Persistence of Mastomys natalensis papillomavirus in multiple organs identifies novel targets for infection

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The high incidence of multiple wart formation and skin cancer in organ-transplant recipients, as well as the question of an involvement of papillomaviruses in a variety of human cancers, require a model system for papillomavirus infections in immunocompetent animals. Such an in vivo model is represented by the multimammate rat Mastomys coucha, which is infected with Mastomys natalensis papillomavirus (MnPV). MnPV primarily induces benign skin tumours, such as papillomas and keratoacanthomas. Here, the incidence of MnPV infections in different skin areas and various organs is described. In situ hybridization showed that hair follicle cells were positive for viral DNA and that the amount of MnPV in normal skin may be considered a predictor for the development of skin tumours. MnPV infection is not restricted to the skin, but can also be detected in inner organs. As the blood and the lymphatic system were temporarily also found to be virus-positive, a haematogenic propagation of MnPV can be assumed. However, MnPV is apparently not transmitted through the germ line, as fetuses and newborns lack viral DNA, despite infection of their mothers. In conclusion, M. coucha is not only useful to study papillomavirus-induced skin carcinogenesis, but may also serve as a model to identify additional, still unknown target cells of papillomavirus infections and the potential pathological impact.

INTRODUCTION

Whilst ‘high-risk’ human papillomaviruses (HPVs) (mostly HPV16 and 18, among others) are generally accepted to be the causative agents of cervical cancer (zur Hausen, 2002), the role of cutaneous HPV types and their aetiology in skin tumours or other forms of human malignancy is still a matter of debate (zur Hausen, 2000; Forslund et al., 2004; Karagas et al., 2006). However, two groups of patients exist where epidemiological data strongly suggest a causal link between the presence of these virus types and non-melanoma skin cancer (NMSC): individuals with the rare, hereditary genetic disorder epidermodysplasia verruciformis (EV) (Majewski & Jablonska, 2002) and organ-transplant recipients under systemic immunosuppression (Stockfleth et al., 2001; Harwood & Proby, 2002). Both groups are extremely susceptible to developing NMSC, such as cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), especially on sun-exposed areas. In EV patients, cutaneous β-HPV types (mostly HPV5 and 8) can be detected in about 80–90% of lesions (Pfister, 2003). Epidermal stem cells suffering inappropriate DNA repair and additional genetic changes are most likely to progress to NMSC (Schmitt et al., 1996; Bouwes Bavinck et al., 2001). As the majority of the tumours appear at chronically sun-exposed locations, UV light is considered to be the most important risk factor. UV-B exposure can lead to multiple DNA damage, which finally provokes an apoptotic programme, resulting in the elimination of deleterious cells (Akgül et al., 2006). Beside p53 induction, one major protective response of UV-irradiated cells is the activation of the pro-apoptotic protein Bak (Thomas & Banks, 1999). In the case of papillomavirus infections, however, the E6 oncoproteins of cutaneous papillomaviruses do not affect p53 stability, but target Bak for proteolytic degradation, thereby enabling survival and accumulation of genetically altered cells (Storey, 2002). Referring to the oncogenic potential of cutaneous HPVs, E6 and E7 of the EV types (HPV8 and 38, respectively) have transforming properties under both in vitro and in vivo conditions, thereby inducing skin cancer in transgenic mouse...
models (Caldeira et al., 2003; Dong et al., 2005; Schaper et al., 2005). Taking into consideration the fact that these animals are immunotolerant against the viral oncoproteins (Doan et al., 1999), it is mandatory to examine the role of cutaneous papillomaviruses in skin carcinogenesis in their natural, immunocompetent host.

Here, we describe the soft-furred, multimammate rat Mastomys coucha [previously assigned to the species Mastomys natalensis (Haag et al., 2000)] as a small laboratory animal that allows the study of skin carcinogenesis in molecular terms. The animal colony at the DKFZ is the only one worldwide that is infected with the *Mastomys natalensis* papillomavirus (MnPv) (Amtmann et al., 1984). As reported almost three decades ago, these animals spontaneously develop multiple benign skin tumours, such as papillomas and keratoacanthomas, with MnPV as aetiological agent (Müller & Gissmann, 1978; Rudolph et al., 1981; Tan et al., 1994). MnPV belongs to the cutaneous papillomavirus types and lacks the E5 open reading frame (ORF) (de Villiers et al., 2004). Analogous to HPV8-induced skin cancer in EY patients (Pfister, 2003), MnPV DNA persists epismally without any evidence of integration (Amtmann et al., 1984). MnPV-induced tumours never regress, and only proceed to SCC after topical application of carcinogens and tumour promoters (Wayss et al., 1981). Moreover, the oncogenic potential of MnPV was recently confirmed in transgenic mice encoding the E6 oncoprotein under the control of the cytokeratin 14 promoter, which targets viral gene expression to the basal layer of the skin (Helfrich et al., 2004).

The focus of the present study was to analyse MnPV distribution and its transcriptional activity in the natural host. We systematically analysed different skin sections of tumour-free and tumour-bearing animals, differing in age as well as location of tumours in various organs, for virus presence and MnPV-specific gene expression. Evidence is provided that viral persistence and viral load are correlated with the development of skin tumours. Moreover, MnPV infection is obviously not restricted to skin, but has also been found in other tissues, indicating that this system can be used as a model to analyse viral spread and persistence in an immunocompetent animal.

**METHODS**

**Animals.** *M. coucha*, previously referred to as *M. natalensis*, from the breeding colony at the DKFZ were kept under conventional conditions at 21–24 °C, 55 % relative humidity and 12–16 air changes h\(^{-1}\). The animals were provided with a standardized mouse diet and allowed to drink water *ad libitum*.

**Dissection and DNA isolation from tissue, blood, embryos and hair bulbs.** To exclude cross-contamination from skin to inner organs, scalpels, scissors and forceps were changed after every resection of an organ and embryo. In total, three pregnant animals, ten fetuses and five newborns shortly after delivery were sacrificed. The fetuses were removed *in utero* at the last week of pregnancy. The tissue was snap-frozen and kept at −80 °C until usage. For DNA extraction, tissue or EDTA-treated whole blood (terminal bleeding) was lysed overnight at 55 °C in DNA lysis buffer (1 % SDS, 1 mM EDTA, 100 μg proteinase K ml\(^{-1}\), 20 mM Tris/HCl, pH 8) and extracted as described previously (Sambrook et al., 1989; Kendall et al., 1991). Approximately 30–50 hairs were removed from the neck. DNA from hair bulbs was isolated by using a QIAamp DNA mini kit (Qiagen) according to the manufacturer’s instructions.

**Southern blotting.** To confirm the PCR results, Southern blotting was performed by standard methods (Sambrook et al., 1989). DNA (2.5–5.0 µg) was digested overnight with HindIII (New England Biolabs), known to cut the MnPV genome only once (Tan et al., 1994). Filters were hybridized with a unit-length MnPV-specific probe (Amtmann et al. 1984).

**RNA extraction and reverse transcription.** Snap-frozen tissue was ground with a chilled mortar and pestle and the tissue powder was extracted with TRIzol reagent (Invitrogen) [100 mg tissue (ml TRIzol)\(^{-1}\)], following the instructions of the manufacturer. RNA was treated with 1–2 µl Turbo DNase (Ambion) prior to reverse transcription. Approximately 2 µg RNA was incubated with 3.5 µM Oligo(dT)\(_{15}\) (Promega) for 10 min at 42 °C. dNTPs (0.5 mM), single-strand buffer (50 mM Tris/HCl, pH 8.3 at room temperature; 75 mM KC1; 3 mM MgCl\(_2\)) and 2.5 mM dithiothreitol were added and left for 10 min at 25 °C. After addition of 200 units SuperScript II reverse transcriptase (Invitrogen), the reaction was incubated at 42 °C for 30 min, followed by a final heating to 70 °C for 15 min.

**PCR/RT-PCR.** PCR was performed with 100–250 ng genomic DNA or 100 ng cDNA. Each amplification mix of 25 µl contained 1.5 mM MgCl\(_2\), 200 µM dNTPs, 0.3 µM each primer, 1 × PCR buffer and 0.5 units recombinant Taq polymerase (Invitrogen). PCRs were run in a Thermocycler MT200 (BioRad) for 25 (DNA) or 28 (cDNA) cycles. The program for all primers was: 4 min at 94 °C; 25–28 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C; final extension for 10 min at 72 °C. The primer sequences are as follows: MnPV E6: sense, 5’-ACTCCTTTGTGGAGCGGCTG-3’; and antisense, 5’-CAATTTCTGACCGTGCCCCTC-3’ (product length, 378 bp); MnPV L1: sense, 5’-TCTACACCGCGTACTTGCCCA-3’; and antisense, 5’-GCCACGAGCTATCITCACCCT-3’ (product length, 379 bp); actin: sense, 5’-ACCCACACTGGCCACATCTACCA-3’ and antisense, 5’-CTTGTCTGATCCACATCTCAGGGA-3’ (two bands).

**In situ hybridization (ISH).** Sections of paraffin-embedded tissue (5 µm) were placed on coated slides and fixed overnight at 56 °C. For deparaffinization, the slides were incubated twice for 10 min in 1 × xylene/99 % ethanol followed by rehydration in a graded ethanol series: 2 × 99 %, 1 × 96 %, 1 × 80 % and 1 × 70 % ethanol in PBS for 1 min each. The slides were boiled for 10 min in 12 mM sodium citrate buffer (pH 6.0) in a microwave. The digestion was performed with proteinase K (8 µg ml\(^{-1}\), 0.05 M Tris/HCl, pH 7.5) for 10 min and the reaction was stopped by transfer to glycerine buffer (50 mM in 1 × TBS). To extinguish residual peroxidase activity, the slides were incubated in 0.3 % H\(_2\)O\(_2\) solution (diluted in PBS) for 30 min, followed by a prehybridization step. For hybridization, slides were covered with hybridization buffer (50 % formamide, 2 × SSC, 1 mg sonicated salmon sperm DNA ml\(^{-1}\); 0.05 M NaH\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\), pH 6.8, 1 mM EDTA), supplemented with 500 ng biotinylated MnPV DNA ml\(^{-1}\) (Biotin–Nick translation kit; Roche) or HPV5 cloned in pBR322 plasmid as negative control, heated at 98 °C and left at 42 °C in a humid chamber overnight. Following the instruction of the TSA amplification kit (Perkin Elmer), slides were incubated with a streptavidin–horseradish peroxidase (HRP) conjugate (dilution 1:500) and the signal was amplified with the biotin derivative and amplification reagent biotinyltyramid (dilution 1:50; TSA kit, Perkin Elmer). MnPV DNA was visualized as a red precipitate of 3-amino-9-ethylcarbazol chromogen substrate (AEC; Dako) activated by HRP (Perkin Elmer). Nuclei were counterstained with haematoxylin.
Quantification of MnPV DNA by real-time PCR. Standard curves were generated by 10-fold dilutions of the MnPV plasmid (Tan et al., 1994) from 10,000 to 10 viral copies in a background of 1 ng viral negative genomic DNA by using MnPV E7 type-specific primers. Each amplification mix of 20 μl contained up to 5 μl template DNA, 500 nM each primer, 2 μl LightCycler FastStart Reaction mix SYBR green I (Roche), 3 mM MgCl₂ and sterile water. The following primers were used: MnPV E7 LC sense, 5’-TCGAGTGCCGTTTTGATAG-3’, and antisense, 5’-CTAGACATTTGCGAATAG-3’; β-actin LC sense, 5’-AGATGACAATCTTGAGGCCATA-3’, and antisense, 5’-ATGCCACAGGATTCCATACC-3’. By using the LightCycler Detection system (Roche), the mixture was amplified with the following steps: 10 min at 95 °C; 10 s at 95 °C, 5 s at 60 °C (MnPV E7 LC) or 63 °C (β-actin LC), and 15 s at 72 °C for 45 cycles. Specific products were verified by melting-curve analysis with a slope of 0.1 °Cs⁻¹ from 65 to 95 °C. Real-time PCR was performed in duplicate and each experiment was performed twice. The amount of genomic Mastomys DNA was converted to the number of cells, calculating an MnPV genome equivalent of 5.7 pg DNA per cell. The value was inferred from complete genome information of mouse (Mus musculus; NCBI Genome Project no. txid10090) and rat (Rattus norvegicus; NCBI Genome Project no. txid10116), as precise data for Mastomys are not available at present.

Electron microscopy. A skin tumour was cut into pieces in 2.5% glutaraldehyde, diluted in 50 mM cacodylate buffer (pH 7.6) and left for fixation on ice. After five or six washing steps in 50 mM sodium cacodylate buffer, the tissue was stained with 2% osmium tetroxide and 0.5% uranyl acetate. The processing for ultrathin sectioning includes dehydration and embedding in Epon (Serva), which was subsequently polymerized at 60 °C for 48 h. Micrographs were taken with a Zeiss EM-10 electron microscope at 80 kV.

RESULTS

MnPV-induced tumours and presence of viral DNA in M. coucha skin

The animals of the DKFZ colony are infected with MnPV, which induces benign tumours such as papillomas and keratoacanthomas to a frequency of 30–40% at about 1 year of age (Amtmann et al., 1984). Lesions were located not only on the dorsum and neck (Fig. 1a), as reported previously (Amtmann & Wayss, 1987), but also at the ventral abdomen, the eyelid and the ear (Fig. 1b–d). Consistent with the notion that papillomavirus replication and virus maturation are linked tightly to terminal differentiation of the host cell (zur Hausen, 2002), electron microscopic examination of papillomas revealed islands of infectious viral particles enclosed by cornified layers of the epithelium (Fig. 2a, b). Although viral particles were not found in normal epidermis, ISH techniques revealed a high viral DNA content in apparently healthy skin of tumour-bearing animals (Fig. 3a, b). Basal cells were not stained, which is in accordance with the concept that, in non-differentiated cells, the viral genomes are maintained in low copy numbers (Middleton et al., 2003), being below the detection sensitivity of the ISH method (McNicol & Farquharson, 1997). In contrast, more differentiated regions (such as the stratum spinosum and the stratum granulosum) showed strong hybridization signals, indicating that MnPV DNA was replicated abundantly. Fig. 3(c) shows a typical cyst-like, endophytic-growing tumour, with a pronounced inner keratinized capsule. Here, an uneven staining pattern of the suprabasal cells could be discerned. Notably, hair follicle cells were also found to contain viral genomes. Different layers of the hair shaft are shown in Fig. 3(d), but viral genomes were only detected in basal cells of the dermal papilla, whereas the interfollicular epidermis was negative.

Analysis of normal tissues and organs by PCR and Southern blot

As it has previously been presumed that MnPV may also persist in other organs (Amtmann et al., 1984), we...
systematically autopsied 21 tumour-free and tumour-bearing animals and screened them for the presence of viral DNA. For this purpose, extreme care was taken to exclude MnPV contamination from organ to organ. PCR analyses were performed with only 25 amplification cycles to avoid non-specific amplicons and the PCR data were verified by Southern blot analyses. Fig. 4 shows representative examples of two animals with different MnPV-positive organs. Whilst in Fig. 4(a), only the skin, lungs, stomach and forestomach were found to be positive, another animal (Fig. 4b) additionally showed the presence of viral DNA both in the liver and in the brain. On inspecting a total of 21 animals with a mean age of 10.5 months (38 % female, 62 % male), MnPV DNA was present in almost all organs, albeit with different incidence rates (Fig. 5; for further details, see also Supplementary Table S1, available in JGV Online). Carrying out PCRs covering two different ORFs (E6 and L1), the graphical overview demonstrates MnPV positivity ranging between 85 and 100 % for skin and skin-related tumours (Fig. 5b, c). Heart, lungs, forestomach and stomach gave signals in around 60–90 % of cases (Fig. 5a). As already depicted in Fig. 4(b), MnPV sequences could even be found in the brain (in >70 % of cases by PCR and in 33 % by Southern blotting), but the pathological consequences are, similar to the other inner organs, still unclear.

On analysing blood from animals without visible lesions, two of six samples were positive. In monitoring extracted DNA obtained from swollen lymph nodes, MnPV was found in three of three animals (Fig. 5a). As it has been reported that HPV can be also transmitted via a maternal–fetal route (Smith et al., 2004), fetuses, which were removed surgically from the uterus, and newborns shortly after delivery were sacrificed. To get a representative insight into all organs, longitudinal sections were performed and the extracted DNAs were analysed by PCR (Fig. 6). Even though the mothers were positive both in the skin (animals...
and 2) and in the tongue (animal 1), the placenta was found to be negative. Accordingly, none of the fetuses or newborns showed any indication of the presence of MnPV sequences. Hence, a transplacental transmission route is unlikely, suggesting that infection occurs after birth.

**MnPv gene expression in different organs**

To examine transcriptional activity, we analysed the mRNA of different organs of two animals (aged 11.5 and 20 months) by RT-PCR (Fig. 7). Two different primers, covering the ORFs representative of the early (E6) and late (L1) stages, were used to monitor the viral life cycle. To avoid false-positive signals, the non-reverse-transcribed RNA was first amplified with L1 primers to check for contaminating viral DNA. We only included RNA in reverse transcription that was negative for L1 even after 30 PCR cycles. Furthermore, tissue was selected that was already shown to be positive for MnPV DNA, as monitored by PCR using L1 primers (Fig. 7, upper row). Although the viral load was similar, abundant transcripts were found only in skin and papilloma samples of animal no. 55 and 58, respectively. Simultaneous expression of early and late viral genes is indicative of a permissive cycle, which ultimately results in the formation of mature virus particles. Notably, although the epidermis is regarded as the primary target of a vegetative MnPV infection, there were still areas in the skin within the same animals where no transcripts could be detected (animal 55). Also, the brain, lungs, forestomach, heart and stomach harboured MnPV DNA, but lacked transcriptional activity. Hence, the absence of detectable mRNA under our RT-PCR conditions implies that the presence of viral DNA was not necessarily linked to transcriptional activity.

**Tumour- but not age-related increase of MnPV DNA in the skin**

As revealed by ISH (Fig. 3d), hair follicle cells harbour MnPV genomes and are therefore suited to investigate viral load over time within the same animal. For follow-up, hairs were plucked from the neck from four 6-month-old *Mastomys* individuals at intervals of 5 weeks. In contrast to punch biopsies, this method does not induce major skin damage and is less invasive.
irritations, thereby avoiding the reactivation of latent infections as a consequence of a strong mechanical stimulus or wound formation (Siegsmund et al., 1991). Hair follicle DNA was extracted and examined by real-time PCR. Supplementary Fig. S1(a) (available in JGV Online) shows that initial copy number ranged from <1 to roughly 151 genome equivalents per cell. Of note, copy numbers in two animals (1 and 2) only transiently increased after 5 and 10 weeks, but finally dropped down to values essentially the same as those found at the beginning of the experiment. Animal 3 maintained approximately the same copy number, whilst animal 4 showed only a slight elevation. To confirm these results, whole-skin samples from five tumour-free animals of different ages (1.5, 3.0, 6.5, 10 and 14 months) were analysed for the presence of MnPV DNA. As expected, all animals maintained low copy numbers in the skin, independent of their age [see Supplementary Fig. S1(b), available in JGV Online]. On the other hand, histologically normal skin of tumour-bearing animals harboured viral loads of 1000–20000 copies per cell [see Supplementary Fig. S1(c), available in JGV Online]. These data indicate that, in tumour-free animals, ageing per se does not lead to a time-dependent increase in number of viral genomes, in contrast to earlier conclusions (Amtmann et al., 1984).

DISCUSSION

M. coucha can be considered a unique model to study papillomavirus-induced skin carcinogenesis in a small laboratory animal, being the natural host of MnPV (Amtmann et al., 1984). Although the animals spontaneously develop only benign tumours, such as papillomas and keratoacanthomas, treatment of the skin with tumour-promoting agents can also lead to malignant transformation (Wayss et al., 1981). The oncogenic potential of the MnPV E6 protein has recently been shown in a transgenic approach (Helfrich et al., 2004). As these animals are immunotolerant against ectopically expressed proteins (Doan et al., 1999), thereby only partially reflecting the in vivo situation, we set out to characterize the prevalence, distribution and transcription of MnPV DNA in its natural host.

Here, we observed MnPV-induced tumours at different sites (Fig. 1), and electron microscopic examination of papillomas revealed paracrystalline patches of mature viruses that were surrounded by cornified layers (Fig. 2). Such concentrated particles at the skin surface can be released, thereby propagating the virus within the colony. By the use of the highly sensitive PCR technique, the presence of MnPV DNA in fetal tissue (Amtmann et al., 1984) could not be confirmed. Although different reports claimed a transplacental transmission of HPV (Cason & Mant, 2005; Medeiros et al., 2005), no placentas, fetuses or newborn animals showed any indication of MnPV DNA, despite infection of the mothers (Fig. 6). As viral DNA was present in virtually all animals, both in females and males older than 4 weeks, assuming an early contagion and persistence. Whilst the route of infection still remains to be clarified, transmission by virus-loaded dust particles or close skin contact between the mothers and their offspring during nursing seems to be most likely. Microlesions caused by scratching or fighting may also provide access of MnPV to basal cells of the epithelium, the primary target of viral infection (Bouwes Bavinck et al., 2001). Scratches and bites not only lead to infection, but may also promote hyperproliferation of tissue during wound healing, in turn favouring reactivation and amplification of latently MnPV-infected cells (Siegsmund et al., 1991). Notably, fights and subsequent wounding of the skin are far more common among males, which is consistent with the observation that tumour incidence in males is about three times higher than that in females of the same age (Nafz, 2007). On the other hand, male animals kept isolated for 1 year did not develop any skin tumours.

**Fig. 6.** Representative PCR analysis of embryos and newborn animals. Pregnant animals were sacrificed in week 2 or at the day of delivery. DNA extracted from five fetuses, two newborns and skin and tongue from mother animals was amplified by using MnPV L1- and mouse actin-specific primers. The upper band shows a pseudogene of β-actin.

**Fig. 7.** RT-PCR analysis of various tissues. Upper lanes: DNA PCR with L1 primers. cDNA was analysed by PCR with two different primer pairs representing an early and a late ORF (E6 and L1, respectively). β-Actin primers were used to check the integrity.
Although the permissive cycle of papillomaviruses is linked tightly to terminal differentiation (Peh et al., 2002), electron microscopy failed to detect viral particles in normal epidermis. However, ISH demonstrates that healthy skin sections from tumour-bearing animals harbour MnPV genomes, whereas skin of animals without lesions was virus-negative (Fig. 3). The staining pattern indicates that extensive viral replication and, in turn, late transcription were taking place, as cells below the epithelial surface (e.g. in stratum spinosum and in stratum granulosum) predominantly showed the highest amount of MnPV DNA (Fig. 3a, b). Remarkably, this correlation could not be observed in skin of animals without tumours, where only low copy numbers were detected (see Supplementary Fig. S1, available in JGV Online). To obtain insight into the immunological surveillance during virus–host interaction, we are currently monitoring the humoral immune response by using a recently established capture ELISA. When analysing the IgG response against MnPV L1 capsid proteins, the absence of tumours correlated with low L1 antibody titres, whereas tumour-bearing animals showed the opposite. This suggests that the animals fail to interfere with MnPV infection and viral DNA accumulation, despite the presence of high L1 antibody titres (Schäfer, 2006).

Of note is the finding that hair follicle cells also contained MnPV genomes [Fig. 3d; Supplementary Fig. S1(a), available in JGV Online], suggesting that these cells have the potential to progress to benign tumours, as described for the cottontail rabbit papillomavirus (Schmitt et al., 1996). As stem cells of hair follicles are also involved in wound healing (Millar, 2002; Ito et al., 2005), reconstitution of an injured tissue, for example after scarification, can subsequently cause the accumulation of MnPV-positive daughter cells, where the viral life cycle is completed during terminal differentiation. This may explain why chronic inflammation of virus-positive skin not only causes hyperproliferation, but also triggers formation of papillomas and keratoacanthomas (Siegmund et al., 1991). As spontaneously developing tumours are non-malignant, MnPV infection per se is not sufficient for a complete progression to malignancy (Amtmann et al., 1984), but requires additional damaging events to interfere with terminal differentiation (zur Hausen, 2000).

It has previously been shown that MnPV may also persist in other organs (Amtmann et al., 1984). To readdress this finding with more refined techniques, we sacrified a total of 21 animals to monitor the presence of viral DNA and transcription by PCR (Figs 4, 5 and 7). The prevalence ranged between 85 and 100% in skin and skin-related tumours, supporting the notion that the epidermis is the major target for maintenance and propagation of an MnPV infection (Fig. 5b, c; see also Supplementary Table S1, available in JGV Online). The tongue also tested positive (Fig. 5b), suggesting that viral particles also infect this epithelium.

Another route of viral spread must be assumed for inner organs (Fig. 5a). Although MnPV was present in the blood, it does not necessarily confer positivity to inner organs, because in animals that had a strongly positive signal in the heart, liver or spleen, respectively, the blood was negative by PCR. It is therefore likely that MnPV presence in the blood is merely transient (temporary viraemia), but may of course finally account for viral spread to inner organs, because no other routes of infections are conceivable. How and when MnPV particles are released into the haematopoietic and lymphatic system remains to be elucidated.

The presence of viral sequences in the brain shows that papillomavirus DNA can also persist in this organ (Figs 5a, 7). Although the choroid plexus is the only region in the brain that is covered by epithelial cells (Rickert & Paulus, 2001), our preliminary ISH studies indicate that this area was not infected (data not shown). We are currently trying to characterize virus-positive target cells in the brain by immunohistochemical means using specific neural marker antibodies. As the brain, similar to cells of inner organs, may represent a non-permissive environment, MnPV infection must be abortive under these conditions. This is consistent with the finding that, in contrast to the skin, MnPV is not transcribed in these organs (Fig. 7). Whether MnPV replication is maintained in these organs by an unidentified potential helper virus or by accessory cellular proteins (Kim et al., 2005) is still unknown. However, it should be noted that replication of papillomaviruses can be mediated even without viral transcription, because cellular proteins such as MCM (minichromosome maintenance protein) may substitute for viral trans-activators (Hoffmann et al., 2006).

Although MnPV positivity of inner organs was not accompanied by obvious pathological changes, *M. coucha* may serve as a model to study the question of a broader causal relationship between the presence of MnPV and other forms of cancer. In fact, besides lesions in the skin (Fig. 1), a certain form of stomach cancer (enterochromaffin-like cell neoplasia) can be induced rapidly in these animals by using H2 receptor blockers (Reubi et al., 1992). It will be worthwhile to study whether MnPV acts as a co-factor in this kind of tumour induction, as stomach and forestomach were also found to be positive to a high percentage (Fig. 5a).

Even though viral DNA was present in different tissue, viral replication and tumour formation were restricted to the skin, where transcripts of both early and late genes could be detected (Fig. 7). This is in line with the concept that the papillomavirus life cycle requires terminal differentiation of the infected cell to be completed (Doorbar, 2005). Moreover, after follow-up of the viral amount within hair follicles by real-time PCR in animals without skin lesions (see Supplementary Fig. S1a, available in JGV Online), viral load may be a useful predictor to identify animals prone to subsequent tumour formation. However, ageing per se, as suggested previously (Amtmann & Ways, 1987), seems not to be responsible for DNA amplification. This can be concluded from the results of tumour-bearing versus
tumour-free animals of the same age, where up to $2.8 \times 10^6$ times higher viral loads were detected in the former [see Supplementary Fig. S1(b, c), available in JGV Online]. To get insight into the role of the immune system in this effect, we are currently monitoring the humoral immune response against MnPV L1 capsid proteins by using a recently developed capture ELISA. Additionally, we will examine whether the immunization of young animals with MnPV L1 virus-like particles prevents tumour formation.

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