correlated with focal increases in epithelial thickness and mitotic rate, demonstrating a link between viral replication and keratinocyte division.

As the papilloma matured through weeks seven and eight, only certain superficial keratinocytes appeared to permit the virus to replicate to a higher copy number (Fig. 3e). This was in contrast to the situation in the lower layers, in which almost every keratinocyte was positive for viral DNA. It was these superficial, strongly-positive keratinocytes, apparently supporting high level genome amplification, which had vacuolated cytoplasm typical of koilocytes. As the papillomas regressed during week nine, less DNA
was detected in the lower layers of the epithelium (Fig. 3f) despite remaining prominent more superficially, until no signal remained by week ten.

**Detection of transcripts from the E2/E4 region**

Probes to the E2 open reading frame were made complementary to regions spanning nucleotides 2694 to 3058 (E2 probe A), and 3460 to 3710 (E2 probe B). These segments flank that part of the E2 region encoding E4 within a different reading frame. E2 probe A did not however allow reliable detection of transcripts and was not extensively used. By contrast, E2 probe B detected abundant transcripts within both experimental and clinical lesions. In the mature clinical lesions, signal was located mostly within the basal layer, but was found also within the other lower epithelial layers and extended superficially within a subset of cells. In the experimental series, signal was abundant basally through weeks five to eight (Fig. 4a) and declined as the wart regressed. Signal was present in a subset of suprabasal and more superficial layers in the maturing wart (Fig. 4b) and was absent by week 10. The E4 riboprobe was complementary to the region between nucleotides 3193 and 3429. The probe detected abundant transcripts within the mature clinical lesions. The signal was abundant in basal layer keratinocytes, rete ridges, and in sporadic cells throughout the lower half of the epidermis but on occasions was confined to the basal layer. Within the experimental series, transcripts from the E4 ORF were first detected at four weeks post-infection in the basal and immediately suprabasal layers (Fig. 4c). The transcripts were found in abundance from week five (Fig. 4d) and throughout wart progression (Fig. 4e), beginning to fade from week nine as the warts regressed, and disappearing by week 10. E2 probe B and the E4 probe are both predicted to detect all early transcripts, as well as transcripts originating from the differentiation dependent promoter, which in other papillomavirus types is situated upstream of E1. The strength of the signal and the similarities between the staining patterns of the two probes suggests both are detecting the E1^E4 message which is expressed at high level by other papillomavirus types.

**Detection of transcripts from the E6/E7 region**

The E7 probe was complementary to a region between nucleotides 575 and 740. The probe detected abundant transcripts in mature clinical papillomas. Signal was present in the basal layer and a
subset of more superficial keratinocytes. In the experimental series, signal was first detected at four weeks post-infection, in only the lowest layers of epithelium. By week five the signal was more abundant, especially in the rete ridges. As the lesions progressed, some keratinocytes in more superficial layers contained abundant transcripts (Fig. 4f). The signal was present in numerous superficial cells by week eight, declining thereafter, until absent by week 10. The E6 probe, complementary to the region between nucleotides 150 and 492, did not reliably detect transcripts and was not extensively studied.

Detection of transcripts from the L1/L2 region

The L1 probe was complementary to a region between nucleotides 7397 and 7755. Naturally-occurring canine oral papillomas contained abundant transcripts from the L1 ORF in only the stratum granulosum or uppermost stratum spinosum. No signal was ever detected below this level. In the experimental series, L1 transcripts were first detected in small amounts at seven weeks post-infection, in the superficial keratinocytes. The transcripts were abundant by week eight (Fig. 4g), and still present at week nine. No transcripts were present at weeks 10 and 11. Transcripts were present in both cytoplasmic and nuclear locations.

The L2 probe spanned a region from nucleotides 5771 to 6110. Transcripts were present in the superficial keratinocytes of mature clinical lesions, but not in keratinocytes of the lower epithelial layers. In the experimental series, signals were not detected until week seven, as for the L1 transcripts. Transcripts were found only in superficial keratinocytes, and became more abundant by week eight (Fig. 4h). Some signal remained at week nine, but none was detected at weeks 10 or 11. Nuclear signal was occasionally seen with the sense control probe, sometimes concentrated over the nucleoli (data not shown).

Expression of the L1 capsid protein during the time course of infection

Although RNA in situ hybridisation provides some insight into the pattern of viral gene expression during wart formation, papillomavirus mRNAs have complex splicing patterns, and the detection of RNA from a viral open reading frame does not necessarily indicate synthesis of viral proteins. Polycistronic transcripts are not necessarily expressed throughout their length, and in other papillomavirus types, post-transcriptional mechanisms preventing protein expression have been reported. To determine the expression
patterns of viral proteins during wart formation and regression, immunohistochemistry was undertaken using antibodies specific to three of the viral proteins - E4, E7 and L1.

The presence of L1 protein correlated well with detection of L1 transcripts, except that the L1 gene products were restricted to the nucleus in the stratum granulosum, becoming cytoplasmic only in the stratum corneum where nuclear disintegration is likely. No L1 protein was seen in pre-infection controls, or from weeks one to six inclusive. An occasional positive nucleus was seen in the stratum granulosum at week seven. By week eight there were numerous positive keratinocyte nuclei in the stratum granulosum, with the signal extending into the stratum corneum (Fig. 5a). Only an occasional positive cell was found during early regression at week nine and no virus capsid was detectable by weeks 10 and 11.

Expression of the viral E4 protein during the time course of infection

E4 is abundantly expressed in lesions caused by several human papillomavirus types. The role of E4 in the virus life cycle is unclear, and its expression pattern during wart formation and regression has not been established. To generate antisera, immunisations were carried out using a glutathione S transferase COPV E4 fusion protein expressed in bacteria. Antibody specificity was confirmed by Western blotting using maltose binding protein (MBP) fusions containing the E4 gene products from COPV, ROPV or HPV 11. Reactivity was apparent only with the COPV E4 fusion (data not shown).

In mature clinical COPV papillomas, immunostaining revealed a predominantly cytoplasmic signal for E4 in sporadic cells of the basal layer and above (Fig. 5b). The E4 staining pattern was very heterogeneous. In some cells the protein existed as cytoplasmic inclusions while in others the staining was diffuse and extended through the nucleus (Fig. 5c). Where nuclear staining was apparent, the E4 protein was concentrated around the periphery of the nucleoli (Fig. 5d). In the experimental series, E4 was first detected at week four - and until week six, staining was apparent in the lower epithelial layers at levels close to the limits of detection (Fig. 6a). By week six, sporadic cells showed high level E4 expression (as apparent from immunostaining), with levels of expression increasing from week seven to week eight (Fig. 6b). In the experimental lesions, E4 could be detected both in the nucleoli as well as the cytoplasm at weeks seven and eight (Fig. 6c). This distribution, which was not apparent in established clinical material
was confirmed by confocal microscopy and phase contrast imaging (Fig. 6d). In both the experimental and clinical material E4 expression was first detected in the basal cells. E4 protein remained at weeks eight (Fig. 6e) and nine, and was apparent at the advanced stages of regression. No E4 was detectable at weeks 10 or 11.

**Correlation of viral protein expression with genome amplification by double staining**

Some reports have indicated that the expression of E4 colocalises exactly with that of L1 [Brown, 1994 #100], whereas more recent work indicates that L1 expression is accompanied by expression of E4, but that the converse is not true, with E4 appearing additionally in lower epithelial layers [Doorbar, 1997 #252]. COPV lesions were first stained for L1 and developed to give a dark purple reaction product. After washing, the same sections were stained for E4 and developed to give a brown reaction product. Although E4 was expressed in the absence of L1 in the lower epithelial layers, L1 expression occurred only in E4 positive cells in the superficial layers (Fig. 6f). These correlations were seen in established clinical biopsies, and from week seven in the experimental time course. To determine whether viral DNA replication and E4 expression coincided during the course of COPV infection, immunolocalisation was carried out on sections previously hybridised to detect viral DNA. The sensitivity of antibody staining was increased by the *in situ* hybridisation step, while the nuclear location of viral DNA and the predominantly cytoplasmic location of E4, enabled double labeling to be interpreted clearly. Expression of E4 correlated closely with viral DNA replication at all time points and both occurred in the basal and more superficial cell layers. These relationships were demonstrated from week four onwards although at early time points (weeks four to six) the level of E4 expression was close to the sensitivity of detection by immunostaining (Fig. 7a,b). At early time points all of the E4-positive cells in the parabasal layers were found to be supporting viral DNA replication. In clinical lesions, and between weeks seven and eight in the experimental warts, sporadic cells could be detected which had higher levels of E4 and higher levels of DNA replication (Fig. 7c,d). As previously reported, high level expression of E4 coincided closely with vegetative viral DNA replication, although occasional cells were apparent which were positive for DNA replication yet negative for E4 and vice versa.
L1 expression did not colocalise with DNA amplification in the lower half of the epithelium, since L1 protein was undetectable despite the presence of abundant viral DNA. Furthermore, viral genome amplification was detectable from week four, whereas L1 was not seen until week seven. In the mature lesions, L1 colocalised with DNA in the upper epithelium, as the L1 signal overlaid the darker DNA signal when enzymatic detection was carried out. This was confirmed by immunofluorescence double staining.

**DISCUSSION**

The delay before COPV lesion appearance is well-documented [Chambers, 1959 #144; Konishi, 1972 #479; Bell, 1994 #52], and a delay of four weeks before COPV DNA is detectable is similar to that described in other models [Sterling, 1990 #884; Stoler, 1990 #891]. COPV infection by injection, rather than scarification, prolongs this lag phase (our unpublished data), presumably because the reduced epithelial trauma provides little stimulus for epithelial repair and stem cell division. Stimulation of viral DNA replication following epithelial trauma has been well-documented [Amella, 1994 #12; Campo, 1994 #123; Whiteley, 1956 #1022; Whiteley, 1957 #1023]. In haired skin, a likely site of stem cells is the follicular bulge region [Cotsarelis, 1990 #200], where early CRPV gene expression was found [Schmitt, 1996 #800], as well as interfollicular epidermis [Miller, 1993 #595]. In non-haired epithelia, stem cells are thought to be located at the tips of rete ridges, based on the detection of slowly-cycling cells in this area [Lavker, 1982 #516; Hume, 1983 #401]. More recent studies have suggested that keratin-19 is a marker for stem cells, with cells within the bulge region of the hair follicle labeling with keratin-19 and retaining a tritiated thymidine label [Michel, 1996 #590]. The same work localised these putative stem cells to the deep tips of rete ridges of non-haired skin. Early COPV lesions showed a distribution of viral DNA and RNA consistent with infection of a stem cell at this site, and the apparent subsequent tracking up the side of the ridge toward the shoulder region resembles proposed trafficking from the stem cell through transient amplifying compartments and into post-mitotic epithelium [Lavker, 1983 #517]. The data presented here are compatible with the idea that the virus infects a basal keratinocyte [McMillan, 1999 #1062; Evander, 1997 #266], most likely a slowly-cycling keratinocyte stem cell [Stanley, 1994 #875].

It has previously been reported that viral DNA amplification does not occur in the basal layer, with viral DNA being detectable in only the more superficial epithelial layers. For instance high copy numbers
of BPV-4 DNA were seen only in the spinous and squamous layers of papilloma fronds [Anderson, 1997 #19], and in recent studies of HPV-1 biopsies no viral DNA or RNA could be detected in basal keratinocytes [Egawa, 2000 #1083]. HPV DNA was detected in the basal layer in CIN 3 lesions, but in a pattern suggesting viral integration [Cooper, 1991 #199] rather than as a normal part of the virus life cycle. In this study, COPV DNA was readily detected in basal keratinocytes, indicating a substantial level of DNA replication at this level. The failure of other studies to detect DNA in the basal layer of the epidermis may be due to technical factors, since the abundant DNA in more superficial keratinocytes gives a strong signal even under sub-optimal conditions. Both protease K digestion and the degree of stringency during washing were critical in detecting DNA in basal keratinocytes. It is possible that not all papillomavirus types produce such abundant DNA, and that basal layer levels are below the sensitivity of detection. Interestingly, COPV is related to the cutaneous HPV types 1 and 63 [Delius, 1994 #228] and abundant low level, although not basal, DNA replication has been described in HPV-1 [Doorbar, 1997 #252; Egawa, 2000 #1083]. It is also possible that established clinical biopsies are taken at a time when basal layer viral replication has been reduced, perhaps in response to moderation by the host defences. We found that post-mature COPV warts in the early phases of regression lost the high levels of viral DNA in the lower epithelium, whilst maintaining abundant DNA more superficially. Interestingly, some in vitro systems have revealed a degree of HPV DNA amplification in cells of the basal layer, and this has been reported as challenging the concept that viral genome amplification is linked to differentiation [Ozbun, 1998 #694]. It should be noted that the basal layer is not made up from a single undifferentiated cell type but includes stem cells, transient amplifying cells and post-mitotic differentiating cells expressing keratin 10 (normally a spinous layer keratin) [Li, 1998 #531]. Caution is required in interpreting such data, since raft cultures differ from normal tissue in their differentiation pattern, with filaggrin expression being seen in basal cells rather than just the granular and cornified layers [Mayer, 1998 #570].

The initial multifocal clustering of COPV DNA-positive cells is consistent with their origin from keratinocyte stem cells, which make up only a few percent of cells in the basal layer. Nearly every cell at the basal and several suprabasal strata of the epidermis appeared permissive for viral replication to a moderately high copy number. Viral DNA in the middle epidermis can act only as template for mRNA, or for further DNA replication, since the absence of capsid proteins prevents its incorporation into viral
particles. Why only a certain fraction of more superficial epithelial cells take DNA replication through to an even higher copy number is not clear, but this presumably reflects either a uniquely permissive environment within these cells or the result of chance factors and competing influences with a bistable outcome. These koilocytes were the cells in which virus was assembled, as revealed by double-labeling for viral DNA and L1 capsid protein. Polyclonal antibodies against GST fusion proteins of full length COPV E2 and E7 also revealed staining in the nuclei of koilocytes of mature papillomas (our unpublished observations). The same patchy distribution of cells with high DNA copy number has been seen in vitro [Ozbun, 1998 #694]. A greater understanding of the factors controlling this permissive environment might provide insight into possible avenues for therapeutic intervention.

The disappearance of viral DNA noted in lower layers of regressing warts, seen also in BPV-4 lesions [Anderson, 1997 #19], could arise through several mechanisms. One possibility is that all the (stem) cells in which viral DNA resides are removed, presumably by the marked cellular immune response seen during the late stages of regression. Destruction of the stable reservoir for viral DNA would result in the infected cells growing out toward the surface without being replaced from below. Another possibility is that viral replication is shut down in the infected cells, either permanently or to be reactivated after a period of latency, a theory supported by detection of COPV DNA in mucosa from sites of regressed papillomas (our unpublished data).

Although thought to have late functions, the pattern of E4 transcription and protein expression seen in this study appeared more typical of a papillomavirus early gene. The transcription pattern had an early onset, and represented the earliest detectable events during the time course of COPV infection. E4 specific transcripts (E1^E4 mRNAs) are the most abundant mRNA species seen in HPV-11 infected human foreskin xenografts, and are located predominantly in the mid-epithelial and more differentiated layers [Stoler, 1990 #891] as found here at late time points. Signals in the basal layers were attributed to the presence of E2 or E6/E7 transcripts spanning the E4 coding region. E2 probe B showed a similar staining pattern to that of the E4 probe, and it is likely that both are detecting the full range of early messages. Identification of the E4 protein at early time points, indicates that at least some of this message serves as a template for the synthesis of E4 protein. A similar distribution was apparent for E7 mRNAs which were found in basal keratinocytes as well as in a subset of cells within the stratum spinosum and stratum granulosum. This is
similar to the pattern previously reported in HPV-11-infected foreskin xenografts where E6-E7 transcripts were detectable from week six, although parabasally at first, and then in the middle epithelial layers [Stoler, 1990 #891]. Similarly, in CRPV-induced lesions, E6-E7 transcripts are detected in the hair follicles at day 11 [Schmitt, 1996 #800] and become stronger in the basal layers by day 45. E7 mRNAs are detected throughout the epithelium in established lesions caused by both HPV-16 and CRPV [Zeltner, 1994 #1046; Higgins, 1991 #381; Higgins, 1992 #382]. The E7 protein has been detected immunohistochemically in BPV-4 lesions [Anderson, 1997 #19], localising to the nucleus in basal and suprabasal cells, and to the cytoplasm in the spinous and squamous layers.

Transcription from late regions was markedly different from that of the other ORFs. A similar pattern of late gene transcription has been seen in HPV-11 infected foreskin xenografts [Stoler, 1990 #891]. L1 antigen was detectable from week eight and correlated with L1 message, as seen here with the COPV lesions. The intensity of staining suggested that the L1 transcripts are more abundant than those of L2. L1 transcripts outnumber L2 transcripts in HPV31-containing keratinocytes cultured organotypically [Ozbun, 1997 #693], and the L1 protein is expressed at higher levels than L2 in cutaneous human warts [Doorbar, 1987 #244]. In the COPV lesions, cells containing L2/L1 transcripts and L1 protein had swollen pale cytoplasm, as reported with BPV-4 [Gaukroger, 1989 #308; Anderson, 1997 #19]. These koilocytic cells additionally contained high levels of viral DNA. Despite the abundant viral DNA and E4 in the lower epithelium, no L1 was detected in the lower half of the epidermis. This is in contrast to HPV-1 lesions, in which L1 transcripts and protein were detected in suprabasal layers [Elzein, 1991 #1067].

RNA in situ hybridisation showed clearly different transcription patterns from the COPV L1 and E4 open reading frames. This is not consistent with earlier reports that L1 and E4 proteins colocalise at the cellular level [Brown, 1994 #100; Brown, 1995 #101]. Interestingly, in the study of Brown et al the E4 protein did not precede the L1 protein, despite the presence of E4 transcripts in lower layers than L1 transcripts [Brown, 1995 #101]. In COPV lesions, good correlation between E4 mRNA and E4 protein was found. E4 was expressed early on during infection, and in cells of the basal layer as well as more superficially. E4 expression preceded L1 expression, although L1 was always accompanied by E4 expression in the same cell.
Although E4 has been reported as being nuclear in location [Palefsky, 1991 #697], recent work with HPV-16 lesions showed that E4 expression, although variable in amount, correlated exactly with DNA replication but not L1 expression, and was cytoplasmic [Doorbar, 1997 #252]. The cytoplasmic location of E4 within cells of the granulosum and upper spinosum strata was confirmed by immunoelectron microscopy of athymic mouse HPV-16 xenografts [Sterling, 1993 #885], with viral capsids detected microscopically and by immunogold staining (L1) in the nucleoplasm of the same cells. The association of E4 with cytoskeletal elements (keratin tonofibrils) was demonstrated in the same report. L1 expression is preceded by E4 expression, but colocalises with E4 in the superficial layers (Doorbar et al., 1997), a finding mirrored in the COPV lesions described in this study. E4 expression was seen in high grade HPV-16 lesions which no longer generated L1. This dissociation of L1 and E4 expression is demonstrated also by the lack of effect of protein kinase C activators on E4 expression in raft cultures, whereas L1 expression is upregulated [Meyers, 1992 #588]. E4 of HPV-31b was detected in rare cells in monolayer culture, but its expression was upregulated upon differentiation of the same cells in raft culture [Pray, 1995 #735]. The E4 protein was located mostly in spinosum and granulosum strata, occasionally extending into the stratum corneum. Whilst occasional (<1%) basal cells were described as expressing E4, none was shown in the report. Basal expression of COPV E4 was clearly present in this study. In the HPV-31b study, as in this study, most L1 positive cells also expressed E1^E4, but the converse was not true.

The finding that E4 is expressed early on in the virus life cycle and in basal cells, and is dissociated from expression of L1, suggests that E4 is an early protein in COPV. These findings do not support the theory that E4 is a late protein with a role in releasing virus from cells [Doorbar, 1991 #248; Doorbar, 1996 #250], although it may be multi-functional. A role for E4 in DNA replication has been suggested [Doorbar, 1997 #252], and a close correlation was found between the detection of high copy number viral DNA and COPV E4 protein both temporarily and spatially. A similar correlation has previously been reported in other species. E4 and L1 are more frequent in genital warts from pregnant women when compared with non-pregnant women or men [Brown, 1992 #98], and this increased expression is associated with a higher HPV DNA copy number. In mucosal lesions caused by HPV-16, and in cutaneous lesions caused by HPV-1 and HPV-63, the onset of genome amplification coincides closely with E4 expression [Doorbar, 1997 #252], while in lesions caused by BPV-4 a similar correlation has been
reported [Anderson, 1997 #19]. Colocalisation of E4 with genome amplification has also been demonstrated in HPV-infected keratinocytes grown in methylcellulose suspension [Ruesch, 1998 #782]. When taken together, these findings suggest a role of E4 in vegetative viral DNA replication, perhaps by sequestering cellular factors which inhibit episomal replication [Breitburd, 1987 #92]. The two proposed functions of E4, cytoskeletal interactions to assist viral egress (a late function) and involvement in DNA replication might not therefore be mutually exclusive. Colocalisation does not of course prove causality, and it has been suggested that vegetative viral DNA replication may permit expression of E4, L1 and L2 by titrating out cellular factors normally inhibiting the late promoter [Frattini, 1996 #290].

The work presented here has established the pattern of events during the formation and regression of COPV-induced papillomas. Following infection, a lag phase of 3-4 weeks occurs with little viral DNA amplification or gene transcription, which we speculate could represent the delay before division of the infected stem cell. Cellular DNA replication begins soon after this, and is probably triggered by E6 and E7 once the viral DNA is passed to the daughter cells. Rapid proliferation of keratinocytes, as illustrated by the increased mitotic count and epithelial thickness is seen from week five. These rapidly dividing keratinocytes pass into the parabasal cell layers carrying viral DNA with them, and a subset initiate genome amplification. These events begin near the stem cell regions of the rete ridges, as revealed by RNA/DNA in situ hybridisation, before the infected cells move up the sides of the rete ridges and spread out to occupy most of the basal layer. At this stage most of the cells are positive for viral DNA by in situ hybridisation, and transcripts for E4, E2 and E7 are detected in abundance. Only a subpopulation of the infected keratinocytes support vegetative viral DNA replication and express E4 at high levels, as illustrated by the patchy distribution of viral DNA, RNA transcripts, and protein, in the spinous and granular layers. Transcription of the late genes (L1 and L2) is not seen below the granular layer, despite high copy numbers of viral DNA and abundant transcripts from the early regions. In human papillomaviruses, the late regions are under control of a differentiation-dependant promoter, which becomes active in the superficial epithelium. Infectious virus is generated in the stratum granulosum as detected by immunohistochemistry and electron microscopy.

By the time the wart reaches maturity, the abundant viral proteins have stimulated host immunity. The cellular immune response causes shut down of viral activity by killing infected keratinocytes or
reducing viral transcription via interferons. A combination of keratinocyte apoptosis and necrosis, coupled with the shut down of viral activity in the lower epithelium, lowers the mitotic rate and reduces the wart volume. This is reflected by the reduced signals for DNA and RNA in situ hybridisation that is seen in the lower epithelium around weeks eight and nine. Transcription in the upper regions of the wart papillae is unaffected by these immune events, as seen by the continued presence of transcripts in the superficial keratinocytes. Eventually all viral-infected cells are replaced from below by new keratinocytes, which may be the progeny of uninfected stem cells from the edge of the lesion. The life cycle is complete. If some cells remain infected with viral DNA (as suggested by PCR), then a latent infection is present, may be reactivated either by immunosuppression or stimulation of the epithelium by wounding.

Although COPV is a mucosal papillomavirus, at the nucleotide sequence level it is more closely related to the cutaneous HPV types 1 and 63, with minimal sequence similarity to mucosatropic HPVs, including the oral HPV types 13 and 32 [Delius, 1994 #228]. The abundant viral replication and expression of E4 low in the epidermis, and the high level of virion production in COPV, are similar to findings in the related human papillomaviruses, HPV-1 and HPV-63, suggesting that the biological properties of papillomaviruses may not correlate well with their preferred target tissues, and that the often-cited distinction between mucosatropic and cutaneous PV types is somewhat arbitrary. Even the closely related HPV-1 and HPV-63 cutaneous HPVs have different E4 staining characteristics, with coalescing intracytoplasmic inclusions in the former and fibrous / granular distribution in the latter [Doorbar, 1997 #252]. It seems that there may be no one ‘typical’ pattern of papillomavirus infection since the different papillomavirus types appear to differ from one another quite markedly with respect to onset of vegetative DNA replication, timing and pattern of E4 expression, and amount of infectious virus produced. It is clearly difficult to generalize about the virus life cycle across so many widely differing viral types, and an understanding of these differences in viral biology could prove important in attempts to develop therapeutic interventions for papillomavirus-related disease.
MATERIALS AND METHODS

Experimental infection

Two female, 10-12 week beagles were housed together in purpose-built, climate-controlled accommodation, in an airspace separate from other dogs in the building, with a virucidal footbath at the common entrance and exit. All staff changed their protective clothing (gown, gloves, boots) on entering the controlled area. Under general anaesthesia, the mucosa of the upper lip was lightly scarified over an area approximately 5 mm x 5 mm, using the tip of a scalpel blade, until a light ooze of blood occurred. Neat COPV wart suspension [Bell, 1994 #52] (a kind gift from A. B. Jenson, Georgetown University, Washington, D. C.) was applied (10 μl) to each site by pipette and allowed to absorb for a few minutes. The site of challenge was marked by Indian ink tattoo points in a triangle centred on the site. Twelve sites were infected in each dog. Control oral mucosal biopsies were taken prior to challenge.

Under general anaesthesia, further weekly biopsies of the oral mucosa were taken using a 6 mm punch biopsy or scalpel followed by suturing as appropriate. Where gross lesions had not yet developed, tissue was taken from the tattoo-marked sites. The biopsies were fixed in 10% neutral buffered formalin then processed after 4-8 hours to paraffin wax for routine (5-7 μm) sectioning. At the end of the experiment all animals were re-homed.

Immunohistochemistry

Immunohistochemistry of formalin-fixed, paraffin-embedded sections was performed using a secondary antibody conjugated to either FITC or Texas red (Amersham, UK), or using an avidin-biotin complex (Vectastain ABC, Vector Laboratories), as previously described [Nicholls, 1999 #1058]. Positive control sections were canine oral papillomas with virions confirmed by transmission electron microscopy. Pre-infection biopsies acted as negative controls. Omission of primary or secondary antibody provided further negative controls.

For the detection of L1, the primary antibody was CAMVIR-1 mouse IgG2a monoclonal [McLean, 1990 #581] diluted 1:1000 in PBS, with biotinylated polyclonal goat anti-mouse IgG2a (Amersham, UK), diluted 1:400 in PBS, as secondary antibody. For the detection of E4, the primary antibody was polyclonal rabbit anti-COPV E4-GST fusion protein diluted 1:2000 in PBS, with biotinylated polyclonal goat anti-
rabbit (DAKO E432), diluted 1:400 in PBS, as secondary antibody. FITC or Texas red conjugated antibodies (Amersham N1031, N2031) were used at a dilution of 1:50. The polyclonal E7 antibody was prepared by immunisation of a rabbit with a full-length COPV-E7 GST fusion protein expressed and purified from E. coli. The E7 antibody was pre-absorbed against GST protein and used at 1:600 in PBS containing 10% normal goat serum, with the same secondary antibodies as for E4 immunohistochemistry.

**Double-labeling immunohistochemistry for L1 and E4**

Sections were treated as for L1 immunostaining, except an alkaline phosphatase tertiary complex (Vectastain ABC, Vector Laboratories) was used for colour development with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT) as the substrate. The reaction was stopped by immersion in TE buffer for 20 min before the second label was applied. For the second label, sections were washed in 0.2% Triton X-100 in PBS for 5 min, followed by washing in PBS (3x 3 min). The second primary antibody (for E4) was applied overnight at 4°C. The sections were washed as before, then the secondary antibody (peroxidase-conjugated swine anti-rabbit (DAKO P217) diluted 1:400 in PBS) was applied for 30 min at room temperature. After further washing, the label was developed with 0.06% 3,3’ diaminobenzidine tetrahydrochloride (DAB), 0.01% hydrogen peroxide in 0.1M Tris (BDH), pH 7.5. The reaction was stopped by washing in water, followed by counterstaining (Carazzi’s haematoxylin, 45 s).

**DNA-DNA in situ hybridisation**

A digoxigenin-labeled, nick-translated (Boehringer Mannheim, Mannheim, Germany) genomic COPV DNA probe was used for DNA-DNA *in situ* hybridisation as described previously [Nicholls, 1999 #1058]. Briefly, after dewaxing and rehydration, sections were digested with proteinase K (50 µg/ml for 15 min at 37°C), washed in PBS, dehydrated and air-dried. The DNA probe in hybridisation buffer was applied, and the coverslipped slides were denatured (6 min at 95°C), quenched on ice, and hybridised overnight at 37°C. Stringency washing at room temperature in 0.5x SSC (3x 5 min), at 55°C in 50% formamide/1x SSC for 10 min, and at 55°C in 0.5x SSC (3x 5 min) was followed by alkaline phosphatase development overnight with BCIP/NBT substrate. Alternatively, *in situ* hybridization was carried out using
a tyramide signal amplification procedure (NEN Life Science Products, USA). The hybridised probe was detected using anti-Dig-HRP and peroxidase activity was detected with coumarin tyramide.

To control for background associated with the different detection systems, such as non-specific binding of the anti-digoxigenin antibody, endogenous alkaline phophatase, or non-specific precipitation of the chromogen, the COPV DNA probe was omitted. To control for non-specific binding of probe DNA by the tissue, an irrelevant DNA probe (HPV-16 or ROPV DNA) was substituted for the COPV probe. To control for other false-positive signals and to establish background signal levels, pre-infection biopsies (week 0) were included. As positive controls, sections known to be positive for COPV virions by immunohistochemistry and electron microscopy were included in each run. Further acceptance criteria included the restriction of positive signals to the epithelium, more specifically to the keratinocyte nucleus, and an absence of signal in adjacent normal tissue. Control sections, not denatured by heat, were used to ensure that double stranded viral DNA, rather than viral RNA, was being detected. An additional control to rule out formation of DNA-RNA hybrids was to digest the section with RNaseA prior to hybridisation.

**Double-labeling of DNA and either L1 or E4 protein**

Sections were processed for detection of viral DNA as described above. After stopping the reaction in TE buffer for 10 min, the sections were washed in 0.2% Triton X-100 in PBS for 5 min before further washing in PBS (3x 3 min). The L1 or E4 primary antibody was applied overnight at 4°C, washed in PBS (3x 3 min) and the appropriate secondary antibody applied for 30 min at room temperature. After washing in PBS, peroxidase-conjugated avidin-biotin complex (Vectastain Elite, Vector Laboratories), or a secondary antibody conjugated to FITC or Texas red, was applied for 30 min at room temperature, and the sections were washed again before visualisation by fluorescent microscopy or development with DAB (peroxidase conjugates). The reaction was stopped by washing in water, followed by counterstaining (Carazzi’s haematoxylin, 45 s).

**Evaluation of mitotic index**

Mitotic figures were counted from haematoxylin and eosin stained sections, using a light microscope with 25x objective. An eyepiece graticule was used to define each field, within which mitoses
were counted. The mean count per field was established by counting all epithelial fields within the section, and repeating the count on a second section from the same time point.

**Generation of RNA probes**

Segments were amplified by PCR from the reference plasmid (COPV genome in pBR322). Probe length was restricted to between 200-500 bp to ensure good tissue penetration whilst maintaining high specificity. The primers added EcoRI and BamH1 as flanking sequences, enabling directional cloning into the vector pGEM3Z (Promega). After transformation of *E. coli* with the riboprobe constructs, small scale DNA preparations were made using the Qiaprep Spin Mini-prep Kit (Qiagen) according to the manufacturer’s instructions. All constructs were verified using the T7 sequencing kit (Pharmacia). The DNA templates were linearised by restriction digestions with either EcoRI or BamHI, and labeled by *in vitro* transcription using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

**RNA-RNA in situ hybridisation**

Sections on Vectabond-coated slides (Vector laboratories) were dewaxed and rehydrated, washed in PBS (2x 3 min) and encircled with a silicone pen (DAKO). The tissues were immersed in 4% paraformaldehyde with 5mM MgCl₂ in PBS for 5 min and re-washed in PBS (2x 3 min). After digestion in RNAse-free DNAse (15 U/ml) in 2x SSC for 1 hour at 37°C in a humid chamber, the sections were washed in PBS (2x 3 min) and digested in Protease K (50 µg/ml) for 15 min at 37°C. Sections were then washed in PBS (2x 3 min) and re-fixed as above for 5 min. After a final wash in PBS (2x 3 min), the sections were dehydrated through graded ethanol and air dried. The section was covered in RNA probe diluted 1:10 or greater in hybridisation buffer (2x SSC, 5% dextran sulphate, 0.2% Marvel (Premier Beverages), 50% deionised formamide), according to labeling efficiency, coverslipped and sealed with cow gum (Cow Proofings Ltd.). Slides were incubated overnight at 42°C.

The gum was removed and the coverslips soaked off in x SSC, 0.1% SDS. Slides were washed at 55°C in 2x SSC, 0.1% SDS for 5 min then incubated in RNAaseA (2 mg/ml in 5x SSC) for 15 min at 37°C. After washing in 0.1x SSC, 0.04% SDS (2x 20 min, 65°C), and soaking in blocking buffer, sections were
developed as for DNA in situ hybridisation. Counterstains were either Carazzi’s haematoxylin (30-45 seconds), or 1% aqueous light green (60 seconds).

To control for false-positive signals due to non-specific binding of the anti-digoxigenin antibody, or from non-specific precipitation of the chromogen, the RNA probe was omitted. To control for non-specific binding of the RNA probe, probes of the opposite orientation (sense probes) were used. Pre-infection tissue acted as a negative control for other false-positive signals and as a means of assessing the level of background signals. To prevent binding to DNA, tissues were not subjected to heat denaturation and were incubated in RNAse-free DNase to ensure that no single-stranded DNA targets were present. Further acceptance criteria included the restriction of signal to the cytoplasm of epithelial cells with no significant signal in adjacent non-infected epithelium or the underlying dermis.

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REFERENCES
Detection of Viral DNA and E4 Protein in Basal Keratinocytes of Experimental Canine Oral Papillomavirus Lesions

Philip K. Nicholls,*1 John Doorbar,† Richard A. Moore,* Woei Peh,† Davina M. Anderson,* and Margaret A. Stanley.*

*Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, United Kingdom; †National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, United Kingdom

1To whom correspondence should be addressed:
Philip K. Nicholls
Division of Veterinary & Biomedical Sciences
Murdoch University
South Street
Murdoch
WA 6150
Australia

Telephone: +61 8 9360 2599
Facsimile: +61 8 9310 4144
E-mail: nicholls@numbat.murdoch.edu.au
ABSTRACT

We studied experimental canine oral papillomavirus (COPV) infection by in situ hybridization, and immunohistochemistry of weekly biopsies. After four-weeks, viral DNA in rete ridges suggested a keratinocyte stem cell target. Abundant viral DNA was seen only in E4-positive cells. E4 was predominantly cytoplasmic but also nuclear, being concentrated in the nucleoli during wart formation. Infected cells spread laterally along the basal layer and into the parabasal layers, accompanied by E7 transcription and increased mitoses. Most of the lower epithelium was positive for viral DNA but, in mature warts, higher levels of E4 expression and genome amplification occurred in only sporadic superficial cells. L1 expression was late and in only a subset of E4-positive cells. During regression, viral DNA was less abundant in deep epithelial layers, suggesting down-regulation of replication prior to replacement of infected cells from beneath. Detection of viral DNA in post-regression tissue indicated latent infection.

Key Words: COPV; gene expression; life cycle; DNA replication; E4

INTRODUCTION

The involvement of human papillomaviruses (HPVs) in cancer of the cervix is well-established, with nearly all cervical carcinomas containing HPV DNA [Walboomers, 1999 #1056]. Furthermore, over 130 HPV types have been cloned or identified by PCR detection of capsid (L1) DNA [de Villiers, 1997 #227], and the association between certain HPV types and cervical cancer is strong enough for these ‘high-risk’ HPVs to have been defined as carcinogens [Anonymous, 1996 #26]. In addition to their association with human epithelial malignancies, papillomaviruses cause a variety of benign tumours of cutaneous and mucosal epithelia [Shah, 1996 #829]. Although benign, many of these warty lesions often respond poorly to a variety of treatments [Beutner, 1997 #60], and are sometimes persistent or progressive, especially in people immunosuppressed from either HIV infection or as a result of organ transplantation [Palefsky, 1998 #698]. In addition to the efforts currently being made to find immunologically-based prophylactic or therapeutic strategies, it is clear from the diseases seen in immunosuppressed individuals that there is a need for non-immunological treatments [Stanley, 1997 #876; Phelps, 1998 #728]. Such antiviral strategies require detailed knowledge of the papillomavirus life cycle. The technical demands of in vitro culture of these viruses, and their species specificity, present difficulties in examining the viral life cycle. Additionally, it
seems that organotypic keratinocyte cultures may not accurately model all aspects of intact epithelia [Mayer, 1998 #570]. Detailed chronological analysis of the papillomavirus infectious cycle is impossible in human clinical material since the time of naturally-acquired infections is difficult to establish, making the chronology of events in subsequent biopsies uncertain. Because multiple sequential biopsies are rarely justified on clinical grounds in the human patient, the experimenter is left with only 'snapshots' of the disease taken from many different patients and lacking in coherence. Although viral DNA and RNA have been examined in biopsies of human clinical material (both warts and neoplasms), similar studies of viral DNA and RNA in the early stages of infection have not been possible because the lesions become macroscopically detectable only after a period of several weeks, and there is a further delay before patients present to clinics for examination and treatment. These problems mean that although molecular biology has enabled great progress to be made in understanding some of the details of virus-cell interactions, there remain large gaps in the understanding of papillomavirus biology. To overcome these difficulties, papillomavirus infections in animals such as the rabbit or ox have been studied, and animal models such as these have been described as the "gold standard" with which in vitro models should be compared [Chow, 1997 #172].

In the CRPV-infected rabbit, analysis of biopsies at different times post-infection has provided important data on the viral life cycle [Schmitt, 1996 #800]. However, CRPV infection involves haired-skin and there are important biological differences, especially with regard to stem cell location, between haired [Cotsarelis, 1990 #200; Michel, 1996 #590] and mucosal epithelia [Lavker, 1982 #516; Hume, 1983 #401]. Since many of the important human papillomavirus infections affect the anogenital mucosa, studies of viral biology in a mucosal model are important. One such model, palatine infection by bovine papillomavirus type 4 (BPV-4) in calves, has been used to study temporal and spatial expression of viral proteins [Anderson, 1997 #19]. However, RNA in situ hybridisation was not undertaken, and viral DNA proved undetectable in basal or suprabasal keratinocytes with the technique used.

Canine oral papillomavirus (COPV), reviewed in [Nicholls, 1999 #1057], has been used for the development of vaccines both historically [Chambers, 1960 #145] and more recently [Bell, 1994 #52; Ghim, 1995 #316]. Furthermore, COPV has been used to establish the efficacy of DNA vaccines (our unpublished data). Some COPV infections have similarities to HPV-associated recurrent laryngeal
papillomatosis [Nicholls, 1999 #1058]. Since the dog is a useful animal model of mucosal papillomavirus infections, it provides an important opportunity for studying the viral life cycle *in vivo*, allowing early biopsies to be taken in a controlled manner at accurately defined intervals after infection. In the COPV model, we have used a chronological series of biopsies, taken from infection through to regression, to analyse the viral life cycle by means of histopathology, *in situ* hybridisation, and immunohistochemistry. We were able to detect viral RNA, DNA and protein during the early stages of infection within the basal and suprabasal keratinocytes of pre-clinical lesions, providing insight into the likely location of the target cell. Double-labeling of viral E4 and L1 proteins revealed both temporal and spatial differences in expression, in distinction to the findings of previous reports using material from human warts [Brown, 1994 #100; Brown, 1995 #101]. Additionally, double-labeling of viral DNA and viral E4 protein showed a strong colocalisation even at early time points, supporting the hypothesis that, in addition to its suggested role late in the viral life cycle [Doorbar, 1991 #248; Doorbar, 1996 #250], E4 may play a role in the earlier stages of infection, perhaps in viral DNA replication [Doorbar, 1997 #252]. The detection of abundant viral DNA and protein in basal keratinocytes of COPV lesions highlights the differences in viral biology between viral types, and indicates that generalizations about the biology of different papillomavirus types should be made with caution.

RESULTS

**Morphological changes during wart development and regression.**

Haematoxylin and eosin stained sections from tissues taken one to four weeks post-infection were morphologically similar to pre-infection controls. At week five, an increased mean mitotic rate (Fig. 1) was associated with expansion of the stratum spinosum (Fig. 2a). After further epithelial thickening, filiform papillae appeared, with koilocytes in spinosum and granulosum strata by week seven (Fig. 2b). Frozen sections revealed a prominent hyperkeratosis, predominantly orthokeratotic. In places, a focal infiltrate of inflammatory cells obscured the dermo-epidermal interface. By week eight, mature papillomas developed multiple projecting papillae rising 5-10 mm above the surrounding mucosal surface (Fig. 2c). Significant numbers of lymphocytes were present intraepithelially and around the interface of the epithelium and lamina propria, in some places obscuring the basement membrane zone. Occasional apoptotic keratinocytes were
seen in the lower layers. In one area the apex of one of the filiform papillae had undergone focal coagulative necrosis. By week nine the infiltration of inflammatory cells, mostly lymphocytes, was more marked and the papilloma had reduced in size (Fig. 2d). There was a prominent influx of neutrophils, with spongiosis and oedema. As the papillomas resolved during week ten, the epithelium reformed, although there were still patchy foci of intraepithelial and subepithelial inflammation. By week eleven, the oral mucosa appeared morphologically normal and the cycle of papilloma progression and regression was complete.

**Analysis of viral location and replication by DNA *in situ* hybridisation**

To identify sites of viral DNA replication during wart formation and regression, the chronological series of biopsies was examined by DNA *in situ* hybridisation using a digoxigenin-labeled COPV genomic probe. No DNA was detectable in pre-infection sections, or from one to three weeks post-infection (Fig. 3a). Viral DNA was first detected at four weeks (Fig. 3b), in only some of the sections examined, and was restricted to scattered foci of positive nuclei in the basal layer or immediately above it. The tight clusters of positive cells, suggested a clonal origin. In places, these early foci were found at the lower tips of the epithelial projections or rete ridges (Fig. 3c). Some sections revealed early foci in the saddle region between the rete ridges. Occasional foci of positive nuclei in a suprabasal position, apparently unconnected to the basal layer, were tracked back to the basal layer or deep rete ridges by *in situ* hybridisation of serial sections.

From week five to week six, more positive cells appeared within the basal and suprabasal layers, until nearly every cell within the basal layer and layers immediately above was positive (Fig. 3d). The foci where viral DNA was detectable correlated with focal increases in epithelial thickness and mitotic rate, demonstrating a link between viral replication and keratinocyte division.

As the papilloma matured through weeks seven and eight, only certain superficial keratinocytes appeared to permit the virus to replicate to a higher copy number (Fig. 3e). This was in contrast to the situation in the lower layers, in which almost every keratinocyte was positive for viral DNA. It was these superficial, strongly-positive keratinocytes, apparently supporting high level genome amplification, which had vacuolated cytoplasm typical of koilocytes. As the papillomas regressed during week nine, less DNA
was detected in the lower layers of the epithelium (Fig. 3f) despite remaining prominent more superficially, until no signal remained by week ten.

**Detection of transcripts from the E2/E4 region**

Probes to the E2 open reading frame were made complementary to regions spanning nucleotides 2694 to 3058 (E2 probe A), and 3460 to 3710 (E2 probe B). These segments flank that part of the E2 region encoding E4 within a different reading frame. E2 probe A did not however allow reliable detection of transcripts and was not extensively used. By contrast, E2 probe B detected abundant transcripts within both experimental and clinical lesions. In the mature clinical lesions, signal was located mostly within the basal layer, but was found also within the other lower epithelial layers and extended superficially within a subset of cells. In the experimental series, signal was abundant basally through weeks five to eight (Fig. 4a) and declined as the wart regressed. Signal was present in a subset of suprabasal and more superficial layers in the maturing wart (Fig. 4b) and was absent by week 10. The E4 riboprobe was complementary to the region between nucleotides 3193 and 3429. The probe detected abundant transcripts within the mature clinical lesions. The signal was abundant in basal layer keratinocytes, rete ridges, and in sporadic cells throughout the lower half of the epidermis but on occasions was confined to the basal layer. Within the experimental series, transcripts from the E4 ORF were first detected at four weeks post-infection in the basal and immediately suprabasal layers (Fig. 4c). The transcripts were found in abundance from week five (Fig. 4d) and throughout wart progression (Fig. 4e), beginning to fade from week nine as the warts regressed, and disappearing by week 10. E2 probe B and the E4 probe are both predicted to detect all early transcripts, as well as transcripts originating from the differentiation dependent promoter, which in other papillomavirus types is situated upstream of E1. The strength of the signal and the similarities between the staining patterns of the two probes suggests both are detecting the E1^E4 message which is expressed at high level by other papillomavirus types.

**Detection of transcripts from the E6/E7 region**

The E7 probe was complementary to a region between nucleotides 575 and 740. The probe detected abundant transcripts in mature clinical papillomas. Signal was present in the basal layer and a
subset of more superficial keratinocytes. In the experimental series, signal was first detected at four weeks post-infection, in only the lowest layers of epithelium. By week five the signal was more abundant, especially in the rete ridges. As the lesions progressed, some keratinocytes in more superficial layers contained abundant transcripts (Fig. 4f). The signal was present in numerous superficial cells by week eight, declining thereafter, until absent by week 10. The E6 probe, complementary to the region between nucleotides 150 and 492, did not reliably detect transcripts and was not extensively studied.

**Detection of transcripts from the L1/L2 region**

The L1 probe was complementary to a region between nucleotides 7397 and 7755. Naturally-occurring canine oral papillomas contained abundant transcripts from the L1 ORF in only the stratum granulosum or uppermost stratum spinosum. No signal was ever detected below this level. In the experimental series, L1 transcripts were first detected in small amounts at seven weeks post-infection, in the superficial keratinocytes. The transcripts were abundant by week eight (Fig. 4g), and still present at week nine. No transcripts were present at weeks 10 and 11. Transcripts were present in both cytoplasmic and nuclear locations.

The L2 probe spanned a region from nucleotides 5771 to 6110. Transcripts were present in the superficial keratinocytes of mature clinical lesions, but not in keratinocytes of the lower epithelial layers. In the experimental series, signals were not detected until week seven, as for the L1 transcripts. Transcripts were found only in superficial keratinocytes, and became more abundant by week eight (Fig. 4h). Some signal remained at week nine, but none was detected at weeks 10 or 11. Nuclear signal was occasionally seen with the sense control probe, sometimes concentrated over the nucleoli (data not shown).

**Expression of the L1 capsid protein during the time course of infection**

Although RNA *in situ* hybridisation provides some insight into the pattern of viral gene expression during wart formation, papillomavirus mRNAs have complex splicing patterns, and the detection of RNA from a viral open reading frame does not necessarily indicate synthesis of viral proteins. Polycistronic transcripts are not necessarily expressed throughout their length, and in other papillomavirus types, post-transcriptional mechanisms preventing protein expression have been reported. To determine the expression
patterns of viral proteins during wart formation and regression, immunohistochemistry was undertaken using antibodies specific to three of the viral proteins - E4, E7 and L1.

The presence of L1 protein correlated well with detection of L1 transcripts, except that the L1 gene products were restricted to the nucleus in the stratum granulosum, becoming cytoplasmic only in the stratum corneum where nuclear disintegration is likely. No L1 protein was seen in pre-infection controls, or from weeks one to six inclusive. An occasional positive nucleus was seen in the stratum granulosum at week seven. By week eight there were numerous positive keratinocyte nuclei in the stratum granulosum, with the signal extending into the stratum corneum (Fig. 5a). Only an occasional positive cell was found during early regression at week nine and no virus capsid was detectable by weeks 10 and 11.

**Expression of the viral E4 protein during the time course of infection**

E4 is abundantly expressed in lesions caused by several human papillomavirus types. The role of E4 in the virus life cycle is unclear, and its expression pattern during wart formation and regression has not been established. To generate antisera, immunisations were carried out using a glutathione S transferase COPV E4 fusion protein expressed in bacteria. Antibody specificity was confirmed by Western blotting using maltose binding protein (MBP) fusions containing the E4 gene products from COPV, ROPV or HPV 11. Reactivity was apparent only with the COPV E4 fusion (data not shown).

In mature clinical COPV papillomas, immunostaining revealed a predominantly cytoplasmic signal for E4 in sporadic cells of the basal layer and above (Fig. 5b). The E4 staining pattern was very heterogeneous. In some cells the protein existed as cytoplasmic inclusions while in others the staining was diffuse and extended through the nucleus (Fig. 5c). Where nuclear staining was apparent, the E4 protein was concentrated around the periphery of the nucleoli (Fig. 5d). In the experimental series, E4 was first detected at week four - and until week six, staining was apparent in the lower epithelial layers at levels close to the limits of detection (Fig. 6a). By week six, sporadic cells showed high level E4 expression (as apparent from immunostaining), with levels of expression increasing from week seven to week eight (Fig. 6b). In the experimental lesions, E4 could be detected both in the nucleoli as well as the cytoplasm at weeks seven and eight (Fig. 6c). This distribution, which was not apparent in established clinical material
was confirmed by confocal microscopy and phase contrast imaging (Fig. 6d). In both the experimental and clinical material E4 expression was first detected in the basal cells. E4 protein remained at weeks eight (Fig. 6e) and nine, and was apparent at the advanced stages of regression. No E4 was detectable at weeks 10 or 11.

**Correlation of viral protein expression with genome amplification by double staining**

Some reports have indicated that the expression of E4 colocalises exactly with that of L1 [Brown, 1994 #100], whereas more recent work indicates that L1 expression is accompanied by expression of E4, but that the converse is not true, with E4 appearing additionally in lower epithelial layers [Doorbar, 1997 #252]. COPV lesions were first stained for L1 and developed to give a dark purple reaction product. After washing, the same sections were stained for E4 and developed to give a brown reaction product. Although E4 was expressed in the absence of L1 in the lower epithelial layers, L1 expression occurred only in E4 positive cells in the superficial layers (Fig. 6f). These correlations were seen in established clinical biopsies, and from week seven in the experimental time course. To determine whether viral DNA replication and E4 expression coincided during the course of COPV infection, immunolocalisation was carried out on sections previously hybridised to detect viral DNA. The sensitivity of antibody staining was increased by the *in situ* hybridisation step, while the nuclear location of viral DNA and the predominantly cytoplasmic location of E4, enabled double labeling to be interpreted clearly. Expression of E4 correlated closely with viral DNA replication at all time points and both occurred in the basal and more superficial cell layers. These relationships were demonstrated from week four onwards although at early time points (weeks four to six) the level of E4 expression was close to the sensitivity of detection by immunostaining (Fig. 7a,b). At early time points all of the E4-positive cells in the parabasal layers were found to be supporting viral DNA replication. In clinical lesions, and between weeks seven and eight in the experimental warts, sporadic cells could be detected which had higher levels of E4 and higher levels of DNA replication (Fig. 7c,d). As previously reported, high level expression of E4 coincided closely with vegetative viral DNA replication, although occasional cells were apparent which were positive for DNA replication yet negative for E4 and vice versa.
L1 expression did not colocalise with DNA amplification in the lower half of the epithelium, since L1 protein was undetectable despite the presence of abundant viral DNA. Furthermore, viral genome amplification was detectable from week four, whereas L1 was not seen until week seven. In the mature lesions, L1 colocalised with DNA in the upper epithelium, as the L1 signal overlaid the darker DNA signal when enzymatic detection was carried out. This was confirmed by immunofluorescence double staining.

**DISCUSSION**

The delay before COPV lesion appearance is well-documented [Chambers, 1959 #144; Konishi, 1972 #479; Bell, 1994 #52], and a delay of four weeks before COPV DNA is detectable is similar to that described in other models [Sterling, 1990 #884; Stoler, 1990 #891]. COPV infection by injection, rather than scarification, prolongs this lag phase (our unpublished data), presumably because the reduced epithelial trauma provides little stimulus for epithelial repair and stem cell division. Stimulation of viral DNA replication following epithelial trauma has been well-documented [Amella, 1994 #12; Campo, 1994 #123; Whiteley, 1956 #1022; Whiteley, 1957 #1023]. In haired skin, a likely site of stem cells is the follicular bulge region [Cotsarelis, 1990 #200], where early CRPV gene expression was found [Schmitt, 1996 #800], as well as interfollicular epidermis [Miller, 1993 #595]. In non-haired epithelia, stem cells are thought to be located at the tips of rete ridges, based on the detection of slowly-cycling cells in this area [Lavker, 1982 #516; Hume, 1983 #401]. More recent studies have suggested that keratin-19 is a marker for stem cells, with cells within the bulge region of the hair follicle labeling with keratin-19 and retaining a tritiated thymidine label [Michel, 1996 #590]. The same work localised these putative stem cells to the deep tips of rete ridges of non-haired skin. Early COPV lesions showed a distribution of viral DNA and RNA consistent with infection of a stem cell at this site, and the apparent subsequent tracking up the side of the ridge toward the shoulder region resembles proposed trafficking from the stem cell through transient amplifying compartments and into post-mitotic epithelium [Lavker, 1983 #517]. The data presented here are compatible with the idea that the virus infects a basal keratinocyte [McMillan, 1999 #1062; Evander, 1997 #266], most likely a slowly-cycling keratinocyte stem cell [Stanley, 1994 #875].

It has previously been reported that viral DNA amplification does not occur in the basal layer, with viral DNA being detectable in only the more superficial epithelial layers. For instance high copy numbers
of BPV-4 DNA were seen only in the spinous and squamous layers of papilloma fronds [Anderson, 1997 #19], and in recent studies of HPV-1 biopsies no viral DNA or RNA could be detected in basal keratinocytes [Egawa, 2000 #1083]. HPV DNA was detected in the basal layer in CIN 3 lesions, but in a pattern suggesting viral integration [Cooper, 1991 #199] rather than as a normal part of the virus life cycle.

In this study, COPV DNA was readily detected in basal keratinocytes, indicating a substantial level of DNA replication at this level. The failure of other studies to detect DNA in the basal layer of the epidermis may be due to technical factors, since the abundant DNA in more superficial keratinocytes gives a strong signal even under sub-optimal conditions. Both protease K digestion and the degree of stringency during washing were critical in detecting DNA in basal keratinocytes. It is possible that not all papillomavirus types produce such abundant DNA, and that basal layer levels are below the sensitivity of detection. Interestingly, COPV is related to the cutaneous HPV types 1 and 63 [Delius, 1994 #228] and abundant low level, although not basal, DNA replication has been described in HPV-1 [Doorbar, 1997 #252; Egawa, 2000 #1083]. It is also possible that established clinical biopsies are taken at a time when basal layer viral replication has been reduced, perhaps in response to moderation by the host defences. We found that post-mature COPV warts in the early phases of regression lost the high levels of viral DNA in the lower epithelium, whilst maintaining abundant DNA more superficially. Interestingly, some in vitro systems have revealed a degree of HPV DNA amplification in cells of the basal layer, and this has been reported as challenging the concept that viral genome amplification is linked to differentiation [Ozbun, 1998 #694]. It should be noted that the basal layer is not made up from a single undifferentiated cell type but includes stem cells, transient amplifying cells and post-mitotic differentiating cells expressing keratin 10 (normally a spinous layer keratin) [Li, 1998 #531]. Caution is required in interpreting such data, since raft cultures differ from normal tissue in their differentiation pattern, with filaggrin expression being seen in basal cells rather than just the granular and cornified layers [Mayer, 1998 #570].

The initial multifocal clustering of COPV DNA-positive cells is consistent with their origin from keratinocyte stem cells, which make up only a few percent of cells in the basal layer. Nearly every cell at the basal and several suprabasal strata of the epidermis appeared permissive for viral replication to a moderately high copy number. Viral DNA in the middle epidermis can act only as template for mRNA, or for further DNA replication, since the absence of capsid proteins prevents its incorporation into viral
particles. Why only a certain fraction of more superficial epithelial cells take DNA replication through to an even higher copy number is not clear, but this presumably reflects either a uniquely permissive environment within these cells or the result of chance factors and competing influences with a bistable outcome. These koilocytes were the cells in which virus was assembled, as revealed by double-labeling for viral DNA and L1 capsid protein. Polyclonal antibodies against GST fusion proteins of full length COPV E2 and E7 also revealed staining in the nuclei of koilocytes of mature papillomas (our unpublished observations). The same patchy distribution of cells with high DNA copy number has been seen in vitro [Ozbun, 1998 #694]. A greater understanding of the factors controlling this permissive environment might provide insight into possible avenues for therapeutic intervention.

The disappearance of viral DNA noted in lower layers of regressing warts, seen also in BPV-4 lesions [Anderson, 1997 #19], could arise through several mechanisms. One possibility is that all the (stem) cells in which viral DNA resides are removed, presumably by the marked cellular immune response seen during the late stages of regression. Destruction of the stable reservoir for viral DNA would result in the infected cells growing out toward the surface without being replaced from below. Another possibility is that viral replication is shut down in the infected cells, either permanently or to be reactivated after a period of latency, a theory supported by detection of COPV DNA in mucosa from sites of regressed papillomas (our unpublished data).

Although thought to have late functions, the pattern of E4 transcription and protein expression seen in this study appeared more typical of a papillomavirus early gene. The transcription pattern had an early onset, and represented the earliest detectable events during the time course of COPV infection. E4 specific transcripts (E1^E4 mRNAs) are the most abundant mRNA species seen in HPV-11 infected human foreskin xenografts, and are located predominantly in the mid-epithelial and more differentiated layers [Stoler, 1990 #891] as found here at late time points. Signals in the basal layers were attributed to the presence of E2 or E6/E7 transcripts spanning the E4 coding region. E2 probe B showed a similar staining pattern to that of the E4 probe, and it is likely that both are detecting the full range of early messages. Identification of the E4 protein at early time points, indicates that at least some of this message serves as a template for the synthesis of E4 protein. A similar distribution was apparent for E7 mRNAs which were found in basal keratinocytes as well as in a subset of cells within the stratum spinosum and stratum granulosum. This is
similar to the pattern previously reported in HPV-11-infected foreskin xenografts where E6-E7 transcripts were detectable from week six, although parabasally at first, and then in the middle epithelial layers [Stoler, 1990 #891]. Similarly, in CRPV-induced lesions, E6-E7 transcripts are detected in the hair follicles at day 11 [Schmitt, 1996 #800] and become stronger in the basal layers by day 45. E7 mRNAs are detected throughout the epithelium in established lesions caused by both HPV-16 and CRPV [Zeltner, 1994 #1046; Higgins, 1991 #381; Higgins, 1992 #382]. The E7 protein has been detected immunohistochemically in BPV-4 lesions [Anderson, 1997 #19], localising to the nucleus in basal and suprabasal cells, and to the cytoplasm in the spinous and squamous layers.

Transcription from late regions was markedly different from that of the other ORFs. A similar pattern of late gene transcription has been seen in HPV-11 infected foreskin xenografts [Stoler, 1990 #891]. L1 antigen was detectable from week eight and correlated with L1 message, as seen here with the COPV lesions. The intensity of staining suggested that the L1 transcripts are more abundant than those of L2. L1 transcripts outnumber L2 transcripts in HPV31-containing keratinocytes cultured organotypically [Ozbun, 1997 #693], and the L1 protein is expressed at higher levels than L2 in cutaneous human warts [Doorbar, 1987 #244]. In the COPV lesions, cells containing L2/L1 transcripts and L1 protein had swollen pale cytoplasm, as reported with BPV-4 [Gaukroger, 1989 #308; Anderson, 1997 #19]. These koilocytic cells additionally contained high levels of viral DNA. Despite the abundant viral DNA and E4 in the lower epithelium, no L1 was detected in the lower half of the epidermis. This is in contrast to HPV-1 lesions, in which L1 transcripts and protein were detected in suprabasal layers [Elzein, 1991 #1067].

RNA in situ hybridisation showed clearly different transcription patterns from the COPV L1 and E4 open reading frames. This is not consistent with earlier reports that L1 and E4 proteins colocalise at the cellular level [Brown, 1994 #100; Brown, 1995 #101]. Interestingly, in the study of Brown et al the E4 protein did not precede the L1 protein, despite the presence of E4 transcripts in lower layers than L1 transcripts [Brown, 1995 #101]. In COPV lesions, good correlation between E4 mRNA and E4 protein was found. E4 was expressed early on during infection, and in cells of the basal layer as well as more superficially. E4 expression preceded L1 expression, although L1 was always accompanied by E4 expression in the same cell.
Although E4 has been reported as being nuclear in location [Palefsky, 1991 #697], recent work with HPV-16 lesions showed that E4 expression, although variable in amount, correlated exactly with DNA replication but not L1 expression, and was cytoplasmic [Doorbar, 1997 #252]. The cytoplasmic location of E4 within cells of the granulosum and upper spinosum strata was confirmed by immunoelectron microscopy of athymic mouse HPV-16 xenografts [Sterling, 1993 #885], with viral capsids detected microscopically and by immunogold staining (L1) in the nucleoplasm of the same cells. The association of E4 with cytoskeletal elements (keratin tonofibrils) was demonstrated in the same report. L1 expression is preceded by E4 expression, but colocalises with E4 in the superficial layers (Doorbar et al., 1997), a finding mirrored in the COPV lesions described in this study. E4 expression was seen in high grade HPV-16 lesions which no longer generated L1. This dissociation of L1 and E4 expression is demonstrated also by the lack of effect of protein kinase C activators on E4 expression in raft cultures, whereas L1 expression is upregulated [Meyers, 1992 #588]. E4 of HPV-31b was detected in rare cells in monolayer culture, but its expression was upregulated upon differentiation of the same cells in raft culture [Pray, 1995 #735]. The E4 protein was located mostly in spinosum and granulosum strata, occasionally extending into the stratum corneum. Whilst occasional (<1%) basal cells were described as expressing E4, none was shown in the report. Basal expression of COPV E4 was clearly present in this study. In the HPV-31b study, as in this study, most L1 positive cells also expressed E1^E4, but the converse was not true.

The finding that E4 is expressed early on in the virus life cycle and in basal cells, and is dissociated from expression of L1, suggests that E4 is an early protein in COPV. These findings do not support the theory that E4 is a late protein with a role in releasing virus from cells [Doorbar, 1991 #248; Doorbar, 1996 #250], although it may be multi-functional. A role for E4 in DNA replication has been suggested [Doorbar, 1997 #252], and a close correlation was found between the detection of high copy number viral DNA and COPV E4 protein both temporarily and spatially. A similar correlation has previously been reported in other species. E4 and L1 are more frequent in genital warts from pregnant women when compared with non-pregnant women or men [Brown, 1992 #98], and this increased expression is associated with a higher HPV DNA copy number. In mucosal lesions caused by HPV-16, and in cutaneous lesions caused by HPV-1 and HPV-63, the onset of genome amplification coincides closely with E4 expression [Doorbar, 1997 #252], while in lesions caused by BPV-4 a similar correlation has been
reported [Anderson, 1997 #19]. Colocalisation of E4 with genome amplification has also been demonstrated in HPV-infected keratinocytes grown in methylcellulose suspension [Ruesch, 1998 #782]. When taken together, these findings suggest a role of E4 in vegetative viral DNA replication, perhaps by sequestering cellular factors which inhibit episomal replication [Breitburd, 1987 #92]. The two proposed functions of E4, cytoskeletal interactions to assist viral egress (a late function) and involvement in DNA replication might not therefore be mutually exclusive. Colocalisation does not of course prove causality, and it has been suggested that vegetative viral DNA replication may permit expression of E4, L1 and L2 by titrating out cellular factors normally inhibiting the late promoter [Frattini, 1996 #290].

The work presented here has established the pattern of events during the formation and regression of COPV-induced papillomas. Following infection, a lag phase of 3-4 weeks occurs with little viral DNA amplification or gene transcription, which we speculate could represent the delay before division of the infected stem cell. Cellular DNA replication begins soon after this, and is probably triggered by E6 and E7 once the viral DNA is passed to the daughter cells. Rapid proliferation of keratinocytes, as illustrated by the increased mitotic count and epithelial thickness is seen from week five. These rapidly dividing keratinocytes pass into the parabasal cell layers carrying viral DNA with them, and a subset initiate genome amplification. These events begin near the stem cell regions of the rete ridges, as revealed by RNA/DNA in situ hybridisation, before the infected cells move up the sides of the rete ridges and spread out to occupy most of the basal layer. At this stage most of the cells are positive for viral DNA by in situ hybridisation, and transcripts for E4, E2 and E7 are detected in abundance. Only a subpopulation of the infected keratinocytes support vegetative viral DNA replication and express E4 at high levels, as illustrated by the patchy distribution of viral DNA, RNA transcripts, and protein, in the spinous and granular layers. Transcription of the late genes (L1 and L2) is not seen below the granular layer, despite high copy numbers of viral DNA and abundant transcripts from the early regions. In human papillomaviruses, the late regions are under control of a differentiation-dependant promoter, which becomes active in the superficial epithelium. Infectious virus is generated in the stratum granulosum as detected by immunohistochemistry and electron microscopy.

By the time the wart reaches maturity, the abundant viral proteins have stimulated host immunity. The cellular immune response causes shut down of viral activity by killing infected keratinocytes or
reducing viral transcription via interferons. A combination of keratinocyte apoptosis and necrosis, coupled with the shut down of viral activity in the lower epithelium, lowers the mitotic rate and reduces the wart volume. This is reflected by the reduced signals for DNA and RNA in situ hybridisation that is seen in the lower epithelium around weeks eight and nine. Transcription in the upper regions of the wart papillae is unaffected by these immune events, as seen by the continued presence of transcripts in the superficial keratinocytes. Eventually all viral-infected cells are replaced from below by new keratinocytes, which may be the progeny of uninfected stem cells from the edge of the lesion. The life cycle is complete. If some cells remain infected with viral DNA (as suggested by PCR), then a latent infection is present, may be reactivated either by immunosuppression or stimulation of the epithelium by wounding.

Although COPV is a mucosal papillomavirus, at the nucleotide sequence level it is more closely related to the cutaneous HPV types 1 and 63, with minimal sequence similarity to mucosatropic HPVs, including the oral HPV types 13 and 32 [Delius, 1994 #228]. The abundant viral replication and expression of E4 low in the epidermis, and the high level of virion production in COPV, are similar to findings in the related human papillomaviruses, HPV-1 and HPV-63, suggesting that the biological properties of papillomaviruses may not correlate well with their preferred target tissues, and that the often-cited distinction between mucosatropic and cutaneous PV types is somewhat arbitrary. Even the closely related HPV-1 and HPV-63 cutaneous HPVs have different E4 staining characteristics, with coalescing intracytoplasmic inclusions in the former and fibrous / granular distribution in the latter [Doorbar, 1997 #252]. It seems that there may be no one ‘typical’ pattern of papillomavirus infection since the different papillomavirus types appear to differ from one another quite markedly with respect to onset of vegetative DNA replication, timing and pattern of E4 expression, and amount of infectious virus produced. It is clearly difficult to generalize about the virus life cycle across so many widely differing viral types, and an understanding of these differences in viral biology could prove important in attempts to develop therapeutic interventions for papillomavirus-related disease.
MATERIALS AND METHODS

Experimental infection

Two female, 10-12 week beagles were housed together in purpose-built, climate-controlled accommodation, in an airspace separate from other dogs in the building, with a virucidal footbath at the common entrance and exit. All staff changed their protective clothing (gown, gloves, boots) on entering the controlled area. Under general anaesthesia, the mucosa of the upper lip was lightly scarified over an area approximately 5 mm x 5 mm, using the tip of a scalpel blade, until a light ooze of blood occurred. Neat COPV wart suspension [Bell, 1994 #52] (a kind gift from A. B. Jenson, Georgetown University, Washington, D. C.) was applied (10 µl) to each site by pipette and allowed to absorb for a few minutes. The site of challenge was marked by Indian ink tattoo points in a triangle centred on the site. Twelve sites were infected in each dog. Control oral mucosal biopsies were taken prior to challenge.

Under general anaesthesia, further weekly biopsies of the oral mucosa were taken using a 6 mm punch biopsy or scalpel followed by suturing as appropriate. Where gross lesions had not yet developed, tissue was taken from the tattoo-marked sites. The biopsies were fixed in 10% neutral buffered formalin then processed after 4-8 hours to paraffin wax for routine (5-7 µm) sectioning. At the end of the experiment all animals were re-homed.

Immunohistochemistry

Immunohistochemistry of formalin-fixed, paraffin-embedded sections was performed using a secondary antibody conjugated to either FITC or Texas red (Amersham, UK), or using an avidin-biotin complex (Vectastain ABC, Vector Laboratories), as previously described [Nicholls, 1999 #1058]. Positive control sections were canine oral papillomas with virions confirmed by transmission electron microscopy. Pre-infection biopsies acted as negative controls. Omission of primary or secondary antibody provided further negative controls.

For the detection of L1, the primary antibody was CAMVIR-1 mouse IgG2a monoclonal [McLean, 1990 #581] diluted 1:1000 in PBS, with biotinylated polyclonal goat anti-mouse IgG2a (Amersham, UK), diluted 1:400 in PBS, as secondary antibody. For the detection of E4, the primary antibody was polyclonal rabbit anti-COPV E4-GST fusion protein diluted 1:2000 in PBS, with biotinylated polyclonal goat anti-
rabbit (DAKO E432), diluted 1:400 in PBS, as secondary antibody. FITC or Texas red conjugated antibodies (Amersham N1031, N2031) were used at a dilution of 1:50. The polyclonal E7 antibody was prepared by immunisation of a rabbit with a full-length COPV-E7 GST fusion protein expressed and purified from *E. coli*. The E7 antibody was pre-absorbed against GST protein and used at 1:600 in PBS containing 10% normal goat serum, with the same secondary antibodies as for E4 immunohistochemistry.

**Double-labeling immunohistochemistry for L1 and E4**

Sections were treated as for L1 immunostaining, except an alkaline phosphatase tertiary complex (Vectastain ABC, Vector Laboratories) was used for colour development with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT) as the substrate. The reaction was stopped by immersion in TE buffer for 20 min before the second label was applied. For the second label, sections were washed in 0.2% Triton X-100 in PBS for 5 min, followed by washing in PBS (3x 3 min). The second primary antibody (for E4) was applied overnight at 4°C. The sections were washed as before, then the secondary antibody (peroxidase-conjugated swine anti-rabbit (DAKO P217) diluted 1:400 in PBS) was applied for 30 min at room temperature. After further washing, the label was developed with 0.06% 3,3' diaminobenzidine tetrahydrochloride (DAB), 0.01% hydrogen peroxide in 0.1M Tris (BDH), pH 7.5. The reaction was stopped by washing in water, followed by counterstaining (Carazzi's haematoxylin, 45 s).

**DNA-DNA *in situ* hybridisation**

A digoxigenin-labeled, nick-translated (Boehringer Mannheim, Mannheim, Germany) genomic COPV DNA probe was used for DNA-DNA *in situ* hybridisation as described previously [Nicholls, 1999 #1058]. Briefly, after dewaxing and rehydration, sections were digested with proteinase K (50 µg/ml for 15 min at 37°C), washed in PBS, dehydrated and air-dried. The DNA probe in hybridisation buffer was applied, and the coverslipped slides were denatured (6 min at 95°C), quenched on ice, and hybridised overnight at 37°C. Stringency washing at room temperature in 0.5x SSC (3x 5 min), at 55°C in 50% formamide/1x SSC for 10 min, and at 55°C in 0.5x SSC (3x 5 min) was followed by alkaline phosphatase development overnight with BCIP/NBT substrate. Alternatively, *in situ* hybridization was carried out using
a tyramide signal amplification procedure (NEN Life Science Products, USA). The hybridised probe was detected using anti-Dig-HRP and peroxidase activity was detected with coumarin tyramide.

To control for background associated with the different detection systems, such as non-specific binding of the anti-digoxigenin antibody, endogenous alkaline phosphatase, or non-specific precipitation of the chromogen, the COPV DNA probe was omitted. To control for non-specific binding of probe DNA by the tissue, an irrelevant DNA probe (HPV-16 or ROPV DNA) was substituted for the COPV probe. To control for other false-positive signals and to establish background signal levels, pre-infection biopsies (week 0) were included. As positive controls, sections known to be positive for COPV virions by immunohistochemistry and electron microscopy were included in each run. Further acceptance criteria included the restriction of positive signals to the epithelium, more specifically to the keratinocyte nucleus, and an absence of signal in adjacent normal tissue. Control sections, not denatured by heat, were used to ensure that double stranded viral DNA, rather than viral RNA, was being detected. An additional control to rule out formation of DNA-RNA hybrids was to digest the section with RNaseA prior to hybridisation.

**Double-labeling of DNA and either L1 or E4 protein**

Sections were processed for detection of viral DNA as described above. After stopping the reaction in TE buffer for 10 min, the sections were washed in 0.2% Triton X-100 in PBS for 5 min before further washing in PBS (3x 3 min). The L1 or E4 primary antibody was applied overnight at 4°C, washed in PBS (3x 3 min) and the appropriate secondary antibody applied for 30 min at room temperature. After washing in PBS, peroxidase-conjugated avidin-biotin complex (Vectastain Elite, Vector Laboratories), or a secondary antibody conjugated to FITC or Texas red, was applied for 30 min at room temperature, and the sections were washed again before visualisation by fluorescent microscopy or development with DAB (peroxidase conjugates). The reaction was stopped by washing in water, followed by counterstaining (Carazzi's haematoxylin, 45 s).

**Evaluation of mitotic index**

Mitotic figures were counted from haematoxylin and eosin stained sections, using a light microscope with 25x objective. An eyepiece graticule was used to define each field, within which mitoses
were counted. The mean count per field was established by counting all epithelial fields within the section, and repeating the count on a second section from the same time point.

**Generation of RNA probes**

Segments were amplified by PCR from the reference plasmid (COPV genome in pBR322). Probe length was restricted to between 200-500 bp to ensure good tissue penetration whilst maintaining high specificity. The primers added EcoRI and BamHI as flanking sequences, enabling directional cloning into the vector pGEM3Z (Promega). After transformation of *E. coli* with the riboprobe constructs, small scale DNA preparations were made using the Qiaprep Spin Mini-prep Kit (Qiagen) according to the manufacturer’s instructions. All constructs were verified using the T7 sequencing kit (Pharmacia). The DNA templates were linearised by restriction digestions with either EcoRI or BamHI, and labeled by *in vitro* transcription using the DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany).

**RNA-RNA in situ hybridisation**

Sections on Vectabond-coated slides (Vector laboratories) were dewaxed and rehydrated, washed in PBS (2x 3 min) and encircled with a silicone pen (DAKO). The tissues were immersed in 4% paraformaldehyde with 5mM MgCl₂ in PBS for 5 min and re-washed in PBS (2x 3 min). After digestion in RNAase-free DNAse (15 U/ml) in 2x SSC for 1 hour at 37°C in a humid chamber, the sections were washed in PBS (2x 3 min) and digested in Protease K (50 µg/ml) for 15 min at 37°C. Sections were then washed in PBS (2x 3 min) and re-fixed as above for 5 min. After a final wash in PBS (2x 3 min), the sections were dehydrated through graded ethanols (30, 50, 70, 90, and 100%, 30 s each) and air dried. The section was covered in RNA probe diluted 1:10 or greater in hybridisation buffer (2x SSC, 5% dextran sulphate, 0.2% Marvel (Premier Beverages), 50% deionised formamide), according to labeling efficiency, coverslipped and sealed with cow gum (Cow Proofings Ltd.). Slides were incubated overnight at 42°C.

The gum was removed and the coverslips soaked off in x SSC, 0.1% SDS. Slides were washed at 55°C in 2x SSC, 0.1% SDS for 5 min then incubated in RNAaseA (2 mg/ml in 5x SSC) for 15 min at 37°C. After washing in 0.1x SSC, 0.04% SDS (2x 20 min, 65°C), and soaking in blocking buffer, sections were
developed as for DNA in situ hybridisation. Counterstains were either Carazzi’s haematoxylin (30–45 seconds), or 1% aqueous light green (60 seconds).

To control for false-positive signals due to non-specific binding of the anti-digoxigenin antibody, or from non-specific precipitation of the chromogen, the RNA probe was omitted. To control for non-specific binding of the RNA probe, probes of the opposite orientation (sense probes) were used. Pre-infection tissue acted as a negative control for other false-positive signals and as a means of assessing the level of background signals. To prevent binding to DNA, tissues were not subjected to heat denaturation and were incubated in RNAse-free DNAse to ensure that no single-stranded DNA targets were present. Further acceptance criteria included the restriction of signal to the cytoplasm of epithelial cells with no significant signal in adjacent non-infected epithelium or the underlying dermis.

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