Abstract

Cloning of full length genomes of herpesviruses as bacterial artificial chromosomes (BAC) has greatly facilitated the manipulation of the genomes of several herpesviruses to identify the pathogenic determinants. We have previously reported the construction of the BAC clone (pRB-1B5) of the highly oncogenic Marek’s disease virus (MDV) strain RB-1B, which has proven to be a valuable resource for elucidating several oncogenic determinants. Despite the retention of the BAC replicon within the genome, the reconstituted virus was able to induce tumours in susceptible chickens. Nevertheless, it was unclear whether the presence of the BAC influenced the full oncogenic potential of the reconstituted virus. To maximize the closeness of BAC-derived virus to the parental RB-1B strain, we modified the existing pRB-1B5 clone by restoring the Us2 and by introducing SV40-cre cassette within the loxP sites of the mini-F plasmid, to allow self-excision of the plasmid sequences in chicken cells. The reconstituted virus from the modified clone showed significant improvement in replication in vitro and in vivo. Excision of the BAC sequences also enhanced the pathogenicity to levels similar to that of the parental virus, as the cumulative incidence of Marek’s disease in groups infected with the recombinant and the parental viruses showed no significant differences. Thus, we have been able to make significant improvements to the existing BAC clone of this highly oncogenic virus which would certainly increase its usefulness as a valuable tool for studies on identifying the oncogenic determinants of this major avian pathogen.

Background

Marek’s disease virus (MDV) is one of the most contagious and highly oncogenic alphaherpesvirus that induces T-cell lymphomas in the chickens [1,2]. Apart from the economic significance to the poultry industry with annual losses ranging between US$ 1–2 billion [3], MD is also a valuable model for studying the principles of virus-induced oncogenesis [4,5]. Studies on understanding the role of viral genes in the biology of MDV have been greatly facilitated by the construction of the bacterial artificial chromosome (BAC) clones of MDV [6,7]. The ability for rapid manipulation of BAC clones using well-established mutagenesis techniques in E. coli [8,9] and easy reconstitution of mutant viruses in transfected chicken cells has made this technique a valuable and efficient tool for studying MDV gene functions. We have previously reported...
the construction of pRB-1B-5, a BAC clone generated from the highly oncogenic RB-1B strain [10] of MDV by inserting the mini-F plasmid into the non-essential Us2 region of the genome [11]. Retention of oncogenicity of MDV (vRB-1B5) reconstituted from this clone has enabled the use of this clone in various studies to examine the oncogenic determinants using natural in vivo models of MD [5,12-15].

Although vRB-1B5 was capable of inducing tumours in susceptible chickens, the parental RB-1B virus appeared to show higher oncogenicity than the recombinant vRB-1B5 measured by the time of onset and incidence of tumours [this study and [12]]. As vRB-1B5 carried the mini-F plasmid sequences in the Us2 locus, we wanted to examine whether the reduced oncogenic property of vRB-1B5 is related to the presence of the extra foreign sequences. For this, we sought to modify the pRB-1B5 clone by introducing a cre-lox site-specific recombination system to excise the mini-F plasmid exploiting the flanking LoxP sites in pRB-1B5. Since the eukaryotic SV40 promoter-driven cre-expression cassette is non-functional in prokaryotes, this method would not affect the replication of the BAC DNA in bacteria. Additionally, the inclusion of an intron sequence within the cre gene further guarantees that no functional cre is expressed in bacteria [16]. Once the MDV BAC DNA is transfected into chicken cells, the eukaryotic SV40 promoter becomes functional and the splicing of the intron leads to the expression of cre, which cleaves at the two loxP sites that flank the BAC backbone cassette, including the cre gene itself. This results in the excision of the mini-F plasmid leaving just one copy of loxP site in the virus genome. The cre/loxP system has been used on BAC clones of other herpesviruses such as pseudorabies virus [16], human cytomegalovirus [17] and rhesus cytomegalovirus [18] to generate self-excisable viruses. In this study, we examined whether the self-excised MDV generated from the modified pRB-1B5 has increased viral replication and pathogenesis in a natural infection model in susceptible chickens.

Findings
The modification of pRB-1B was carried out as shown in Fig IA. Briefly, the Us2-Us3 sequence was amplified from the wtRB-1B virus-infected chicken embryo fibroblasts (CEF) genomic DNA by PCR using primers Us2F (5’-GTTAATTAACGACAGACCTACTTGCTACCA) and Us3R (5’-CTCGAGGATGCCATGGTGTTCTCTA) that contained the PacI and Xhol restriction sites respectively. The Xhol-PacI fragment containing partial mini-F sequence released from the plasmid pDS-HA1 [8] was linked to the above Us2-Us3 fragment through PacI-Sall site. The SV40-cre fragment released from pYD-C66 (kindly provided by Thomas Shenk, Princeton, USA) as EcoRI-Xhol fragment was blunt-ended and inserted into Pmel site of the above plasmid. Finally a blunt ended FRT-Kan cassette from pKD13 was further inserted into BstEII site (blunt ended) of the above plasmid. The resulting construct pPartial-F-Kan-cre-Us2-Us3 was used for making self-excisable BAC clone of RB-1B virus by lambda Red mutagenesis [19] by co-transforming with pRB-1B5 in bacterial strain EL250 and selecting in Luria-Bertani (LB) plates containing chloramphenicol (30 µg/ml) and kanamycin (50 µg/ml) at 32°C for 24–36 hours. Targeted recombination in the kanamycin and chloramphenicol-resistant colonies was confirmed by the detection of a 3.5-kb product by PCR using primer pairs Us2-F (5’-GGAATACATTCGACGCCCAA) & Us7-R (5’-CTATAAGCAGATGCGCCCGA) located in the Us2 and Us7 respectively. The Kan cassette flanked by the FRT sequences was flipped off by adding 0.2% L-arabinose for 1 hour and selecting on chloramphenicol-containing LB plates. DNA extracted from one of the chloramphenicol-resistant clones (pRB-1B*X6) was checked for infectivity by transfection into primary CEF using Lipofectamine (Invitrogen, Paisley, United Kingdom). The virus reconstituted virus from this clone, designated vRB-1B*X6, was grown up on CEF, titrated and stored in liquid nitrogen.

The integrity of the pRB-1B*X6 clone was initially checked by EcoRI restriction digestion (not shown) followed by Southern blot analysis using digoxigenin (DIG)-labelled DNA probes (Roche Applied Sciences, Hertfordshire, United Kingdom) using procedures described before [11]. The membrane was sequentially hybridized with probes specific for MEQ, Us2 region and the E. coli guanine phosphoribosyl transferase (gpt) sequence in the pDS-PHAI [8]. As expected, a single 2.4 kb MEQ specific band was present in all lanes including the DNA from pRB-1B5, pRB-1B*X6, vRB-1B5, vRB-1B*X6, as well as the wtRB-1B virus (Fig. 1B). Stripping and reprobing the membrane with the Us2 probe showed no signals pRB1B-5 and vRB-1B5 (lanes 1 and 3), confirming the deletion of the Us2 gene in these constructs [11]. Detection of the Us2-specific band of the similar size in the wtRB-1B (lane 5) both in pRB-1B*X6 (lane 2) and vRB-1B*X6 (lane 4) confirmed the repair of the Us2 deletion in this construct. When the same membrane was further stripped and probed with the gpt probe, specific signals were detected in pRB-1B5 (lane 1), pRB-1B*X6 (lane 2) and vRB-1B5 (lane 3), demonstrating the presence of the mini-F plasmid in these DNA samples. The absence of gpt signals in the DNA sample from vRB-1B*X6 (lane 4), similar to the wtRB-1B virus (lane 5), confirms the self-excision of the mini-F plasmid during the growth of the virus in the CEF, following the expression of the functional cre in these cells. Thus the data from the Southern blot hybridization showing the presence of MEQ and Us2 and absence of mini-F plasmid confirmed that the genome structure of the vRB-1B*X6 is similar to the wtRB-1B virus. We also
examined the status of expression of Us2 by reverse transcription PCR in the cells infected with the 3 viruses. As expected, both vRB1B-X6 and wtRB-1B virus-infected cells amplified a PCR product of the expected size, cells infected with vRB1B-5 virus was negative (data not shown).

In order to examine whether the above modifications have given any growth advantage to the vRB-1B*X6 over vRB-1B5, we first compared its in vitro growth of the two viruses in CEF. As shown in the Fig 1C (1), at all the time points after 24 hours post infection vRB-1B*X6 showed higher titres than vRB-1B5, demonstrating that the modification did have a positive effect on virus replication in
viruses. We then asked whether the increased in vitro replication of the vRB-1B*X6 is also reflected in the replication in vivo. For this, we monitored the MDV genome copy numbers in the peripheral blood leukocytes (PBL) of 5 line P SPF (specific-pathogen-free) chickens infected with 1000 p. f. u. of vRB-1B5 and vRB-1B*X6 viruses at different days up to 28 days post infection using real-time quantitative PCR [20]. MDV genome copy numbers of both viruses reached the plateau at 14 days post infection, after which the titres were maintained. The genome copy numbers of vRB-1B*X6 virus was at a higher level than that of vRB-1B5 at all time points demonstrating higher in vivo replication in PBL. In this respect, vRB-1B*X6 virus showed a replication kinetics almost identical to the wtRB-1B virus [Fig 1C (2)]. The differences in the replication trend between the 3 viruses in the PBL was also reflected in the feather DNA samples [Fig 1C (2)].

We then asked whether the increased replication ability of vRB-1B*X6 is associated with higher pathogenicity in an infection model. For this, groups (n = 10) of one-day old MD-susceptible specific-pathogen-free line P (B19/B19) chickens lacking maternal antibodies to MDV were infected intra-abdominally with 1000 p. f. u. of vRB-1B5, vRB-1B*X6 and wtRB-1B viruses. All procedures on experimental birds were approved by the Institute for Animal Health Ethical Committee and carried out in accordance with the Project Licence 30/2145 issued by the United Kingdom Home Office. All the groups of infected birds, together with a group of uninfected control birds, were maintained in isolation and observed for 90 days. Cumulative occurrence of MD for the different groups, based on the incidence gross or histological lesions, was used to calculate the survival rates, and the statistical differences between the different groups were calculated using Kaplan-Meier log rank test for survival [21]. The survival curves showed significant increase in the median time to death between the birds infected with vRB-1B*X6 and vRB-1B5 viruses [Fig 1C (3)]. By including the wtRB-1B virus-infected group in the experiment, we were able to examine the pathogenicity of the two recombinant cloned viruses with that of the wtRB-1B virus stock. There were significant differences in the survival rates between birds infected with wtRB-1B virus and vRB-1B5 viruses (p < 0.0001), as well as between the birds infected with wtRB-1B virus and vRB-1B*X6 viruses (p = 0.0064). Thus our studies demonstrate that the excision of the mini-F plasmid can generate recombinant viruses with increased pathogenicity. These observations were supported in a recent study where the excision of the mini-F sequences was achieved using a different strategy [22]. Thus, we have been able to achieve a significant improvement to the existing BAC clone of this highly oncogenic virus to generate virus stocks with very close biological properties as the parent wtRB-1B virus. This would certainly increase its usefulness as a valuable tool for studies on genome manipulation to identify the oncogenic determinants of this major avian pathogen.

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors’ contributions**

YZ contributed to design, perform the experiment and draft the manuscript. LP participated in the experiment. LPS and SB conducted animal experiments and quantitative PCR analysis. VN supervised the study and drafted the manuscript.

**Acknowledgements**

We would like to thank Dr. Shenk (Princeton University, USA) for pYD-C66 plasmid containing SV40-cre, Dr. Copeland (NCI Frederick MD, USA) for the EL250 cells, EAH staff for assisting in the animal experiments, and Mick Gill for the digital imaging. This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), and the Department of Environment, Food & Rural Affairs (DEFRA), United Kingdom.

**References**

1. Calnek BW: Pathogenesis of Marek’s disease virus infection. Curr Top Microbiol Immunol 2001, 255:25-55.
2. Davison F, Nair V: Marek’s disease: An Evolving Problem London: Elsevier Academic Press; 2004.
3. Morrow C, Feller F: Marek’s disease: a worldwide problem. In Marek’s disease, An Evolving Problem Edited by: Davison F, Nair V. Oxford: Elsevier Academic Press; 2004:49-61. [Pastoret P-P (Series Editor): Biology of animal infections].
4. Calnek BW: Marek’s disease: a model for herpesvirus oncology. CRC Crit Rev Microbiol 1986, 12:293-320.
5. Osterrieder N, Kamil JP, Schumacher D, Tischer BK, Trapp S: Marek’s disease virus: from miasma to model. Nat Rev Micro 2006, 4:283-294.
6. Osterrieder K, Vautheroit JF: The genome content of Marek’s disease-like viruses. In Marek’s disease, An Evolving Problem Edited by: Davison F, Nair V. Oxford: Elsevier Academic Press; 2004:17-31. [Pastoret P-P (Series Editor): Biology of animal infections].
7. Želník V: Marek’s disease virus research in the post-sequencing era: new tools for the study of gene functions and virus-host interactions. Avian Pathol 2003, 32:323-334.
8. Schumacher D, Tischer BK, Fuchs W, Osterrieder N: Reconstitution of Marek’s disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-negative MDV-1 mutant. J Virol 2000, 74:11088-11098.
9. Tischer BK, von Einem J, Kaufer B, Osterrieder N: Two-step Red-mediated recombination for versatile, high-efficiency markerless DNA manipulation in Escherichia coli. BioTechniques 2006, 40:1-6.
10. Schat KA, Calnek BW, Fabricant J: Characterisation of two highly oncogenic strains of Marek’s disease virus. Avian Pathol 1982, 11:593-605.
11. Petherbridge L, Brown AC, Baigent SJ, Howes K, Sacco MA, Osterrieder N, Nair VK: Oncogenicity of virulent Marek’s disease virus cloned as bacterial artificial chromosomes. J Virol 2004, 78:13376-13380.
12. Brown AC, Baigent SJ, Smith LP, Chattoop JY, Petherbridge LJ, Hawes P, Allady MJ, Nair V: Interaction of MEQ protein and C-terminal binding protein is critical for induction of lymphomas by Marek’s disease virus. PNAS 2006, 103:1687-1692.
13. Jarosinski KW, Osterrieder N, Nair VK, Schat KA: Attenuation of Marek’s Disease Virus by Deletion of Open Reading Frame RLORF4 but Not RLORF5a. J Virol 2005, 79:11647-11659.

14. Kamil JP, Tischer BK, Trapp S, Nair VK, Osterrieder N, Kung Hj; vlIP, a viral lipase homologue, is a virulence factor of Marek’s disease virus. J Virol 2005, 79:6984-6996.

15. Trapp S, Parcells MS, Kamil JP, Schumacher D, Tischer BK, Kumar PM, Nair VK, Osterrieder N: A virus-encoded telomerase RNA promotes malignant T cell lymphomagenesis. J Exp Med 2006, 203:1307-1317.

16. Smith GA, Enquist LW: A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. Proc Natl Acad Sci USA 2000, 97:4873-4878.

17. Yu D, Smith GA, Enquist LW, Shenk T: Construction of a self-excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene. J Virol 2002, 76:2316-2328.

18. Chang WL, Barry PA: Cloning of the full-length rhesus cytomegalovirus genome as an infectious and self-excisable bacterial artificial chromosome for analysis of viral pathogenesis. J Virol 2003, 77:5073-5083.

19. Lee EC, Yu D, Martinez de Velasco J, Terrasrollo L, Swing DA, Court DL, Jenkins NA, Copeland NG: A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics 2001, 73:56-65.

20. Baigent SJ, Petherbridge LJ, Howes K, Smith LP, Currie RJ, Nair VK: Absolute quantitation of Marek’s disease virus genome copy number in chicken feather and lymphocyte samples using real-time PCR. J Virol Methods 2005, 123:53-64.

21. Altman DG: Practical Statistics for Medical Research London: Chapman and Hall, 1991.

22. Jarosinski KW, Margulis NG, Kamil JP, Spatz SJ, Nair VK, Osterrieder N: Horizontal transmission of Marek’s disease virus requires US2, the UL13 protein kinase, and gC. J Virol 2007, 81:10575-10587.