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Papillomavirus DNA complementation *in vivo*

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ABSTRACT

Recent phylogenic studies indicate that DNA recombination could have occurred in ancient papillomavirus types. However, no experimental data are available to demonstrate this event because of the lack of human papillomavirus infection models. We have used the cottontail rabbit papillomavirus (CRPV)/rabbit model to study pathogenesis and immunogenicity of different mutant genomes *in vivo*. Although the domestic rabbit is not a natural host for CRPV infection, it is possible to initiate infection with naked CRPV DNA cloned into a plasmid and monitor papilloma outgrowth on these animals. Taking advantage of a large panel of mutants based on a CRPV strain (Hershey CRPV), we tested the hypothesis that two non-viable mutant genomes could induce papillomas by either recombination or complementation. We found that co-infection with a dysfunctional mutant with an E2 transactivation domain mutation and another mutant with an E7 ATG knock out generated papillomas in rabbits. DNA extracted from these papillomas contained genotypes from both parental genomes. Three additional pairs of dysfunctional mutants also showed similar results. Individual wild type genes were also shown to rescue the function of corresponding dysfunctional mutants. Therefore, we suggest that complementation occurred between these two non-viable mutant PV genomes *in vivo*.

1. Introduction

The cottontail rabbit papillomavirus (CRPV)/rabbit model has been used extensively in pathogenesis and vaccine development studies (Nicholls and Stanley, 2000; Christensen, 2005). This model has also played an important role in the development of virus-like particle (VLP) vaccines for clinical application (Breitburd et al., 1995; Christensen et al., 1996; Lowy and Schiller, 2006). One unique characteristic of this model system is that the infection can be initiated with CRPV DNA (Kreider et al., 1995; Brandsma and Xiao, 1993). This feature has allowed us and others to generate large numbers of mutants for pathogenesis investigations (Nasseri et al., 1989; Wu et al., 1994; Salmon et al., 2000; Jeckel et al., 2002; Hu et al., 2002). Our previous studies have demonstrated a high capacity of the CRPV genome for modification without loss of viability (Hu et al., 2007). However, some mutant genomes were defective and incapable of tumor formation in rabbits. These mutants were identified as non-viable or dysfunctional genomes.

A novel virus that was recently detected in papillomas and carcinomas of the endangered western barred bandicoot exhibits genomic features of both papillomaviridae and polyomaviridae. This observation suggests a recombination event might have occurred between these two ancient viruses (Woolford et al., 2007). Other phylogenetic studies have suggested that recombination may have occurred in variants from the same papillomavirus type (Pushko et al., 1994; Varsani et al., 2006; Narechania et al., 2005; Angulo and Carvajal-Rodriguez, 2007; Bravo and Alonso, 2004). To demonstrate recombination of human papillomaviruses *in vivo* is challenging because these viruses are generally considered to be highly specific for their hosts although bovine papillomaviruses are able to cause nonproductive infections in horses and other only distantly related mammals (Bloch et al., 1994; Koller and Olson, 1972). To date, the lack of immunocompetent *in vivo* laboratory animal models has hindered the direct studies of HPV infection. Our dysfunctional mutants generated from the Hershey progressive strain of CRPV (H.CRPV, also identified as wild type CRPV) are all members of the same papillomavirus type. We postulated that one dysfunctional mutant CRPV genome (with a mutation in one gene) could rescue the function of another dysfunctional CRPV genome (with a mutation in another gene) by co-infection *in vivo*. The readout would be papilloma formation in rabbits. To avoid the argument that infections might have arisen from wild type CRPV contamination, we used mutants that contained a functional point mutation in the E6 gene in addition to defective mutations in either the E2 or E7 genes. These E6 point mutant genomes have been shown to be functional in previous studies (Hu et al., 2002). Among the pairs of

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dysfunctional genomes tested in New Zealand White (NZW) rabbits, four pairs were able to induce papillomas. DNA sequencing demonstrated that the genotype of the viral DNA extracted from these papillomas showed both parental genotypes. Therefore, we propose that *in vivo* complementation events have occurred between these two non-viable papillomavirus DNAs.

2. Materials and methods

2.1. Constructs

Hershey progressive strain CRPV (H.CRPV) cloned into PUC19 at SalI was identified as wild type CRPV and used as the backbone for all mutants (Hu et al., 2002). For convenience of cloning and handling, each selected individual viral gene was cloned into PUC19 for subsequent modification. Point mutations and deletions were introduced into each gene by using a QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Other insertions and replacements were made with routine restriction digest and ligation technology as described previously (Hu et al., 2007). All the mutations were confirmed by DNA sequence analysis at the Core Facility of Pennsylvania State University Hershey Medical Center. To avoid possible contamination by the wild type DNA, the mutants used for the complementation/recombination studies all contained a point mutation in E6 as an extra biomarker to distinguish them from the wild type E6. H.CRPVE6, E7 and E2 were also individually cloned into the expression vector PCR3 containing a CMV promoter (Invitrogen) for complementation studies. A tandem repeat construct that contained a dysfunctional mutant genome in sense orientation within a BglII fragment of CRPV DNA that contained the functional site was also generated (Fig. 1). The constructs were purified by ultra-centrifugation on cesium chloride gradients and adjusted to 200 μg/ml in 1× TE buffer (Hu et al., 2002, 2007) prior to challenge on animals.

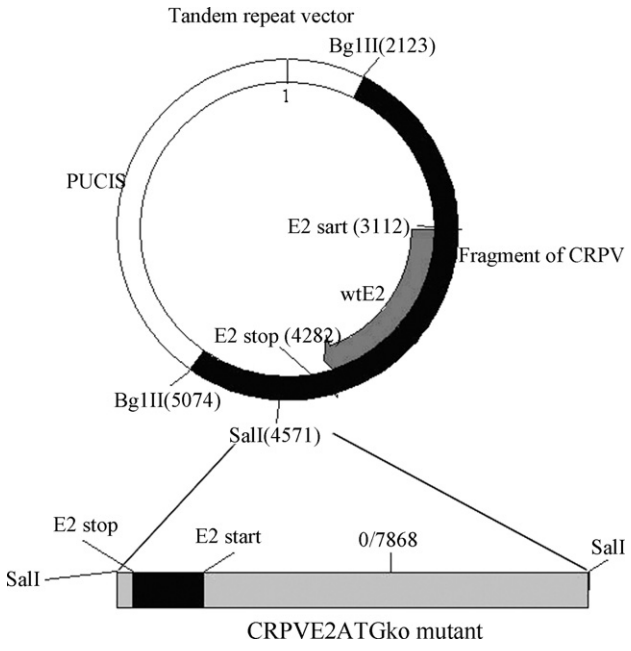

Fig. 1. The BglII fragment (2123–5074 bp) from the wild type CRPV genome cloned into PUC19 was used as the template for insertion of a whole CRPVE2ATGko mutant genome. A wild type CRPV E2 (3112–4282 bp) gene was located inside this fragment. The CRPV E2ATGko dysfunctional genome was inserted in sense orientation at enzyme site SalI (4571 bp) included inside this BglII fragment. The final construct contains one wild type CRPVE2 and one CRPVE2ATGko in sense orientation.

2.2. DNA challenge and monitoring of tumors

New Zealand White rabbits were maintained in the animal facility of the Pennsylvania State University College of Medicine. The studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University. For application of viral DNA and viruses, rabbits were sedated using ketamine/xylazine anesthesia (Cladel et al., 2008). Back skin of the animals was scarified with a scalpel blade and superficially scratched. Three days later, again under sedation, each site was then challenged with 10 μg Hershey progressive CRPV DNA (wtCRPV) or a combination of two dysfunctional mutants (5 μg/5 μg) in 50 μl of 1× TE buffer. The infected sites were then scratched 20 times with a 21G needle to introduce small abrasions into the scab (Cladel et al., 2008). Monitoring of papilloma outgrowth began three weeks after the infection and continued weekly until week 12. Papillomas were harvested for DNA isolation and histological examination.

2.3. DNA sequence confirmation from papillomas

Total genomic DNA was isolated from 20 mg of each papilloma biopsy using the DNeasy Tissue Kit (QIAGEN) according to the manufacture's protocol. DNA extracts were then used as templates to amplify specific regions and also the region from the E6 to the E2 gene. The primers for the E6 and E7 region were: upstream primer 5′ GAC-AAC-TGC-CTG-CCG-CGG-TCG-CTA-GAG-AAG and down stream primer 5′ CCG-GAT-CCC-AGT-CAT-CGA-TAG-GGT-CTG-TAC and the primer pairs to amplify the E2 region were: upstream primer 5′ GGT-GAC-GAT-GGA-GGC-TCT-CA and down stream primer 5′ GTT-TTC-GTT-GCT-TTG-CCG. The primers for E6-E2 large fragment (about 4 kb) were: upstream primer 5′ GAC-AAC-TGC-CTG-CCG-CGG-TCG-CTA-GAG-AAG and down stream primer 5′ GCGGAATTCCTAAAGCCCATAAAAA TTCCC 3′. The E6-E2 long fragment was also cloned into PUC19. Clones from each mixture were sequenced to analyze for recombination. The PCR products for E6, E7 and E2 regions were purified with PCR purification kit (QIAGEN) and also sent for sequencing at the Core Facility of Pennsylvania State University Hershey Medical Center. The sequences were analyzed using the DNAMAN software (Lynnon BioSoft) and representative sequence regions were cropped with Chromas 2 (www.technelysium.com.au).

2.4. Histology

Papillomas induced by wild type CRPV DNA and mutant mixtures were harvested and fixed in 10% formalin. The tissue was processed as reported previously (Hu et al., 2006).

2.5. Statistics

Papilloma size was determined by calculating the cubic root of the product of length × width × height of individual papillomas in millimeters to obtain a geometric mean diameter (GMD). Data were represented as the means (±SEMs) of the GMDs for each test group. Statistical significance was determined by unpaired *t*-test comparison ($P < 0.05$ was considered significant).

3. Results

3.1. Dysfunctional mutant genome mixtures induced persistent papillomas in rabbits

Among the dysfunctional mutant genomes we tested, four combinations were able to induce papillomas in rabbits (Table 1). One combination [PJF192 (E6/G907A and E2/CTC del, a deletion in the E2 transactivation domain) plus PJF185 (E7/ATGko)] has been tested