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Removal of Inserted BAC after linearizatiON (RIBON)—a novel strategy to excise the mini-F sequences from viral BAC vectors

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ABSTRACT. The bacterial artificial chromosome (BAC) technology has been a mainstay approach for generating recombinant viruses, and several methods for excision of the mini-F sequences from the viral BAC vectors have been developed. However, these strategies either require complicated procedures or leave scars of inserted sequences. To overcome these problems, a new method to excise the mini-F sequences from viral BAC vectors based on the Removal of Inserted BAC after linearizatiON (RIBON) strategy was developed in this study for herpesvirus of turkeys (HVT). Enhanced green fluorescent protein (eGFP) DNA and the mini-F sequences were inserted into the gene encoding HVT thymidine kinase (TK) by homologous recombination in chicken embryo fibroblasts (CEFs), and the constructed HVT-BAC vector was used to transform *Escherichia coli* (pHVT-BAC). To remove the inserted eGFP and mini-F sequences, pHVT-BAC was linearized using a homing endonuclease I-*Sce*I and used to cotransfect CEFs together with a plasmid containing the TK gene of HVT. The obtained viruses (44%) did not express eGFP, and DNA sequencing of isolated clones revealed that they were completely free of the inserted BAC sequences. Moreover, growth kinetics and plaque morphology of reconstituted viruses were comparable with those of the parental HVT. The results of this study demonstrate that the novel RIBON approach to remove mini-F sequences from the viral genome is simple and effective.

KEY WORDS: bacterial artificial chromosome, herpesvirus of turkeys, mini-F sequences

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Herpesviruses have frequently been used for generating recombinant vaccines, because of their capacity to accommodate foreign genes and maintain persistent infection in the host. In the past, recombinant herpesviruses had been constructed by laborious homologous recombination in eukaryotic cells; however, the adaptation of the bacterial artificial chromosome (BAC) technology for viruses in 1993 [7] provided the mainstay approach to generate recombinant viruses.

The use of the BAC technology for virus cloning requires the excision of the minimal fertility factor replicon (mini-F), the backbone of the BAC vector, from the viral genome-containing construct. To achieve this, four methods have been mainly used [11]. The most popular one utilizes the Cre/*loxP* or FLP/*FRT* recombination systems [1, 3]. In these systems, *loxP* or *FRT* sites are added to either end of the mini-F sequences; then, one of each of the *loxP* or *FRT* sites and sandwiched mini-F sequences are removed by Cre or FLP enzymes via recombination. For this reaction, the virus BAC should be either incubated with Cre or FLP enzymes *in vitro* or transferred into eukaryotic cells together with the Cre or FLP expression plasmids. Although the method is easy, it introduces one 34-bp *loxP* or *FRT* sequence, which can compromise the development of commercial vaccines and may change the expression of viral genes if inserted into open reading frames or gene regulatory regions. In addition, some reconstituted viruses retain the mini-F sequences; then, selective purification of mini-F-negative viruses is required, because the FLP and Cre reactions tend to approach an equilibrium, resulting in the same emergence rate for mini-F-negative and -positive viruses. The second method uses the recombination mechanism of eukaryotic cells and a repair vector or PCR product substitute for the mini-F sequences [10]. This method requires the repair vector or PCR product homologous to the original sequence upstream and downstream of the insertion site for the mini-F sequences. The repair vector or PCR product and the virus BAC are cotransferred into eukaryotic cells, where the mini-F sequences are removed via recombination between homologous sequences of the repair vector or PCR product and the virus BAC. To obtain a homogeneous mini-F-negative viral population, laborious purification steps are required, but no residual mini-F sequences are left at the insertion site. The third and fourth methods use the recombination mechanism of eukaryotic cells and the sequence overlapping the mini-F replicon; these methods do not require laborious purification steps and leave no scar. In the third method, the mini-F is sandwiched between homologous sequences [13], which recombine with each other and remove the mini-F during virus replication in eukaryotic cells. The drawback of this strategy is the instability of virus BAC in *Escherichia coli* due to duplication of the viral sequence. To overcome this problem, the fourth method utilizes two sets of inverted duplicated sequences [4], providing stable maintenance of the mini-F in *E. coli*.

Although the fourth method seems to be an ideal strategy, the construction of the virus BAC is complicated by inverted

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sequence duplications, making this strategy difficult to use once the virus BAC is constructed by other methods. In this study, we developed a simple method that did not leave mini-F scars and could be used after virus BAC construction; it is called the Removal of Inserted BAC after linearizatiON (RIBON) and is based on the excision of the mini-F cassette using the recombination mechanism of eukaryotic cells, a repair vector and linearized virus BAC DNA.

MATERIALS AND METHODS

Chicken embryo cells and herpesvirus: Chicken embryo fibroblasts (CEFs) were obtained from specific pathogen-free (SPF) 10-day-old chicken embryos (Nissei Bio Co., Hokuto, Japan) and maintained in a 1:1 mixture of Leibovitz's L-15 and McCoy's 5A Medium (Life Technologies Corp., Tokyo, Japan) (LM) supplemented with 4% calf serum (CS). The parental herpesvirus of turkeys (HVT) FC126 strain [16] was obtained from Dr R. L. Witter at the Avian Disease and Oncology Laboratory (East Lansing, MI, U.S.A.).

Construction of pUC18-HVT-TK: A 3.3-kb DNA fragment of the HVT FC126 genome comprising the region from the UL22 to UL25 gene (nucleotides [nts] 45,700 to 48,967 of the FC126 genome, GenBank Accession # AF291866) was cloned by PCR using primers F-HVT-*Sal*I-45700 and R-HVT-*Sac*I-48967 (Table 1) and FC126 DNA as a template. The amplified fragment was digested with *Sal*I and *Sac*I and cloned into the pUC18 vector (Takara, Otsu, Japan), resulting in pUC18-HVT-TK (Fig. 1A).

Construction of pUC18-HVT-TK-SfiI: The pUC18-HVT-TK-*Sfi*I plasmid also comprises the UL22-UL25 region, with the *Sfi*I recognition site added between nts 47,316 and 47,317. Two primer pairs, F-HVT-*Sal*I-45700/R-HVT-*Sfi*I-47316 and F-HVT-*Sfi*I-47317/R-HVT-*SacI*-48967 (Table 1), were used for amplification of the UL22–UL25 region with the *Sfi*I site. The resultant 3.3-kbp fragment was cloned into the pUC18 vector (Takara) digested with *Sal*I and *Sac*I, resulting in pUC18-HVT-TK-*Sfi*I (Fig. 1B).

Construction of pUC18-HVT-BAC: The CMV promoter of pBK-CMV (Stratagene, Tokyo, Japan) with the added *Sfi*I restriction site and the enhanced green fluorescent protein (eGFP) gene of pEGFP-1 (Takara) with added *Sal*I and *Sfi*I restriction sites were amplified by PCR using two primer pairs (F-*Sfi*I-*LoxP*-CMV/R-eGFP-CMV and F-CMV-eGFP/R-*Sfi*I-*LoxP*-*Sal*I-eGFP, respectively, Table 1). The products were mixed and used as a template for the following PCR with primers F-*Sfi*I-*LoxP*-CMV/R-*Sfi*I-*LoxP*-*Sal*I-eGFP, producing the CMV-eGFP fragment. As a result, the *Sfi*I recognition site and *loxP* sequence were added to the 5′ end, while the *Sal*I site, another *loxP* sequence, and *Sfi*I site were added to the 3′ end of the fragment, which was then cloned into the T-cloning site of the T-vector pMD19 (Takara) to generate pCMVeGFP. The mini-F and chloramphenicol resistance cassette obtained from pBeloBAC11 (New England BioLabs, Tokyo, Japan) by *Sal*I digestion were cloned into the *Sal*I restriction site of pCMVeGFP, resulting in pCMVeGFP-BAC.

To insert the I-*Sce*I recognition site and 50-bp duplication sequence (nts 47,317–47,366 of FC126) adjacent to the *Sfi*I recognition site of pUC18-HVT-TK-*Sfi*I (Fig. 1B), a 100-bp DNA fragment was amplified without a template using complimentary primers, F-TK-duplication and R-TK-*Sfi*I-I-*Sce*I (Table 1). The amplified fragment was cloned into the *Nae*I site of pUC18-HVT-TK-*Sfi*I using the In-Fusion HD Cloning Kit (Takara), resulting in the pUC18-HVT-TK-I-*Sce*I-*Sfi*I plasmid (Fig. 1C), which contained the 45,700–48,967 fragment with the 50-bp duplication sequence, I-*Sce*I site and *Sfi*I site inserted between nts 47,316 and 47,317.

Then, CMV promoter, eGFP, mini-F and chloramphenicol resistance sequences obtained from pCMVeGFP-BAC by *Sfi*I digestion were cloned into the *Sfi*I site of pUC18-HVT-TK-I-*Sce*I-*SfiI*, resulting in pUC18-HVT-BAC (Fig. 1D).

Construction of HVT-BAC: CEFs (1×10^7) were co-transfected with 1 μg of pUC18-HVT-BAC and 2 μg of the FC126 genomic DNA by electroporation using Nucleofector II (Lonza, Basel, Switzerland), diluted in 20 m*l* LM, and seeded in two 96-well tissue culture plates. Five days post-transfection, eGFP-positive plaques were identified, and the cells were detached by trypsinization, mixed with fresh CEFs in 10 m*l* LM and seeded in a 96-well plate. After three rounds of purification by limited dilution, recombinant virus clones (HVT-BAC) were isolated, and HVT-BAC DNA was extracted from CEFs infected with the purified viruses as previously described [9] and used to transform *E. coli* GS1783 strain [12] (obtained from Dr G. Smith, Northwestern University, Chicago, IL, U.S.A.) by electroporation at 1.6 kV, 25 μF and 200 ohm using Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The transformed bacteria were grown with agitation for 1 hr at 30°C, planted on Luria-Bertani (LB) agar containing 20 μg/m*l* chloramphenicol and incubated overnight at 30°C. The emerged colonies were inoculated into liquid LB medium, and circular HVT-BAC DNA (pHVT-BAC; Fig. 2A) was purified by the alkaline lysis method and analyzed by band pattern after digestion with *Eco*RI and separation on 0.8% agarose gels.

Reconstitution of HVT and HVT-SfiI: pHVT-BAC DNA extracted from 3 m*l* of overnight culture of GS1783 cells was digested with a homing endonuclease I-*Sce*I (New England Biolabs) and used together with 1 μg of pUC18-HVT-TK-*Sfi*I or pUC18-HVT-TK to cotransfect CEFs by electroporation. The transfected cells (1×10^7) were diluted with 20 m*l* LM and seeded into two 96-well plates. Five days after transfection, plaques with or without eGFP expression were identified.

Identification of BAC excision: Reconstituted (rc) HVT-*Sfi*I viruses (rcHVT-*Sfi*I) rescued by cotransfection of linearized pHVT-BAC and pUC18-HVT-TK-*Sfi*I were passaged in CEFs 10 times and examined for eGFP expression. DNA was extracted from CEFs infected with rcHVT-*Sfi*I at the second passage using the QIAamp DNA Mini Kit (Qiagen, Limburg, The Netherlands) and subjected to further analysis. Extracted DNA carrying or not carrying eGFP, mini-F and chloramphenicol resistance sequences were identified by PCR; three primer pairs (F-eGFP/R-BAC, F-cm/R-HVT-47750 and F-HVT-46898/R-HVT-*Sac*I-48967; Table 1) were used to

Table 1. Primers used for generating plasmids and *in vitro* stability analysis

Primer	Sequence (5'-3')
F-HVT-*Sal*I-45700	GCGTCGACTTGTCGGGGTGGCCA
R-HVT-*Sac*I-48967	GCGAGCTCTCCAAAGGTCTGAGTCTGC
R-HVT-*Sfi*I-47316	TAGGCCGGGGGGGCCGGCACCCTGTGG
F-HVT-*Sfi*I-47317	CCGGCCCCCCCGGCCTATCCAGCATTAAT
F-*Sfi*I-*LoxP*-CMV	GGCCCCCCCGGCCATAACTTCGTATAGCATACATTATACGAAGTTATAAGGCTGCAGAGTTATTAATAGTAA
R-eGFP-CMV	GCCCTTGCTCACCATGGATCTGACGGTTCACT
F-CMV-eGFP	AGTGAACCGTCAGATCCATGGTGAGCAAGGGCGAGGAGCT
R-*Sfi*I-*LoxP*-*Sal*I-eGFP	GGCCGGGGGGGCCATAACTTCGTATAATGTATGCTATACGAAGTTATGTCGACCCCCAGCTGGTTCTTTCCG
F-TK-duplication	CCGCCACAGGGTGCCTATCCAGCATTAATATAATTGCTGGAGTATCGCATATTTCTATTTTTCC
R-TK-*Sfi*I-I-*Sce*I	TAGGCCGGGGGGGCCATTACCCTGTTATCCCTAAGGAAAAATAGAAATATGCGATACTCCAGCA
F-eGFP	GTGAGCAAGGGCGAGGAG
R-BAC	GGGTAACGATTATCGCCCAAC
F-cm	GTACTGCGATGAGTGGCAG
R-HVT-47750	CCTCGAAGACAATTGCCAGC
F-HVT-46898	AATGGCCAGGAGAGTTCGG

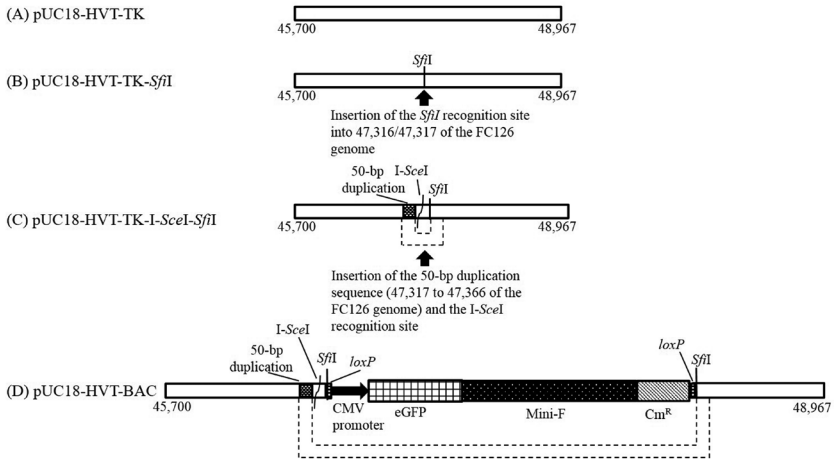

Fig. 1. Schematic diagrams of the plasmids. (A) pUC18-HVT-TK. The region from 45,700 to 48,967 nucleotides (nts) of the herpesvirus of turkeys (HVT) FC126 strain genome was cloned into pUC18. (B) pUC18-HVT-TK-*Sfi*I. The *Sfi*I recognition site was introduced between nts 47,316 and 47,317 of the FC126 genome, and the 45,700–48,967 region was cloned into pUC18. (C) pUC18-HVT-TK-I-*Sce*I-*Sfi*I. A 50-bp duplication sequence (nts 47,317–47,366 of the FC126 genome) and the I-*Sce*I recognition site were inserted before the *Sfi*I site. Dashed lines show homologous sequences. (D) pUC18-HVT-BAC. *LoxP*, eGFP, mini-F and chloramphenicol resistance cassette sequences were inserted into the *Sfi*I site of pUC18-HVT-TK-I-*Sce*I-*Sfi*I. Dashed lines show homologous sequences. Cm^R indicates the chloramphenicol resistance gene.

amplify the regions between the eGFP-encoding gene and mini-F sequences, the chloramphenicol resistance cassette and insertion site of the FC126 genome, and the insertion site region of the FC126 genome, respectively. FC126 genomic DNA was also used as a template for a positive control. The products amplified with primers F-eGFP/R-BAC and F-cm/R-HVT-47750 were directly separated on 0.8% agarose gels, while the fragment amplified with primers F-HVT-46898/R-HVT-*Sac*I-48967 was first digested with *Sfi*I before separation in the same gel. The *Sfi*I-undigested fragments were excised, and DNAs were extracted using the NucleoSpin Gel and PCR Clean-up kit (Takara) and were sequenced using F-HVT-46898 or R-HVT-*Sac*I-48967 primers and a CEQ 2000XL sequencer (Beckman Coulter, Tokyo, Japan).

Isolation of rcHVT: To purify the cloned virus, CEFs infected with rcHVT rescued by cotransfection with pHVT-BAC and pUC18-HVT-TK were trypsinized and sonicated in sucrose, phosphate, glutamate and albumin (SPGA) buffer [2]. Cell-free viruses in SPGA were inoculated into fresh CEFs in 96-well plates and incubated for 2 hr at 37°C; then, the supernatant was removed, and LM supplemented with 4% CS was added to CEF monolayers. Five days after infection, cells with or without plaques and eGFP expression were identified; DNA was extracted and analyzed by PCR using primer pairs F-eGFP/R-BAC, F-cm/R-HVT-47750 and F-HVT-46898/R-HVT-*Sac*I-48967 (Table 1) described above.

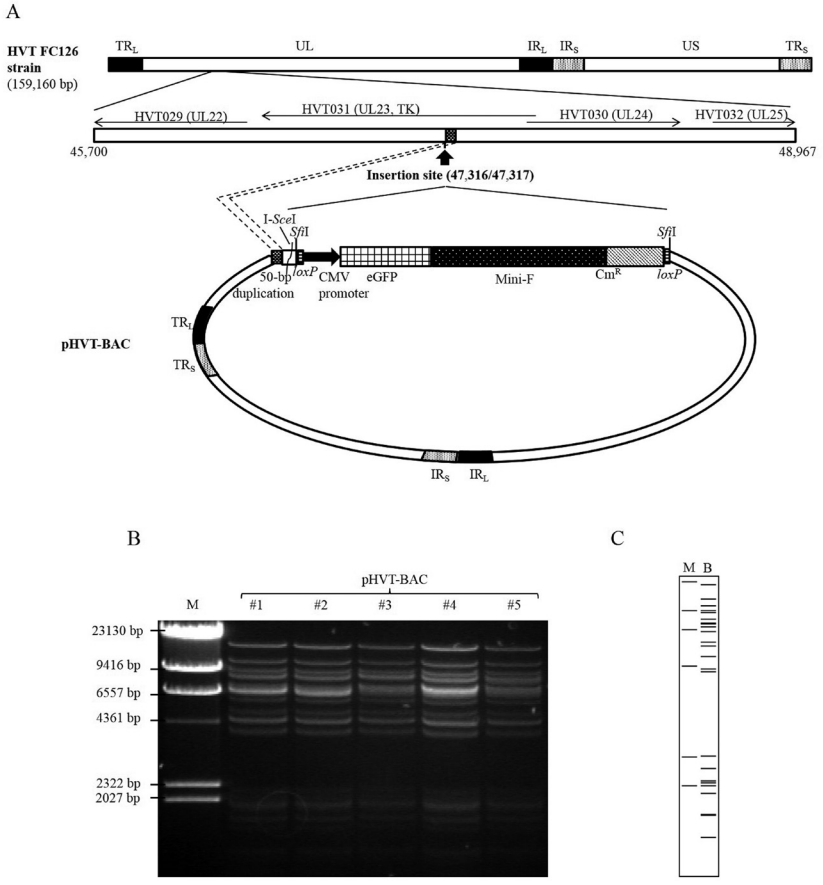

Fig. 2. Construction of pHVT-BAC. (A) A schematic diagram of pHVT-BAC. Duplication sequence of FC126, I-*Sce*I recognition sequence, *lox*P, eGFP, mini-F and chloramphenicol resistance cassette were inserted between nts 47,316 and 47,317 of the FC126 genome. Cm^R indicates the chloramphenicol resistance gene. Dashed lines show homologous sequences. (B) *Eco*RI digestion pattern of pHVT-BAC in 0.8% agarose gel. Numbers indicate virus clones. (C) Expected band pattern of pHVT-BAC constructed using the Gene Construction Kit. B indicates pHVT-BAC. M, molecular weight markers.

Growth kinetics: CEFs plated in 6-well plates (9.5×10^5 cells/well) were infected with rcHVT or FC126 at the multiplicity of infection (MOI) 0.001. At 0, 24, 48, 72 and 96 hr after infection, CEFs were harvested, and virus titers were determined by the plaque assay. The data were obtained from two independent experiments. Viral growth kinetics was evaluated by Student's *t*-test.

Plaque assay: CEFs seeded in 12-well plates (1×10^6 cells/well) were infected with serial 10-fold dilutions of virus-carrying trypsinized cells and grown in LM supplemented with 4% CS for 4 days. Cells were then fixed with methanol:acetone mixture (1:2) and incubated with anti-FC126 monoclonal antibody L78 diluted 1:500 [6] for 1 hr at 37°C; biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA, U.S.A.) was used as a secondary antibody. The signal was detected using the VECTASTAIN ABC-AP kit (Vector Laboratories) for signal enhancement and NBT/BCIP solution (Roche Applied Science, Penzberg, Germany) for development. Plaque numbers were determined macroscopically, and plaque morphology was evaluated. The average size of 50 plaques was calculated using the CellSens standard program (Olympus, Tokyo, Japan). Plaque sizes were compared using Student's *t*-test.

RESULTS

Construction of HVT-BAC: After insertion of eGFP, chloramphenicol resistance cassette and mini-F sequences into the TK region of FC126 by homologous recombination in CEFs, four independent clones of HVT-BAC were isolated, and the extracted viral DNAs were used to transduce *E. coli* GS1783 producing a total of 25 colonies. The analysis of DNA extracted from *E. coli* (pHVT-BAC; Fig. 2A) revealed two band patterns shared by clones #1, #2 and #4, and clones #3 and #5, respectively (Fig. 2B), although all band patterns were similar to that of pHVT-BAC (Fig. 2C). Therefore, clones pHVT-BAC#1 and pHVT-BAC#3 were selected for further analysis as representatives of the two band patterns.

Reconstitution of HVT-SfiI and identification of BAC excision: To excise eGFP, chloramphenicol resistance cassette

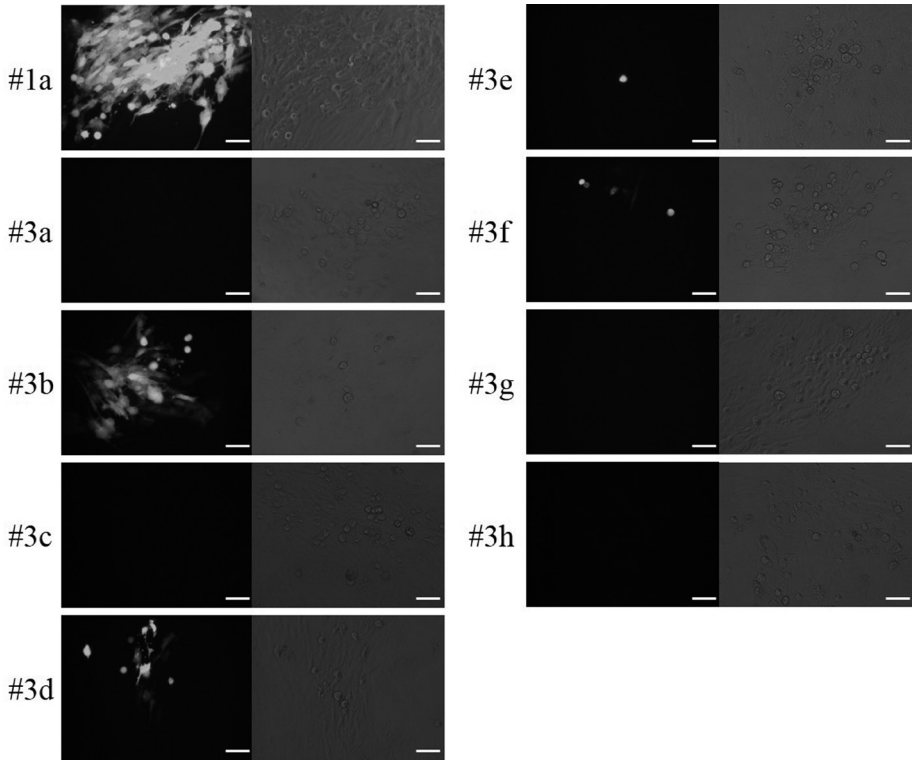

Fig. 3. Plaques produced by reconstituted HVT-*Sfi*I in chicken embryo fibroblasts. Cells were transfected with linearized pHVT-BAC and pUC18-HVT-TK-*Sfi*I and analyzed for plaque formation and eGFP expression five days after transfection. Left panels, eGFP fluorescence; right panels, bright field microscopy. Scale bars, 50μm.

Table 2. eGFP expression by reconstituted HVT-*Sfi*I

Clone number	Passage 0	Passage 1	Passage 2	Passage 9	Passage 10
rcHVT-*Sfi*I #1a	+	+	+	+	+
rcHVT-*Sfi*I #3a	−	−	−	−	−
rcHVT-*Sfi*I #3b	+	partial	partial	partial	−
rcHVT-*Sfi*I #3c	−	−	−	−	−
rcHVT-*Sfi*I #3d	partial	−	−	−	−
rcHVT-*Sfi*I #3e	partial	−	−	−	−
rcHVT-*Sfi*I #3f	partial	−	−	−	−
rcHVT-*Sfi*I #3g	−	−	−	−	−
rcHVT-*Sfi*I #3h	−	−	−	−	−

+, eGFP expression in all plaques; partial, eGFP expression in some plaques; −, no eGFP expression.

and the mini-F, pHVT-BAC DNA was first digested with the homing enzyme I-*Sce*I, which has a single recognition site of 18-bp in pHVT-BAC adjacent to the 50-bp duplication site (Fig. 2A). Linearized pHVT-BAC #1 or #3 and pUC18-HVT-TK-*Sfi*I were used to cotransfect CEFs. In this study, pUC18-HVT-TK-*Sfi*I carrying the *Sfi*I site in the TK region was used to distinguish the viruses recombined with this plasmid from those reconstituted by self-recombination of pHVT-BAC via the 50-bp duplication sequence.

Five days after transfection, one plaque was produced by pHVT-BAC#1 (rcHVT-*Sfi*I#1a) and eight plaques—by pHVT-BAC#3 (rcHVT-*Sfi*I#3a, 3b, 3c, 3d, 3e, 3f, 3g and 3h). Four of the nine viruses were not fluorescent (44%, rcHVT-*Sfi*I#3a, 3c, 3g and 3h), while two (rcHVT-*Sfi*I#1a and #3b) demonstrated green fluorescence in all infected cells and the other three (rcHVT-*Sfi*I#3d, 3e and 3f) were partially fluorescent (Fig. 3 and Table 2).

To identify BAC excision, the reconstituted viruses were serially passaged. Green fluorescence disappeared in the three clones with partial eGFP expression after the first passage (rcHVT-*Sfi*I#3d, 3e and 3f; Table 2), and in rcHVT-*Sfi*I #3b clone, it decreased after 10 passages, but was still observed in all rcHVT-*Sfi*I#1a plaques after multiple passages. PCR analysis of this clone at the 2nd passage demonstrated amplification of eGFP and mini-F sequences (Region 1; Fig. 4A and B (a)) and the bridge between the chloram-

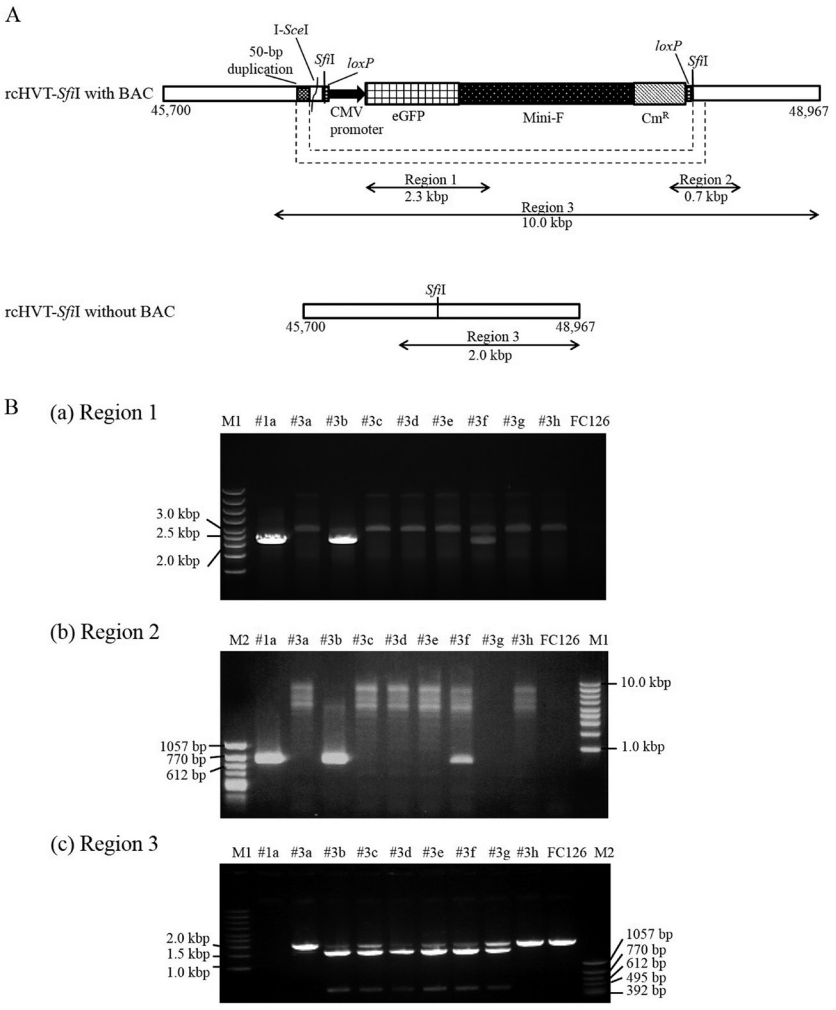

Fig. 4. PCR analysis of rcHVT-*Sfi*I. (A) Schematic diagram of the amplified region of rcHVT-*Sfi*I with and without BAC. The size of the region is indicated below the arrow. Dashed lines show homologous sequences. (B) PCR analysis of rcHVT-*Sfi*I. DNA extracted from chicken embryo fibroblasts infected with rcHVT-*Sfi*I at the 2nd passage or FC126 DNA was used as templates. (a) A region between the eGFP-encoding gene and mini-F sequences (Region 1). (b) A region between chloramphenicol resistance cassette and the insertion site of the FC126 genome (Region 2). (c) The insertion site region of the FC126 genome (Region 3). PCR-amplified Region 3 fragments were digested with *Sfi*I and analyzed by agarose gel electrophoresis. M1 and M2, molecular weight markers.

phenicol resistance cassette and HVT (Region 2; Fig. 4A and B (b)), indicating the presence of the inserted sequences. rcHVT-*Sfi*I#3b and 3f also retained eGFP, chloramphenicol resistance cassette and the mini-F (Fig. 4A and B (a, b)). Then, the insertion site region of the FC126 genome (Region 3; Fig. 4A) was analyzed by PCR, which was supposed to amplify a 2.0-kbp fragment if the viral genome did not carry eGFP, chloramphenicol resistance cassette and the mini-F in the TK region. In addition, the amplified fragment (Region 3) should be digested by *Sfi*I into 1.6-kbp and 400-bp fragments if recombination occurred between the virus genome and pUC18-HVT-TK-*Sfi*I, since there was no *Sfi*I site in the TK region of parent FC126, while pHVT-BAC should be digested by *Sfi*I into 8.0-kbp, 1.6-kbp and 500-bp fragments (Fig. 4A). The results showed that rcHVT-*Sfi*I#3a and 3h were not digested with *Sfi*I and rcHVT-*Sfi*I#1a did not show the 2.0-kbp band (Fig. 4B (c)), which is consistent with its eGFP expression in CEFs. Other clones showed 2.0-kbp, 1.6-kbp and 400-bp bands. Sequencing of the 2.0-kbp fragments revealed that rcHVT-*Sfi*I#3a and 3h did not have the *Sfi*I recognition site in the TK region and carried sequences homologous to the intact TK region, while rcHVT-*Sfi*I#3b, 3c, 3d, 3e, 3f and 3g had the *Sfi*I site.

Growth kinetics and plaque morphology of the reconstituted viruses: To analyze characteristics of the reconstituted viruses, pHVT-BAC #1 and #3 were digested with I-*Sce*I and transferred to CEFs together with pUC18-HVT-TK. Plaques with and without green fluorescence (rcHVT-BAC (+) and rcHVT-BAC (−), respectively) were obtained from both #1 and #3 clones, and the respective viruses were isolated

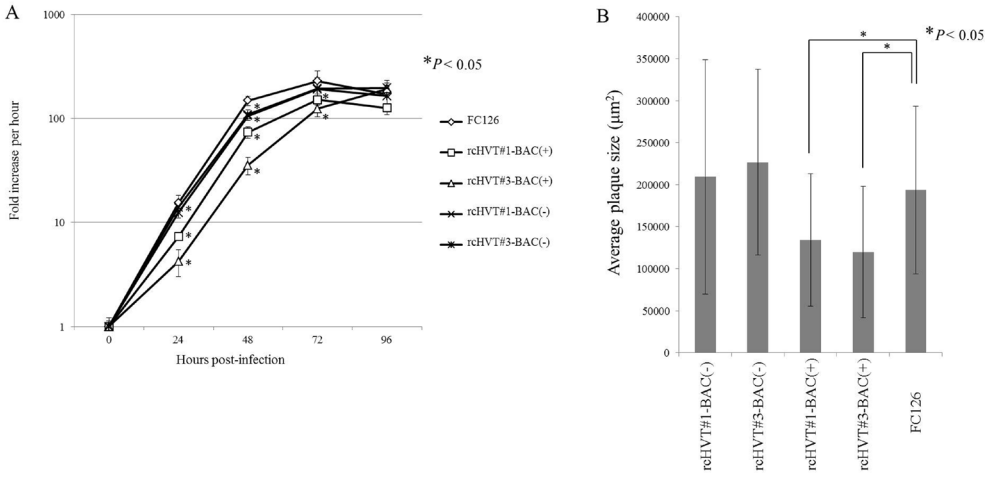

Fig. 5. Growth characteristics of rcHVT. (A) Growth kinetics of rcHVT and parental FC126. Chicken embryo fibroblasts were infected with rcHVT-BAC or FC126 at the MOI of 0.001 and analyzed at the indicated times post-infection for viral titers by the plaque assay. *P<0.05 was considered statistically significant. (B) The average plaque size produced by rcHVT and FC126. Cells were infected as above for four days and analyzed for plaque size by immunocytochemistry. The data are expressed as the mean ± SD (n=50 plaques); *P<0.05 was considered statistically significant.

(rcHVT#1-BAC (+), rcHVT#3-BAC (+), rcHVT#1-BAC (−) and rcHVT#3-BAC (−)). Growth kinetics and plaque morphology of reconstituted viruses were compared with those of the parental FC126 strain (Fig. 5). While non-fluorescent clones showed growth almost similar to that of the FC126 strain, significant differences were observed between the parental FC126 strain and rcHVT#1-BAC (−) at 48 hr, and between FC126 and rcHVT#3-BAC (−) at 24 and 48 hr after infection (Fig. 5A). In contrast, the titers of fluorescent viruses were lower than those of the parental strain, and significant differences were observed between the FC126 strain and fluorescent viruses at 24, 48 and 72 hr after infection. Similar results were obtained for plaque morphology: there was no significant difference between plaque sizes of non-fluorescent clones rcHVT #1-BAC (−) and rcHVT#3-BAC (−) and the FC126 strain, while the average plaque size of fluorescent viruses was significantly smaller (Fig. 5B).

DISCUSSION

In this study, we demonstrated a new strategy to excise BAC cassette from the viral BAC vector by cotransfecting eukaryotic cells with a linearized virus-BAC construct and a homologous plasmid. Traditional methods have certain inherent problems, such as leaving scars of one *loxP* or *FRT* sequence, laborious purification, complicated construction of virus BAC or instability of the construct [1, 3, 4, 10, 13]. Our strategy termed RIBON (Removal of Inserted BAC after linearizatiON) is simple and does not need laborious purification steps: it only requires transfection of eukaryotic cells with the linearized virus BAC and homology plasmid. Furthermore, it does not involve the generation of complex virus BAC structures and leaves no scars of the BAC sequence in the reconstituted virus. Moreover, by inserting I-*Sce*I site adjacent to the mini-F sequences in *E. coli* using the Red-Recombinase technology, this method can be easily applied to the cases when the virus BAC has been constructed with the aim to be used with other BAC-excision methods. Combined with two-step selection [14] procedures, such as *galK* positive/negative selection for gene insertion or modification, the RIBON strategy enables to generate viruses without scars. In addition, the RIBON strategy can be applied with "en passant" mutagenesis [12] to modify or insert genes, if other homing enzymes, such as I-*Ceu*I, PI-*Psp*I and PI-*Sce*I, are used for linearization.

After transfection of CEFs, some clones still retained eGFP fluorescence, possibly because of transient expression of linearized but not recombined pHVT-BAC or traces of circular I-*Sce*I-undigested pHVT-BAC. Reconstituted HVT-*Sfi*I#1a was probably generated from circular pHVT-BAC, as indicated by the retention of eGFP expression after 10 rounds of passaging and the presence of BAC sequence confirmed by PCR. On the other hand, in rcHVT-BAC-*Sfi*I clones #3b, 3d, 3e and 3f, which lost eGFP expression after several passages, linearized but not recombined or circular pHVT-BAC DNA may coexist with the correctly recombined virus. As growth of the BAC-carrying virus is slower than that of the BAC-free virus, the latter would be selected in culture after several passages if these viruses are co-cultured. Therefore, it should be useful in the RIBON strategy to insert the BAC cassette into the region important for viral replication, so that BAC-carrying viruses would undergo negative selection if co-cultured with BAC-excised viruses.

PCR analysis revealed that the lack of eGFP expression may not indicate the loss of BAC sequence, since not only rcHVT-BAC-*Sfi*I#1a and 3b expressing eGFP, but also eGFP-negative 3f retained BAC DNA at the second passage. However, no BAC sequence was detected in any clone

except rcHVT-*Sfi*I#1a at the 10th passage (data not shown), and the disappearance of eGFP expression corresponded to the absence of BAC-carrying viruses. Thus, while viruses with partial green fluorescence probably lost BAC DNA after several passages, it will be safer to choose the clones without fluorescence after transfection.

Sequencing of PCR amplicons revealed that rcHVT-*Sfi*I#3b, 3c, 3d, 3e, 3f and 3g had a *Sfi*I recognition site and that they had correctly recombined with pHVT-TK-*Sfi*I. The uncut 2.0-kbp bands were probably a result of incomplete *Sfi*I digestion, since this restriction enzyme requires paired recognition sites for cleavage, and the presence of only one site decreases DNA digestion by *Sfi*I [15]. In contrast, rcHVT-*Sfi*I#3a and 3h did not have the *Sfi*I restriction site, despite the presence of the site in both of the original pHVT-BAC and pUC18-HVT-TK-*Sfi*I constructs. The sequences of the two amplicons were homologous to that of the intact TK region lacking the *Sfi*I site, which may be eliminated via recombination between the 50-bp duplication sequences flanking the BAC cassette and *Sfi*I recognition sites during the reaction of linearized pHVT-BAC with pUC18-HVT-TK-*Sfi*I, or during self-recombination of linearized pHVT-BAC.

There is significant difference in growth kinetics between the parental FC126 strain and BAC-positive as well as BAC-negative viruses. The difference between FC126 and BAC-negative viruses may be a result of HVT-BAC construction rather than BAC excision. Thus, genomic mutation in the reconstituted virus-BAC has been reported for Marek's disease virus [8]; besides, HVT-BAC is typically derived from a single population of the parental FC126 strain, although FC126 is not a clonal virus. *In vivo* studies of the reconstituted viruses are required, considering that changes in the pathogenic profile were detected for reconstituted Marek's disease viruses [5].

In this study, the I-*Sce*I recognition site, eGFP, chloramphenicol resistance cassette, mini-F, *loxP* and 50-bp duplication sequences were inserted into the virus-BAC construct. However, *loxP* and 50-bp duplication sequences were inserted to be used with other BAC excision approaches and were not necessary for the RIBON strategy. Further studies of the RIBON method applied to other herpesviruses without *loxP* and the 50-bp duplication sequence are needed. In these cases, the RIBON strategy would be used with other homing enzyme, such as I-*Ceu*I, PI-*Psp*I and PI-*Sce*I. Furthermore, this novel strategy may be also applied for gene insertion, if an appropriate transfer plasmid carrying the gene flanked with sequences homologous to the insertion region of the BAC cassette is used for transfection. Thus, the RIBON strategy should be a useful method for the excision of the BAC cassette and gene insertion.

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