NON-ESSENTIAL LOCI IN THE *Bam*HI-I AND -F FRAGMENTS OF THE HVT FC126 GENOME

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Summary. – The sequence of *Bam*HI-I fragment of the herpesvirus of turkeys (HVT) FC126 strain DNA was analyzed for the presence of potential open reading frames (ORFs). Four complete (ORFs 2 to 5) and 2 partial ORFs (ORFs 1 and 6) were detected. ORFs 2 and 3 were homologous to the HSV-1 UL55 and the EHV-1 gene 3, respectively. The ORF 6 was already partially sequenced by Smith *et al.* (*Virology* **207**, 205-216, 1995), and was homologous to a Marek's disease virus (MDV) ORF located in a similar position (ORF 21; Ross *et al., Virus Genes* **7**, 33-51, 1993a). No significant homology was found for the other ORFs. ORF 4 was antisense to ORF 3. Two HVT recombinants having an expression cassette inserted into two intergenic sites were generated and tested for viremia in chickens. Results demonstrated that these 2 intergenic loci are non-essential for *in vitro* and *in vivo* HVT replication. A 650 bp deletion in the repeat region flanking U_L (TR_L and IR_L (*Bam*HI-F)) has been identified in some DNA molecules of HVT FC126 strain. This deletion covers the entire truncated pp38 homologous ORF and the N-terminus of a small ORF which has no detectable homology with any known gene. Our results indicate that (1) this genomic region including the HVT pp38 homologue was not essential for *in vitro* and *in vivo* growth of HVT, and (2) this deletion had no apparent effect on Marek's disease (MD) protection induced by HVT.

Key words: HVT; insertion locus; recombinant virus; pp38

Introduction

HVT is widely used in chickens as a vaccine against MD. It has been recently developed as a viral vector for poultry (Morgan *et al.*, 1992; Ross *et al.*, 1993b; Sondermeijer *et al.*, 1993). Recent data obtained in our laboratory suggested that the locus of insertion of the foreign expression cassette into the HVT genome is an important factor for HVT recombinant-induced protection. Indeed, vHVT5 which contained an infectious bursal disease virus (IBDV) VP2 expression cassette inserted into the UL13 locus induced equivalent protection against IBD with a dose 10 times lower than that of vHVT2, which contains the same expression cassette inserted into the gI (US7 homologue) locus (Darteil *et al.*, 1995; Bublot *et al.*, 1996). However, none of these recombinants were able to induce a detectable viremia in infected chickens suggesting that the level of *in vivo* replication is low. The objective of the current study was to find other non-essential sites in the HVT genome that could be used as insertion loci for the generation of new HVT recombinants without affecting too much their *in vivo* replication.

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Materials and Methods

Viruses and cells. The HVT FC126 strain (Witter *et al.*, 1970) was propagated in chicken embryo fibroblasts (CEFs). The structure and generation of vHVT5 has been described by Bublot *et al.* (1996).

Construction of the donor plasmids. The HVT *Bam*HI-I fragment (Igarashi *et al.*, 1987) was cloned into pBS SK+ vector (Stratagene) and sequenced. Insertion plasmids for the intergene 1 and 2 loci were constructed by subcloning the *Bam*HI-I fragment, and by inserting a linker (using PCR) containing unique restriction sites into the intergenic regions. A double expression cassette was then introduced into these unique restriction sites in order to construct the donor plasmids. The double expression cassette had an head-to-head orien-

tation and contained (1) the murine cytomegalovirus (mCMV) immediate early promoter followed by the IBDV VP2 gene and the SV40 poly-adenylation signal, and (2) the MDV RNA 1.8 promoter followed by the MDV gB gene including the gB gene polyadenylation signal. The donor plasmids were linearized before transfection.

Generation of HVT recombinants. HVT DNA preparation, transfection and isolation of HVT recombinants were performed as previously described by Darteil *et al.* (1995).

Viremia. One-day-old SPF chickens (white Leghorn chickens from Lohmann) were inoculated intramuscularly (im) with 10^3 PFU of cell-associated HVT recombinants. Blood was sampled at 7, 14, and 21 days after inoculation and treated as previously described by Darteil *et al.* (1995).

Cloning of the HVT FC126 strain. A frozen cell-associated batch of the FC126 strain (17th passage) was sonicated and centrifuged at 2,000 x g for 10 mins at 4°C. The supernatant was filtrated through a 0.2 µm filter. Limiting dilutions were then performed in 96 well plates. The plates were replicated after 3 days in order to amplify the virus clones. The supernatant of some wells positive for cytopathic effect was tested by PCR for the presence of the deletion.

PCR reaction to detect the deletion in BamHI-F fragment. The sequences of the primers used in the PCR are reported in Table 1. The supernatants of positive wells were incubated with proteinase K (1 mg/ml) for 1 hr at 55°C, and then for 15 mins at 95°C in order to inactivate proteinase K and to denature DNA. Five µl of the samples was tested by PCR in a 50 µl standard reaction.

MD challenge. One-day-old SPF chickens (white Leghorn chickens from Lohmann) were vaccinated im with 10 or 10,000 PFU of cell-associated HVT clones. Five days after vaccination, they were challenged intraperitoneally with 100 µl of diluted blood collected from chickens infected with the MDV RB1B strain. Birds were observed on a daily basis after challenge. Dying animals were euthanized by intracardiac injection of barbiturates, and tissues were examined for gross tumors. Ten weeks post-challenge, surviving birds were euthanized and necropsied. All animal protocols were approved by the Institutional Animal Care and Use Committee of Merial Laboratoire de Lyon Gerland.

Results and Discussion

Non-essential loci in the HVT BamHI-I fragment

The *Bam*HI-I fragment of the HVT FC126 strain was cloned and sequenced (5,838 bp). Its location in the U_L (Igarashi *et al.*, 1987) and map are presented in Fig. 1. Four complete (ORFs 2 to 5) and two partial (ORFs 1 and 6) ORFs were detected in this fragment. ORFs 2 and 3 were homologous to the HSV-1 UL55 (McGeoch *et al.*, 1988) and the EHV-1 gene 3 (Telford *et al.*, 1992) respectively. The ORF 6 was already described by Smith *et al.* (1995), and was homologous to an MDV-1 ORF located in a similar position (designated ORF 21 by Ross *et al.*, 1993a). No significant homology was found for partial ORF 1, ORF 4 and ORF 5. ORF 4 was antisense to ORF 3 (Fig. 1).

Two HVT recombinants having a double expression cassette (see Materials and Methods) inserted into two intergenic sites were generated: vHVT9 containing the expression cassette into the intergene 1 locus (between ORF 2 and ORFs 3 & 4), and vHVT10 containing the same expression cassette into the intergene 2 locus (between ORFs 3 & 4 and ORF 5) (Fig. 1). Both HVT recombinants were shown to express the IBDV VP2 and the MDV gB genes by indirect immunofluoescence assay. Their genomic structure was verified by Southern blot analysis (data not shown). No difference in *in vitro* virus growth could be detected between these recombinant viruses and the parental HVT virus.

The two HVT recombinants were tested for viremia induction in one-day-old SPF chickens. The HVT recombinant vHVT5 containing an IBDV VP2 expression cassette in the UL13 locus (Bublot *et al.*, 1996) was included in this experiment as a control. Results are reported in Table 2. In contrast to vHVT5, vHVT9 and vHVT10 induced a detectable level of viremia in all animals inoculated. These results demonstrated that these 2 intergenic loci are non-essential for *in vitro* and *in vivo* HVT replication and therefore are good candidates for deriving HVT recombinants.

Table 1. Sequences of the primers used in the PCR

Name	Sequence (5'-3')
CD59	AAC GCT GAC GCG TTC GCC TTG
CD60	GCC AGA TCG TAC AAA GTC GCG
CD76	TTT CCG CCT CGC TCA GTG TCA ACG
CD77	ACC GCC ACG AAC CAC ATG ATG GCG
CD78	ATG GTT GAG CAT CTG TTC TAG ATC
CD79	CAG TTC AGT CAT TTA CTA CAC ACC

Non-essential loci in the HVT BamHI-F fragment

Southern blot analysis of the parental HVT FC126 strain DNA performed with the *Bam*HI-F fragment as a probe has detected the presence of a 650 bp deletion localized in the repeat region flanking U_L (TR_L and IR_L) in some genomic molecules. The sequence of this genomic region (from an undeleted molecule) was already published by Smith *et al.* (1995). The approximate location of the deletion was determined by PCR using different pairs of primers based on the published sequence. PCR-amplified DNA fragments containing the deletion were cloned, and their sequences were compared to the published undeleted sequence in order to precisely localize the deletion. The observed deletion was 656 bp long and covered the entire truncated pp38 homologous ORF, as well as the N-terminus of a small ORF which had no detectable homology with any known gene (ORFs 3 and 2 respectively described by Smith *et al.*, 1995; ORFs 7 and 8 respectively in Fig. 1). The 5'-limit of the deletion was localized 270 bp downstream of the U_L/R_L junction,

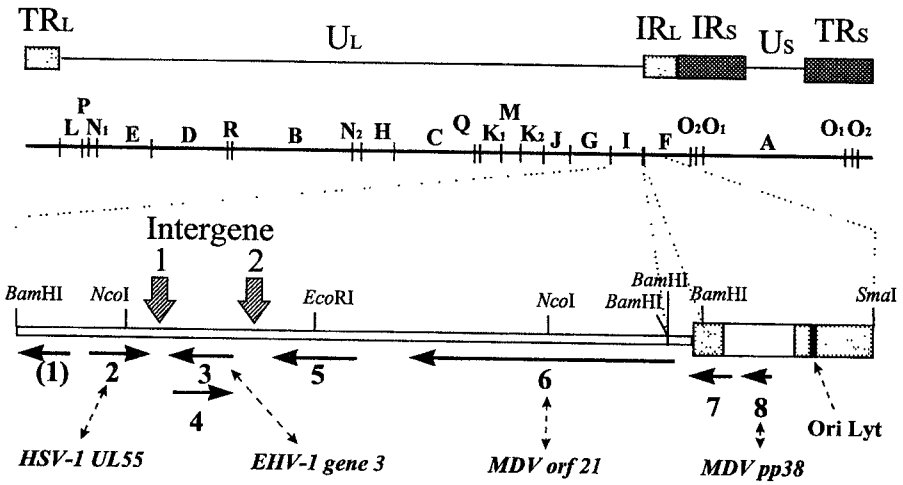

Fig. 1

Genomic structure and *Bam*HI restriction map of HVT (upper part), and restriction map of the *Bam*HI-I and the 5'-end of *Bam*HI-F fragments (lower part)

A. The map is based on data of Igarashi *et al* (1987).

B. The map is based on the sequence of the *Bam*HI-I fragment obtained in our laboratory and on the sequence of 5'-end of the *Bam*HI-F fragment published by Smith *et al* (1995). Horizontal arrows indicates the location and orientation of the 8 potential ORFs; ORF number in brackets (1) indicates that the ORF is partial. Homology with genes of other herpesviruses are indicated by double-sided arrows. The location of the two loci of insertion called intergenes 1 and 2 is indicated by two vertical hatched arrows. The deleted region in the *Bam*HI-F fragment is shown as an open box. The potential lytic origin of replication (Ori_{Lyt}) is shown as a black box.

Table 2. Levels of viremia induced by vHVT9 and vHVT10

Virus[a]	Locus[b]	Day 7[c]	Day 14[c]	Day 34[c]
vHVT5	UL13	0 (0/5)	0 (0/5)	0 (0/5)
vHVT9	Intergene 1	5.3 ± 6.2 (4/5)	12.2 ± 16.8 (5/5)	1.6 ± 0.9 (6/7)
vHVT10	Intergene 2	3.4 ± 4.6 (4/5)	5.1 ± 2.1 (5/5)	0.8 ± 1.4 (3/8)

[a]One-day-old SPF chickens were inoculated im with 10^3 PFU of cell-associated recombinant virus.

[b]The expression cassette inserted into the UL13 locus of vHVT5 contained the HCMV IE promoter followed by the IBDV VP2 gene and the SV40 polyadenylation signal. The double expression cassette inserted into the intergene 1 and 2 loci of vHVT9 and vHVT10, respectively, is described in Materials and Methods.

[c]Mean number of plaques/10^6 leukocytes ± SD (numbers of positive/total animals).

and its 3'-limit 135 bp upstream of the putative origin of replication (Ori Lyt) described by Smith *et al.* (1995).

The HVT FC126 strain stock was cloned in order to isolate a pure population of deleted viruses and to analyze the effect of this deletion on the virus replication. The PCR reaction used in order to test the different FC126 clones was set up with 3 primers (CD59, CD60 and CD76; see Table 1 and Fig. 2 for the sequence and localization of the primers, respectively). It allowed the differentiation between the clones containing undeleted R_L only (2 bands of 315 and 872 bp) or deleted R_L only (1 band of 216 bp) from those containing undeleted and deleted R_L (3 bands of 216, 315, and 872 bp). Among the 42 clones tested, 37 (88%) did not show any deletion, 4 (10%) had deleted and non-deleted repeats, and 1 (2%) had both repeats deleted. For the 4 clones that contained deleted and non-deleted R_L, the molarity of the band having the deletion (the 216 bp band amplified with primers CD76 and CD59) was equivalent to that of the band containing non-deleted R_L (the 315 bp band amplified with primers CD60 and CD59), suggesting that those clones had one of their RL deleted and the other undeleted.

Additional PCR reactions were made with two different primer pairs (CD79/CD77 and CD78/CD77) on the 4 samples that contained deleted and non-deleted repeats in order to identify which repeat contained the deletion. The localization and sequences of the primers are shown in Fig. 2 and Table 1, respectively. Only the band corresponding to the deletion (368 bp) could be observed in the 4 samples after amplification with primers CD79/CD77. Primers CD78/CD77 amplified mainly the undeleted band (1024 bp), but a faint band corresponding to the deletion (368 bp) could be also observed in all samples. These results indicated that most of the DNA molecules of these 4 samples had the R_L near *Bam*HI-I fragment (corresponding to IR_L in the isomer shown in the *Bam*HI map of on Fig. 1) deleted, and the R_L near *Bam*HI-L fragment (corresponding to TR_L in

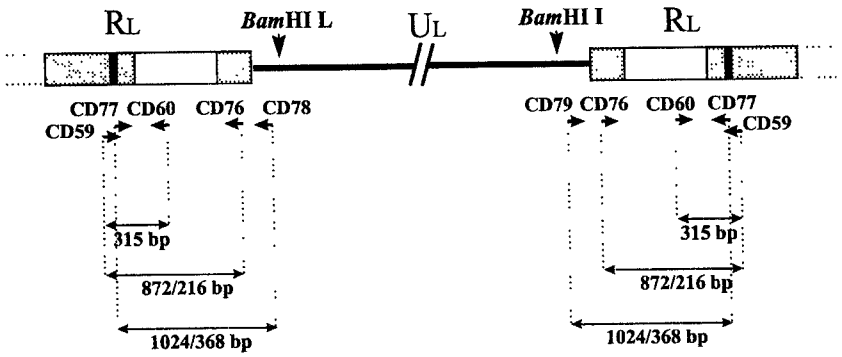

Fig. 2
Location of primers used in PCR

Only the junctions between both ends (*Bam*HI-I and -L fragments) of the U_L and R_L are shown. The deletion is shown as an open white box in the R_L. The black box of the repeat corresponds to the potential Ori_{Lyt}. The primers are represented by small arrows. The sizes of the amplified fragments are included. When two sizes are separated by division sign, the first one corresponds to the amplified undeleted R_L, and the second one to the amplified R_L with deletion.

the isomer shown in the map on Fig. 1) undeleted. These samples contained also DNA molecules deleted in both repeats. These results suggested that the initial viral particle of those 4 clones had a deleted R_L located near *Bam*HI-I fragment and the other R_L undeleted. Recombination events which occurred during the replication of this particle resulted in the generation of a small percentage of DNA molecules deleted in both repeats, and probably also in the generation of other DNA molecules with undeleted R_L located near *Bam*HI-I (not detected by our PCR reaction). This hypothesis is supported by the results of a similar cloning experiment of one HVT recombinant virus generated in our laboratory whose stock contained mainly DNA molecules with one R_L deleted and the other R_L undeleted: 87% (131/151) of the clones obtained from this stock contained one of the R_L deleted, 11% (17/151) contained both R_L deleted, and 2% (3/151) contained both R_L undeleted.

This deletion probably occurred in one of the two R_L (probably the one near *Bam*HI-I fragment) of one DNA molecule during an early passage of the HVT FC126 strain and has been amplified and conserved during the successive passages of that strain.

Two non-deleted (clones 2 and 5) and one deleted (clone 3) clones were amplified. No difference in *in vitro* virus growth could be detected between these clones and the parental HVT virus, indicating that the deleted region is not essential for *in vitro* replication. These clones were tested for their ability to protect SPF chickens from a MDV challenge. Results are reported in Table 3. No significant difference in the levels of MD protection could be detected between the different groups in this experiment suggesting that the deletion does not impair significantly the *in vivo* replication of HVT.

In summary, the genomic region including the HVT pp38 homologue located in the inverted repeats flanking U_L (R_L) is not essential for *in vitro* and *in vivo* growth of HVT and therefore could be used as a locus of insertion for the generation of recombinant HVT viruses.

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Table 3. Protection against Marek's disease induced by different clones of the HVT FC126 strain

Vaccine	Deletion[a]	Dose (PFU)[b]	MD lesion[c]	Protection index[d]
Clone 2	No	10^4 10	4/10 5/10	58% 47%
Clone 5	No	10^4 10	1/10/ 8/10	89% 16%
Clone 3	Yes	10^4 10	2/10 7/10	79% 26%
Parental HVT	Mix	10^4 10	4/10 8/10	58% 16%
Control	–	–	19/20	0%

[a]„No" means that all DNA molecules of particular clone have both TR_L and IR_L undeleted; „Yes" means that all DNA molecules of particular clone have both TR_L and IR_L deleted, „Mix" means that less than 15% of DNA molecules contained at least one repeat deleted.

[b]One-day-old SPF chickens were vaccinated im with 10 or 10^4 PFU of cell-associated HVT.

[c]Chickens were challenged 5 days post-vaccination and observed daily during 10 weeks after challenge. Tissues of dead and euthanized birds were examined for tumors. Numbers of MDV-positive/total animals in the group.

[d]The protection index (PI) was calculated by the formula: PI = {[(MD response in unvaccinated chickens) – (MD response in vaccinated chickens)]/MD response in unvaccinated chickens} x 100.

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