VIRUS GENES 6:3, 281–290, 1992
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Quantitative Assessment of Poxvirus Promoters in Fowlpox and Vaccinia Virus Recombinants

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Received July 22, 1991
Accepted November 19, 1991

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Key words: vaccinia virus, fowlpox virus, promoters, cowpox virus, β-galactosidase

Abstract

A comparison was undertaken of poxvirus promoters in vaccinia and fowlpox virus (FPV) recombinants using the level of β-galactosidase expressed from the *LacZ* gene as a measure of promoter function. In this study a comparison was made of the vaccinia virus promoters, P 7.5 and P L11, the major late promoter of cowpox virus, P CPX (expressing the abundant inclusion body protein), and the FPV promoters, P E/L and P L. In vaccinia virus recombinants the FPV P E/L promoter expressed one-third to one-half the level of β-galactosidase expressed by the P L11 promoter. In comparison with the P 7.5 promoter, the FPV P E/L promoter expressed four to five times the level of β-galactosidase. In FPV recombinants β-galactosidase activity expressed was equal for the P E/L and P CPX promoters. Levels expressed by P L11 and P L were one-half and one-fifth that level, respectively. The temporal regulation of the promoters was maintained in both vaccinia virus and FPV recombinants. The P E/L promoter of FPV has the TAAATG sequence characteristic of late poxvirus promoters at the transcription initiation site. In an attempt to enhance the utility of this promoter for the expression of foreign genes in FPV and vaccinia virus recombinants, the effect upon promoter function of changing the G of the ATG to A, T, or C was determined using transient expression assays with vaccinia virus. Substitution of A, T, or C for the G abolished promoter function. Because of its early/late function, the level of expression and the presence of the oppositely oriented late P L promoter, the FPV P E/L promoter will be valuable for the expression of foreign genes in poxvirus recombinants.

Introduction

A variety of poxvirus promoters have been used to express foreign genes in vaccinia and fowlpox virus (FPV) recombinants (1–3). We have used the vaccinia virus promoters, P 7.5 and P L11, to express genes in FPV recombinants, although the levels of gene product expressed by the FPV recombinants are substantially lower than the levels produced with the same promoter-gene combination in vaccinia virus recombinants (4). The reasons for this apparent difference in promoter function in different poxviruses have not been studied; however, they may be related to subtle differences in promoter sequences from the different poxviruses and the recognition of those sequences by the DNA-dependent RNA polymerases of the different viruses.

In an endeavor to optimize FPV recombinants for the delivery of vaccine antigens to poultry, we have undertaken the identification and characterization of promoters from FPV for use in FPV recombinants (2,5). One of the promoters identified in these studies is an early/late promoter (P E/L), which, on the basis of mRNA abundance in FPV infected cells and transient expression experiments in vaccinia virus, appears to be a strong pox virus promoter (2,6). In addition, contained within the 42-bp sequence of this promoter is another late promoter (P L) in the opposite orientation to the early/late promoter (2). For these reasons this promoter appears to be potentially valuable for the construction of FPV recombinant vaccines and may be useful for the expression of genes in vaccinia virus recombinants.

Here we describe a comparison of this early/late promoter from FPV with promoters from vaccinia virus, P 7.5 (7) and P L11 (8), and from cowpox virus, P CPX (the major late promoter of cowpox expressing the abundant inclusion body protein) (9,10). The promoters were assessed by comparison of the levels of β-galactosidase expressed in FPV and vaccinia virus recombinants with the *LacZ* gene under the control of these promoters. The results show that the P E/L promoter expresses the highest levels of product of any of the poxvirus promoters tested in FPV recombinants. Whilst in vaccinia virus recombinants the levels of β-galactosidase expressed by the FPV P E/L promoter are substantially higher than levels expressed by the P 7.5 promoter, about half the levels are expressed by the P L11 promoter.

Materials and Methods

Vaccinia virus recombinants

The vaccinia virus recombinants expressing the *LacZ* gene under the control of the P L11 promoter (VV-P L11-LacZ) and the FPV P E/L promoter (VV-P E/L-LacZ) have been described previously (2,4). A vaccinia virus recombinant with the *LacZ* gene expressed by the P 7.5 vaccinia virus promoter (VV-P 7.5-LacZ) was constructed using plasmid pTP4 (see below). The plasmid was used in

established protocols for the construction of TK-vaccinia viruses. Recombinants expressing β-galactosidase were plaque purified by staining with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (XGAL) in the overlays (11). Stocks of recombinant viruses were prepared in 143B or CV1 cells, and the genome arrangement was confirmed by restriction enzyme analyses and Southern hybridization.

Fowlpox virus recombinants

The FPV recombinants expressing the *LacZ* gene under the control of the P L11 promoter (FPV-P L11-LacZ), the FPV P E/L promoter (FPV-CAT1), and the FPV P L late promoter (FPV-CAT2) have been described previously (2,4). A FPV recombinant with the *LacZ* gene under the control of the cowpox virus promoter, P CPX, was constructed by established protocols (1) using the plasmid pLM2 described below. Recombinant FPV expressing β-galactosidase was selected by staining with XGAL in the overlays.

Construction of recombinant plasmids

Recombinant plasmids were constructed using well-established techniques. For insertion of the *LacZ* gene into vaccinia virus under the control of the P 7.5 promoter, the P 7.5 promoter of vaccinia virus was subcloned from pGS20 (12) into pUC-J (13) to construct plasmid pTP3. The *LacZ* gene from pGH101 (14) was cloned as a *Bam*HI fragment into the unique *Bam*HI site of pTP3. The resulting plasmid, pTP4, contains the TK gene of vaccinia virus interrupted at the *Eco*RI site by the *LacZ* gene under the control of the P 7.5 promoter and in opposite orientation to the TK gene.

For insertion of the *LacZ* gene into FPV under control of the cowpox virus inclusion body late promoter, the mutated P CPX promoter was subcloned as a *Taq*I fragment from p2046 (15) into pUC18 (*Acc*I digested). This facilitated the addition of unique restriction enzyme sites downstream of the promoter (plasmid pTP16). The P CPX promoter as an *Eco*RI/*Hin*dIII fragment was inserted into a plasmid for the construction of FPV recombinants, pDB18 (1) (*Eco*RI/*Hin*dIII digested), and the *LacZ* gene was inserted downstream of the promoter. The resultant plasmid, pLM2, contains the FPV TK gene interrupted at the unique *Nco*I site by the *LacZ* gene under the control of the P CPX promoter, in addition to the *E. coli* xanthine-guanine phosphoribosyl transferase (Ecogpt) selectable marker expressed under the control of the P 7.5 promoter.

β-galactosidase assays

Confluent monolayers of 143B or CV1 cells for vaccinia virus and chicken embryo skin cells for FPV were infected at a multiplicity of 10 PFU per cell. Virus was

allowed to adsorb for 1 hr at 37°C, after which the inoculum was removed and the monolayers were gently washed to minimize background levels of β-galactosidase from the inoculum. Medium was added and the cultures were incubated at 37°C. When cytosine arabinoside (Ara-C) was used to inhibit DNA replication, the cells were pretreated for 30–60 min prior to infection and were incubated in 40 μg/ml of Ara-C after infection.

Estimations of β-galactosidase activity were made at various times after infection using the method of Miller (16). Infected cells were resuspended in the overlay medium using a rubber policeman and were lysed by vortexing in the presence of sodium dodecyl sulfate and chloroform. Overlay medium, together with cells, was assayed, as a previous report (11) showed that significant amounts of β-galactosidase were lost into the overlay medium from vaccinia virus LacZ recombinant infected cells due to cell lysis late in infection. Enzyme assays were performed at 28°C using o-nitrophenyl-β-D-galactoside (ONPG) as a substrate. A_{420} readings were recorded and the levels of enzyme produced were recorded as micromoles of o-nitrophenol (ONP) produced per 2×10^6 cells per 30 min.

Mutagenesis of the FPV P E/L promoter ATG codon

Mutagenesis of the FPV P E/L promoter was carried out in plasmid pSK15 (2) by the polymerase chain reaction to amplify most of the plasmid using two synthetic oligonucleotides in opposite orientations (Fig. 1) (17). pSK15 contains the FPV TK gene interrupted at the unique *Nco*I site by the vaccinia virus P 7.5 promoter expressing the Ecogpt selectable marker and by the FPV P E/L promoter with an attached multiple cloning site (MCS). The G of the initiating ATG of the P E/L promoter gene product was mutated to give all possible variants at this position, i.e., ATG, ATA, ATT, and ATC. The first oligonucleotide primer overlapped the P E/L promoter and MCS, and the second was from within the Ecogpt gene. The oligonucleotide overlapping the promoter was redundant at the G position of the ATG and contained modifications to the MCS to facilitate future use

Primer from the Ecogpt gene - positions 329 to 345

5' CTGGCGCGTGAACTGGG 3'

Primer from the P E/L promoter

← P L   P E/L →

5'AATTCAGATCTTAG *cat* TTAGTATCCTAAAATTGAATTGTAATTATCGATAATAA *atn* GACGGATCCCGGGTCGACTGCAGAAGCTT 3'

n = G, A, T or C.

Fig. 1. Oligonucleotide primers used for mutagenesis of the FPV P E/L promoter ATG codon.

of the promoter. pSK15 was digested with *Eco*RI and *Kpn*I; then 10 ng was used in a PCR reaction with 0.2 μM of each primer. The reaction conditions were 94°C for 1 min, 50°C for 2 min, and 72°C for 6 min with a 30-sec extension per cycle for 25 cycles. The reaction product was purified by Gene Clean, treated with the Klenow fragment of DNA polymerase to remove any extra bases added (17), and then ligated to itself. Plasmids carrying the appropriate modifications to the ATG and the MCS were selected by sequence determination with a primer from within the TK gene. These plasmids were designated pAF01:G,A,T,C. The *LacZ* gene as a *Bam*HI fragment from pGH101 (14) was subcloned into these plasmids at the *Bam*HI site to construct plasmids pAF02:G,A,T,C.

Transient expression assays for promoter function

Transient expression assays using plasmids containing mutated forms of the FPV promoter P E/L and the *LacZ* gene as a reporter gene were carried out as described by Chakrabarti et al. (11) using 25 μg of plasmid DNA and 3×10^6 CV1 cells/plate. β-galactosidase activities were determined as above, and the function of modified P E/L promoters was expressed as a percentage of the unmodified promoter.

Results

β-galactosidase expression by vaccinia virus recombinants

A quantitative assessment of promoters, P L11, P E/L, and P 7.5 was made by comparing the levels of β-galactosidase expressed from the *LacZ* gene by vaccinia virus recombinants in single-step growth experiments. The temporal regulation of the promoters was also assessed by using Ara-C to inhibit DNA replication. Both the P L11 and P 7.5 promoters have previously been well characterized in vaccinia virus recombinants (7,8). The level of β-galactosidase activity expressed by the P E/L promoter was one-third to one-half the level expressed by the P L11 promoter and four to five times the level expressed by the P 7.5 promoter (in the absence of Ara-C). In the presence of Ara-C, early promoter function of P E/L represented 50–60% of the total promoter function (Fig. 2).

β-galactosidase expression by FPV recombinants

In FPV recombinants at 72 hr after infection, β-galactosidase activity expressed was equal for the P E/L and P CPX promoters. Levels of β-galactosidase expressed by P L11 and P L were one-half and one-fifth that level, respectively (Fig. 3). The inhibition of promoter function in the presence of Ara-C for P L11,

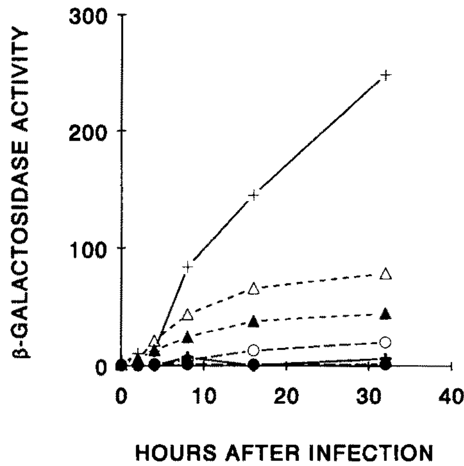

Fig. 2. β-galactosidase expression by vaccinia virus recombinants. β-galactosidase activity (micromoles of ONP/2 × 10^6 cells/30 min) was determined from single-step growth experiments of cells infected with vaccinia virus recombinants expressing the *LacZ* gene under the control of poxvirus promoters. Promoters P L11 (+), P E/L (triangles), and P 7.5 (circles) in the presence (filled symbols) or absence (open symbols) of Ara-C.

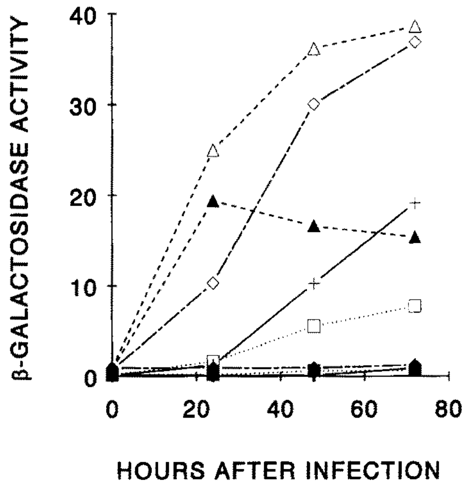

Fig. 3. β-galactosidase expression by FPV recombinants. β-galactosidase activity (micromoles of ONP/2 × 10^6 cells/30 min) was determined from single-step growth experiments of cells infected with FPV recombinants expressing the *LacZ* gene under the control of poxvirus promoters. Promoters P L11 (+), P E/L (triangles), P L (squares), and P CPX (diamonds) in the presence (filled symbols) or absence (open symbols) of Ara-C.

P CPX, and P L showed that all operated as late promoters in FPV recombinants. In contrast, 30–50% of activity expressed by the P E/L promoter remained in the presence of Ara-C, confirming that this promoter has both early and late function.

There were differences in the recognition of promoters by vaccinia and FPV, since in vaccinia virus recombinants P L11 produced two to three times more β-galactosidase activity than P E/L; whilst in FPV the reverse was apparent, with P E/L producing two to three times more than P L11. A comparison of the β-galactosidase activity produced by P E/L in vaccinia and FPV showed that the maximum level produced at 32 hr by vaccinia was twice the maximum level produced by FPV at 72 hr after infection (Figs. 2 and 3). This promoter operated as an early/late promoter in both FPV and vaccinia virus recombinants.

Transient expression assessment of modified FPV P E/L promoter

The P E/L promoter of FPV has at the transcription initiation site the TAAATG sequence characteristic of late poxvirus promoters (18–20). Early transcripts from this promoter also mapped to this region (2). For the expression of foreign genes in FPV recombinants using this promoter, it is necessary to ensure that the initiation codon of the gene is in frame with the ATG of the TAAATG sequence. In an attempt to enhance the utility of this promoter for the expression of foreign genes in FPV and vaccinia virus recombinants, we have determined the effect of changing the G of the ATG to A, T, or C upon promoter function using site-directed mutagenesis and transient expression with vaccinia virus. Substitution of A, T, or C for the G of the ATG abolished promoter function in the presence and absence of Ara-C (Fig. 4). Less than 20% of promoter function remained when G was replaced by A and less than 10% for the replacement of G by T or C. The residual promoter activity operated early, since Ara-C had little or no effect on promoter function. In contrast, for the wild-type promoter early and late function contributed equally to the total promoter activity.

Discussion

The successful expression of immunogenic antigens in poxviruses is dependent upon the availability of *cis*-acting transcriptional control elements—promoters. Both the level and the temporal regulation of foreign gene expression appear to have effects upon the successful use of poxvirus recombinants for vaccine vectors. The early/late promoter from FPV appears to be particularly valuable, since it expresses foreign gene products at high levels in vaccinia virus and FPV, and has early promoter function.

Our earlier studies showed substantial similarities between FPV and vaccinia virus transcriptional controls (2,4). The ability of vaccinia virus to recognize transcriptional control elements from the Entomopox Spheroidin gene promoter

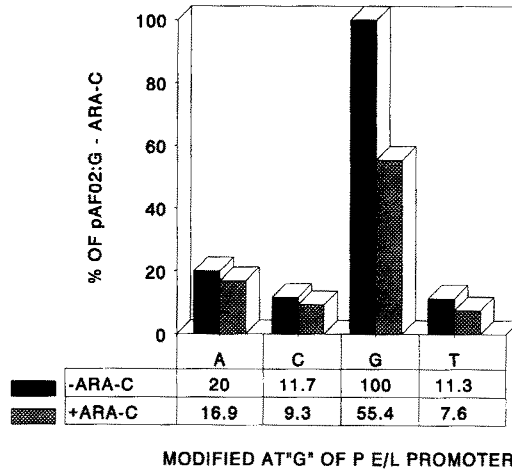

Fig. 4. Transient expression assessment of modified FPV P E/L promoter. β-galactosidase activity in vaccinia virus transient expression assays of modified forms of P E/L promoter. Activity of promoters with the G of ATG modified were expressed as a percent of activity of the wild-type promoter (pAF02:G) in the absence of Ara-C. Assays were carried out in the presence (hatched) or absence (filled) of Ara-C.

suggests that transcriptional mechanisms are highly conserved among the poxviruses (21). This conservation appears to extend to the distantly related cytoplasmically replicated African Swine Fever virus (ASFV), with vaccinia virus apparently able to recognize ASFV promoters (22). Data presented here suggest that the efficiency of promoter recognition may not be uniform, with expression levels higher in the homologous virus-promoter recombinants than in the heterologous virus-promoter recombinants.

In a number of systems studied the induction of cell-mediated immune responses to the foreign antigen expressed by vaccinia virus is dependent upon the early expression of gene product. Genes expressed solely by late poxvirus promoters induce low levels of CMI or fail to induce any CMI response, e.g., influenza virus HA and NP (23,24), herpes simplex virus gD (25), and human papilloma virus major coat protein (26). In the case of HSV gD expressed by a late promoter, the failure to induce a CMI response was reflected in a failure to protect against disease, even though good antibody responses were induced. This can be overcome by use of a promoter having both early and late function (25). The mechanisms involved may be related to the processing of antigens by vaccinia-virus-infected cells, e.g., the shutdown of host protein synthesis by vaccinia virus infection or specific inhibition of antigen processing by vaccinia-encoded genes, perhaps serine protease inhibitors (27,28). In addition, the removal of early poxvirus transcription termination signals, TTTTTNT, from within the coding sequences of genes expressed by early promoters helps ensure early expression of full-length gene products to induce CMI responses (26,29).

The level of antigen expression appears to influence the level of antibody response (23,29). For this reason high-level promoters, e.g., P L11 and P CPX, have been characterized in an endeavor to optimize the immunogenicity of poxvirus recombinants. Although this may induce higher levels of antibody responses, the expression of gene products by late promoters may be deterimental to the induction of protective CMI responses.

For these reasons the FPV P E/L promoter should prove valuable for the expression of vaccine antigens in vaccinia virus and FPV recombinants. Contained within the P E/L promoter sequence and oriented in the opposite direction is a late promoter whose level of expression is one-fifth the E/L promoter. This provides the ability to express two genes under the control of these oppositely oriented promoters or the ability to use the P L promoter to express a selectable marker, e.g., β-galactosidase, for rapid construction of recombinants carrying a gene expressed by the oppositely oriented P E/L promoter.

The inability to modify the G of the TAAATG sequence shows that the sequence is crucial for both late and early function of this promoter. Although the utility of the promoter may have been enhanced had it been possible to maintain high-level function with the G modified, the polymerase chain reaction allows rapid modification of genes to be inserted downstream of this promoter to ensure the correct reading frame for optimal gene expression.

Acknowledgments

Technical assistance was provided by Mr. A.D. Pye, Mr. A. Foord, Mrs. M.A. Anderson, and Ms. L. Morton.

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