Characterization of Promoters Integrated in the Genome of Bovine Herpesvirus-1 (BHV-1)

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(Received 3 September 1998/Accepted 24 December 1998)

ABSTRACT. Bovine herpesvirus-1 (BHV-1) has been used as a vector of live recombinant vaccines for cattle which express the genes of other pathogens. Because of the importance of the choice of the promoter which allows the efficient expression of the foreign genes in the BHV-1 vector, we compared the relative efficacy of various promoters integrated in the BHV-1 genome. The promoter sequences of the BHV-1 thymidine kinase (tk), gB, gC, SV40 early, and pseudorabies virus (PRV) immediate early (IE) genes were placed at the upstream of the open reading frame of the chloramphenicol acetyl transferase (CAT) gene and the promoter-CAT sequences were integrated into the tk gene of BHV-1 by homologous recombination. The promoter activity was assayed by measuring the CAT activity in the extracts of Madin Darby bovine kidney (MDBK) cells infected with the recombinant BHV-1. The PRV IE promoter was activated earlier and maintained at a higher level activity than the BHV-1 gB or gC promoters throughout the most of the growth phase of BHV-1. At the late phase, however, the activities of the BHV-1 gB and gC promoters reached the higher level. The BHV-1 tk promoter activity was low and the SV40 early promoter was hardly activated when integrated into the BHV-1 genome.—KEY WORDS: CAT assay, gene expression, promoter, recombinant BHV-1.


MATERIALS AND METHODS

Viruses and cells: The virus strain used in this study was BHV-1 Los Angeles (LA) strain re-cloned in our laboratory. BHV-1 recombinants BHV-1/PRVIECAT and BHV-1/SV40CAT were derivatives of LA (wild type) strain.

The thymidine kinase (tk) minus, gC minus deletion mutant of the BHV-1 LA strain, IBRVdltkdlgIII was obtained from American Type Culture Collection (ATCCVR-2181). BHV-1/TF7-9 was a derivative of IBRVdltkdlgIII, in which the PRV tk gene was inserted at the site of the original tk gene of the parental virus [21]. BHV-1 recombinants containing the BHV-1-promoter-CAT sequences were constructed using BHV-1/TF7-9 as a parental virus of BHV-1/tkCAT, BHV-1/gBCAT, BHV-1/ggCAT, BHV-1/sciCAT because PRV tk is more sensitive to the inhibitory effect of arabinosyl thymine (Ara-T) than BHV-1 tk, and thus the tk minus recombinants derived from BHV-1/TF7-9 are more effectively selected by Ara-T [21]. The deletion of the gC gene in the BHV-1 recombinants did not affect the growth behavior in Madin Darby bovine kidney (MDBK) cells because gC is not essential for viral replication in vitro [13, 14].

MDBK and RK13 cells were cultured in Eagle's MEN (autoclavable, Nissui) containing heat inactivated 7.5% fetal...
calf serum (FCS) and 60 µg/ml kanamycin.

Construction of BHV-1 recombinants: The tk promoter of the BHV-1 tk gene used in this study was the 364 bp BamHI-Apal fragment [19]. The 355 bp AccI fragment [18, 31], located at the 5' end of the coding region of BHV-1 gB, and the 405 bp BglII-BamHI fragment, at the 5' end of the coding region of BHV-1 gC [5], were used as the gB and gC promoters, respectively. The SV40 early promoter was the 323 bp PvuII-HindIII fragment [6]. The PRV IE promoter was obtained as the 570 bp sequence by a double-digestion with HindIII and BamHI [28]. The PRV IE promoters of the BHV-1 tk, gB, gC, SV40 early and PRV IE genes were inserted at the HindIII site of the pHdCAT [10, 27]. Resultant plasmids were digested with PstI and the PstI fragments, which contained the coding region of the CAT gene flanked by the promoter sequences, were purified and inserted at the BglII site of the transfer vector pBHVTk [21]. The transfer vector containing the promoter-CAT sequences were used to transfer them to the tk gene site of BHV-1/TF7–9 by the homologous recombination method described previously [21]. Recombinant viruses were selected with Ara-T or 5-iodo-2-deoxyuridine (IDU) [12] and plaque purified. The recombinants with the BHV-1 tk, gB, gC, SV40 early, PRV IE promoters were designated as BHV-1/TkCAT, BHV-1/gBCAT, BHV-1/gCCAT, BHV-1/SV40CAT and BHV-1/PRV IECAT, respectively. BHV-1/semiCAT was the recombinant which contained the CAT gene without any promoter sequence.

CAT assay: MDBK cells grown in a 6 well plate were infected with these recombinant viruses at a moi 5 and incubated at 37°C. The CAT assay was carried out as described in the earlier reports [6, 20]. At various times after infection, cells were washed with PBS, collected and suspended in 100 µl of 0.25 M Tris-HCl pH 7.8. Cells were broken by 3 times of freezing (−80°C) and thawing (37°C) followed by heat treatment (at 65°C for 15 min) to inactivate cellular enzymes other than CAT. Then extracts were centrifuged for 2 min at 15,000 rpm and supernatants were stored at −80°C. The acetyltransferase assays were carried out at 37°C in the 250 µl reaction mixtures containing 10 mM Tris-HCl pH 7.8, 1 mM chloramphenicol, 10 µl cell extract, 10 µl of 10 µCi/ml, 2.5 µM/ml [14C]acetil CoA. CAT activities were determined using the solvent partition method and presented as the net dpm of products formed per hour.

Transient CAT assay in plasmid transfected cells: The effector plasmid, pLAEH-1, were constructed by cloning the HindIII-EcoRI fragment of BHV-1 which contained the promoter, leader, splicing and open reading frames of BICP4 and BICP0 [25] in pUC19. The reporter plasmid, pSV40CAT, was constructed by inserting the AccI-BamHI fragment of pSV2CAT [6], which contained the SV40 early promoter-CAT gene-SV40 polyA sequence, was inserted at the HindIII site of pUC19. RK13 cells were cotransfected with 0.5 µg of the reporter plasmid (pSV40CAT) and 0, 0.1, 0.5, 2.5, or 5 µg of the effector plasmid (pLAEH-1). At 24 hr post transfection, cells were harvested, washed with PBS and CAT activities were determined.

RESULTS

Construction of BHV-1 recombinants: In order to compare the activities of various promoters quantitatively, we chose the bacterial CAT gene as a reporter gene. The promoter sequences of the BHV-1 tk (as a representative of early promoters), BHV-1 gB (late promoter), BHV-1 gC (late promoter), SV40 early, PRV IE genes were placed upstream of the CAT gene and these promoter-CAT combinations were integrated at the tk gene locus of the BHV-1 genome. Figure 1 shows the schematic diagrams of the recombinants. To confirm the structure of the recombinants Southern blot analysis was carried out as described [19]. The EcoRI digestion of the recombinant DNA yielded 37 and 25 kbp fragments which hybridized with the 32P labeled CAT gene probe (data not shown). The result agrees with the structure that the CAT gene is integrated at the TK gene site of the EcoRI A fragment of BHV-1 genome since the CAT gene contained one EcoRI site [4, 29, 32].

Promoter activities in viral genome: MDBK cells were infected with the BHV-1/CAT recombinants at moi 5. At indicated times post infection (p.i.), cells were harvested and CAT activities were assayed. Results are shown in Fig. 2. The recombinant which contained the PRV IE-CAT sequence demonstrated the highest CAT activity among the recombinants tested here. BHV-1 tk promoter, demonstrated low level activity compared to the BHV-1 gB, gC or PRV IE promoter. The PRV IE promoter was activated earlier than the promoters of the BHV-1 gB or gC genes probably reflecting the fact that the former is the immediate early promoter and the latter is the late promoters. In our
experiments the gB and gC promoters of BHV-1 behaved more or less in the same manner. In HSV, however, the gB gene was classified as the late gene and the gC gene as the delayed late gene because the induction of the gC gene required viral DNA replication while the gB gene was activated without DNA replication [30]. We examined if the activation of the BHV-1 gC promoter needed viral DNA replication. MDBK cells were infected with BHVgCCAT with or without phosphonoacetic acid (PAA) (400 µg/ml) and the CAT activity was assayed. As shown in Fig.3, the BHV-1 gC promoter was activated even in the presence of PAA, at which no BHV-1 DNA replication took place (data not shown). The CAT activity was lower with PAA at 24 hr p. i., 34% of the control without PAA, but at 48 hr p. i. it was 92% of the control. It would appear that the BHV-1 gC promoter did not necessarily require virus DNA replication for activation. We used the 405 bp BgII-BamHI fragment as the BHV-1 gC promoter and the promoter-CAT complex was integrated at the tk gene site. It might be possible that the original BHV-1 gC promoter sequence may contain some other regulatory sequences outside the region which may bestow the delayed late gene characteristics. However, when BHV-1 infected cells were examined by the indirect immuno-fluorescent assay using the monoclonal antibodies against BHV-1 gC, it was confirmed that BHV-1 gC protein was synthesized even in the presence of 400 µg/ml PAA at which concentration no viral DNA replication took place (data not shown). Therfore it can be concluded that even the gC promoter in the original site without any modification is activated without viral DNA replication.

Inhibition of the SV40 early promoter activity by BICP4: The SV40 early promoter has clusters of sp-1 binding site and has a high promoter activity in many cell lines, and has been used as an effective promoter to express foreign genes [3]. However when integrated into BHV-1 genome, the SV40 promoter was hardly activated (Fig. 2). It was reported that activity of the SV40 early promoter was inhibited by the presence of Vmw175 (ICP4) when the SV40 promoter was integrated at the tk gene locus in the HSV-1 genome [24]. The transient CAT assay was performed to examine whether the SV40 promoter activity was inhibited by BICP4, the homologue of HSV-1 ICP4. When RK13 cells were transfected with the effector plasmid pLAEH-1,

![Fig. 2. CAT activity in MDBK cells infected with BHV-1 recombinants which contains the promoter-CAT sequence. MDBK cells were infected with BHV-1/TKCAT, BHV-1/gBCAT, BHV-1/gCCAT, BHV-1/SVCAT, BHV-1/PRVIECAT or BHV-1/seiCAT and at indicated times, cell extracts were prepared and CAT activities were measured.](image1)

![Fig. 3. Effect of PAA on the gC promoter activity. Cells were infected with BHV-1/gCCAT with PAA or without any inhibitor. At 24, 36, 48 hr post infection, cells were harvested and subjected to CAT assay.](image2)

![Fig. 4. Effect of immediate early genes of BHV-1 on the SV40 early promoter activity. RK13 cells were co-transfected with 0.5 µg of reporter plasmid (pSV40CAT) and with 0, 0.1, 0.5, 2.5 5 µg of effecter plasmid (pLAEH-1) which contained whole sequence of BICP4 and BICP0. At 24 hr post transfection, cells were harvested and subjected to CAT assay.](image3)
which contained the promoter, splicing and open reading frames of BICP4 and BICP0. BICP4 protein was detectable by western blot analysis (data not shown). RK13 cells were co-transfected with 0.5 µg of the reporter plasmid (pSV40CAT) and 0, 0.1, 0.5, 2.5, or 5 µg of the effector plasmid (pLAEH-1). At 24 hr post transfection, cells were harvested and subjected to CAT assay. As shown in Fig. 4, the CAT activity of the transfected reporter plasmid was inhibited when co-transfected with the effector plasmid: 85% inhibition at the effector/reporter ratio (E/R ratio) of 5 or 10. The result suggests that the SV40 promoter is not activated in BHV-1 because it is inhibited by BICP4.

DISCUSSION

The expression of the genes of alphaherpesvirus is regulated in a coordinated fashion and sequentially ordered in a well-described cascade manner [8, 15, 17, 26]. When infected, the first group of the genes, the IE genes, are transcribed in the absence of de novo protein synthesis. They are positively regulated by α gene trans-inducing factor (α-TIF) [1, 2] and negatively regulated by its own product [22, 23]. We considered the BHV-1 IE promoter not suitable to express foreign genes integrated into BHV-1 genome, because the promoter activity would be inhibited as soon as the BHV-1 IE gene product accumulated in the infected cells. Therefore we tested the PRV IE promoter instead. It was activated earlier and higher than the BHV-1 gB or gC promoters. The activity was maintained up to 36 hr p.i. suggesting that it was not inhibited by the BHV-1 IE product.

The BHV-1 gB and gC promoters demonstrated the high activities in the late phase of the virus growth. It has been known that the activation of the HSV-1 gC gene requires the viral DNA replication and the HSV-1 gC gene is classified as a delayed late gene [30]. However, the BHV-1 gC promoter appeared to be not the delayed late gene because it was activated without viral DNA replication. The 364 bp BamHI-Apol fragment which we used as the BHV-1 tk promoter was activated earlier than the BHV-1 gB or gC promoters, probably reflecting the fact that the tk gene is classified as an early gene. However, the activity level of the tk promoter was very low indicating that the 364 bp fragment did not contain all the elements of the BHV-1 tk promoter, such as an enhancer sequence, which was necessary for the full activation.

The SV40 early promoter has been used successfully to express foreign genes in canine herpesvirus and other herpesviruses [16, 33]. However, this promoter was not activated when integrated into the BHV-1 genome probably because it was inhibited by BICP4. The possibility that the SV40 promoter underwent mutations when integrated into the BHV-1 genome and lost the promoter activity was ruled out because other BHV-1 recombinants which contained SV40 promoter and other reporter genes failed to express the reporter genes (data not shown). We also observed the Raus sarcoma virus LTR promoter, which had been used to express foreign genes in various systems [34], failed to show the promoter activity in the BHV-1 genome (unpublished observation).

In this study we measured the promoter activities in BHV-1 genome quantitatively using CAT assay. We also constructed BHV-1 recombinants containing the combination of various promoters and coding region of the *Theilella sergenti* p32 gene [11] and measured the transcription of the p32 gene semi-quantitatively. The results were similar to those obtained by using the CAT assay (data not show). Among the promoters tested in this study PRV IE promoter appeared to be the best choice to express foreign genes integrated in the BHV-1 genome. The BHV-1 gB and gC promoters may also be useful to express foreign genes at the late phase of the virus growth.

ACKNOWLEDGEMENTS. We are grateful to Dr. Adachi (Tokushima University, Tokushima, Japan) for providing pHuCAT plasmid. pSVCAT was provided by Y. Kawaguchi (Tokyo Medical and Dental School, Tokyo, Japan) and T. Mikami (Research Center for Protozoan Molecular Immunology, Obihiro University, Hokkaido, Japan). This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES