Effect of in vitro Mutations in a Vaccinia Virus Early Promoter Region Monitored by Herpes Simplex Virus Thymidine Kinase Expression in Recombinant Vaccinia Virus

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SUMMARY

The location of a promoter (PF) in the HindIII F region of the vaccinia virus genome was mapped by introducing deletions into this region of the DNA. Modified promoters were fused to the herpes simplex virus (HSV) thymidine kinase (TK) gene in plasmids facilitating the construction of recombinant vaccinia viruses, and promoter function was monitored by the ability of such plasmids to rescue TK+ vaccinia viruses from cells infected with TK- virus. Deletions from the 3' end of the promoter region produced mutants for which function was either not inhibited or abolished, allowing the 3' promoter boundary to be defined to within 13 nucleotides. As indicated by the presence of the PF transcript in early RNA and the kinetics of HSV TK expression in recombinant vaccinia viruses, transcription from PF occurred primarily at early times during infection. The major transcript was initiated at a site within 20 nucleotides of the 3' end of the promoter and nine bases upstream of the probable translation initiation codon. In one mutant for which a small but reproducible increase in promoter function was detected, the transcription start site was deleted. Nevertheless, transcription still appeared to begin at the equivalent position with respect to the promoter, despite the altered nucleotide sequence. The location of the start site for the PF transcript indicated that the HSV TK gene, inserted at the BamHI site following the promoter, was preceded by an initiation codon which could potentially attenuate expression of the inserted gene. Conversion of this ATG codon to TAG did not significantly improve HSV TK expression.

INTRODUCTION

Poxviruses are large DNA viruses which replicate in the cytoplasm of infected cells (Dales & Pogo, 1981). Vaccinia virus (VV), the best characterized member of the orthopoxvirus genus, is a useful vector for the expression of heterologous genes inserted into non-essential regions of the viral genome (Mackett & Smith, 1986). Transcription of foreign genes by viral DNA-dependent RNA polymerase is regulated by VV promoter sequences which are distinct from those recognized by eukaryotic RNA polymerase II. Therefore, expression of a foreign gene in a VV recombinant requires the location of a VV promoter 5' to the inserted sequence. On the other hand, viral mRNAs, including transcripts from the inserted DNA, are translated and protein products are modified (e.g. glycosylated) via the normal mechanisms of the host cell.

Promoters which regulate the expression of genes not essential for viral replication can be used in their natural position in the viral genome (Panicali & Paolletti, 1982; Panicali et al., 1983). Ideally, foreign DNA is inserted between the regulatory sequence and the initiation codon of the wild-type gene. Alternatively, a chimeric promoter–gene fragment can be translocated to a non-essential region of the VV genome such as the thymidine kinase (TK) gene...
A promoter region (PF) derived from the HindIII F fragment of VV DNA has been used to express a number of foreign proteins including herpes simplex virus (HSV) TK and glycoprotein D, hepatitis B virus surface antigen and influenza virus haemagglutinin either by insertion of foreign DNA at the BamHI site (Panicali & Paoletti, 1982; Panicali et al., 1983; Paoletti et al., 1984) or by transposition of the promoter to the VV TK gene (Boyle et al., 1985; Coupar et al., 1986). We have begun to map the extremities of the PF promoter region by introducing deletions into this region of the DNA. Mutated promoters were spliced to the HSV TK gene in plasmids and promoter function was monitored by the ability of such plasmids to rescue recombinant TK+ VV from cells infected with TK− VV. We obtained mutants which maintained, or which lacked, promoter activity, allowing the 3′ boundary to be defined to within 13 nucleotides.

The major start site for early transcription from PF was also determined. This revealed that foreign genes inserted at the BamHI site would be preceded by a potential initiation codon. Since the presence of upstream initiation codons can attenuate initiation at downstream, out-of-phase sites in mammalian cells (Kozak, 1984; Liu et al., 1984) the possibility was considered that the reduced efficiency of expression from PF compared with P7-5 or PL11 (Coupar et al., 1986), was due to a cryptic initiation codon. Mutations removing the codon were therefore introduced in attempts to improve expression of the inserted HSV TK gene.

**METHODS**

**Enzymes, plasmids and DNA manipulations.** Restriction endonucleases and DNA-modifying enzymes were obtained from several commercial suppliers and used according to the manufacturers’ instructions or those described in detail by Maniatis et al. (1982). pXI contained a 3.4 kb BamHI fragment of HSV DNA including the TK gene, inserted at the BamHI site of pBR322 (Wagner et al., 1981). pFB contained a modified 2.6 kb pX1-HindIII fragment (Boyle et al., 1985) from the HindIII F fragment of VV WR strain, cloned in pUC9 (Vieira & Messing, 1982). A BamHI–Smal–EcoR1 linker was inserted at the BamHI site in a series of manipulations to be described in detail elsewhere (B. E. H. Coupar & D. B. Boyle, unpublished).

Construction of PF promoter mutant plasmids carrying the HSV TK gene. The PF promoter region in pBCB03 (Boyle et al., 1985) was mutated as follows (Fig. 1). (1) pBCB03 was made linear with BamHI and digested with Bal31 nuclease. Molecules were made flush-ended with DNA polymerase (Klenow), BamHI linkers were added using T4 DNA ligase and recut. Circular plasmids were re-formed by ligation and transformed into E. coli RR1. Miniprep DNA from colonies was screened by nucleotide sequencing (Maxam & Gilbert, 1977) to identify plasmids with appropriate deletions. (2) From these plasmids, AccI–BamHI fragments of approx. 120 bp containing the putative promoter region were prepared. (3) The presence of a second AccI site between the BamHI and Clal sites in pFB required separate preparation of AccI–Clal and Clal–BamHI fragments. (4) These were ligated with AccI–BamHI fragments to form plasmids pFB2 and pFB27. (5) Similarly, to remove the putative promoter sequence, pFBdel was made by combining these two fragments with a synthetic double-stranded oligonucleotide 5′ ATACTACCTAGG 3′ (plus strand), which had AccI/BamHI compatible ends. (6) The HSV TK gene (a BglII–EcoRI fragment) prepared from pXI was inserted downstream of potential promoter sequences in the mutated pFB plasmids cut with BamHI and EcoR1 to construct pFB2-TK, pFB27-TK and pFBdel-TK. pFBter-TK, in which the ATG codon immediately preceding the BamHI site (Fig. 2) was changed to a TAG codon, was constructed as follows. (7) An EcoR1–NruI fragment of approx. 300 bp containing the PF promoter was prepared from plasmid pFB and subcloned into BamHI/Smal-cut bacteriophage M13mp9. Single-stranded DNA was prepared and used as a template for the elongation of a synthetic oligonucleotide, 5′ GGGATCCCTAGACGAGCGT3′ (negative sense) by Klenow DNA polymerase in the presence of DNA ligase (Nisbet & Beilharz, 1985). Double-stranded DNA produced was transformed into E. coli TG1 cells and mutant plaques were identified by differential hybridization of the 32p-labelled oligonucleotide at temperatures near the Tm. (8 and 9) The AccI–BamHI fragment of 122 bp was prepared from one mutant and ligated with the AccI–Clal and Clal–BamHI fragments from pFB to form pFBter. Finally, (10), the HSV TK gene was incorporated as the BglII–EcoRI fragment into pFBter cut with BamHI and EcoR1.

(11) The HSV TK gene (BglII–PstI fragment) was incorporated into pFB between the BamHI and Smal sites to form pFB-TK. The sequence of all plasmid constructions was finally checked by determining the nucleotide sequence of the relevant regions (Maxam & Gilbert, 1977).
Viruses and cell lines. An L929 cell-adapted VV WR strain (VV-WR) has been described previously (Boyle & Coupar, 1986). A TK− mutant (VV-WR-TK−), was provided by Dr B. Moss, NIH, Bethesda, Md., U.S.A. Human 143B cells, a TK− variant of cell line R970-5 (Rhim et al., 1975), were obtained from Dr K. Huebner, Wistar Institute, Philadelphia, Pa., U.S.A. and maintained as described previously (Boyle & Coupar, 1986).

Analysis of RNA transcripts. Early and late cytoplasmic RNAs were prepared from virus-infected or uninfected 143B cells and analysed by Northern hybridization as described previously (Boyle et al., 1987). The 5′ ends of the mRNAs expressed from the PF promoter were mapped by primer extension using a 5′ end-labelled 17 base synthetic oligonucleotide (5′ GAATCTACCACAGG 3′; negative strand) or a 95 bp HindIII fragment as primer as described previously (Boyle et al., 1987). In some experiments, dideoxynucleoside triphosphates were used to determine the sequence of the extended products (Sanger et al., 1977).

Marker rescue-recombination. Marker rescue was performed as described by Weir et al. (1982) and by Boyle & Coupar (1986). TK+ recombinant viruses were plaque-purified three times on 143B (TK−) cell monolayers in medium containing 3 μM-methotrexate, 15 μM-thymidine, 50 μM-adenosine, 50 μM-guanosine and 10 μM-glycine (MTAGG). Stocks of viruses were grown in CV1 cells without selection and DNA was prepared from virus pellets as described by Nakano et al. (1982).

Preparation of cell lysates and TK assays. Extracts from infected 143B cell monolayers were prepared as described previously (Boyle & Coupar, 1986). Where indicated, cells were incubated with 40 μg/ml of cytosine arabinoside (araC) for 15 min before infection with the virus. AraC was included in the incubation medium at the same concentration. Enzyme assays were carried out essentially as described for HSV TK (Jamieson & Subak-Sharpe, 1974) and fowlpox virus TK (Boyle & Coupar, 1986).

RESULTS

5′ End mapping of RNA transcripts from the VV PF promoter

The nucleotide sequence 5′ to a BamHI site in the HindIII F region of VV-WR has been described (Boyle et al., 1985). The additional downstream sequence (Fig. 2) continues in the reading frame beginning at ATG codon 192 to 194. A second in-frame ATG is present nearby (bases 204 to 206). To characterize mRNA species transcribed from this region, cytoplasmic
RNA prepared from uninfected cells or from VV-WR-infected cells at early (4 h in the presence of cycloheximide) or late (8 h in the absence of inhibitors) times, was hybridized with a 17 nucleotide primer (boxed, Fig. 2) which was extended with reverse transcriptase. A major cDNA product of 33 nucleotides, terminating at base 195 in Fig. 2, was copied from early transcripts of infected cells (Fig. 3). Minor transcripts initiating at adjacent bases in early RNA are indicated by dots in Fig. 2. Late transcripts from infected cells were extended to 39 and 45 bases i.e. to positions 189 and 183 respectively (Fig. 3). Initiation of early transcripts at base 195 was confirmed when a 95 base HindIII primer (underlined, Fig. 2) was extended in the presence and absence of chain-terminating inhibitors. Late transcripts similarly extended had minor cDNA products terminating at bases 183, 156 and 132 (data not shown). Thus for the PF promoter, most RNAs are transcribed early during infection initiating at or near base 195, indicating that the ATG at bases 204 to 206 (Fig. 2) is the first potential initiation site for translation of the F mRNA species. Downstream ATG codons are out of frame and followed by termination codons.

A single mRNA species of about 500 nucleotides was identified when early transcripts from infected cells were analysed by Northern hybridization using a 300 bp (BamHI-XbaI) probe derived from pFB (Fig. 1). No discrete species of late RNA which hybridized with the probe were detected.

**Mutation of the PF sequence and assessment of promoter activity by expression of HSV TK**

To determine whether the PF promoter function was localized within the AccI–BamHI fragment of pFB DNA, this region was deleted and replaced by a synthetic oligonucleotide linker of 12 bases in pFBdel. The linker introduced a stop codon immediately prior to the BamHI site. The wild-type PF promoter and the modified promoter region in pFBdel were
Vaccinia virus early promoter

Fig. 3. Mapping of the 5' end of PF mRNA expressed by VV-WR. Early (E) and late (L) cytoplasmic RNA from VV-WR-infected or uninfected (U) 143B cells hybridized with the 17 base primer was extended with reverse transcriptase. Arrows indicate positions equivalent to nucleotides as numbered in Fig. 2. DNA fragments from HpaII-cut pBR322, end-filled with [α-32P]dCTP were used as markers (M).

ligated to the HSV TK gene as shown in Fig. 1(6). Promoter function was assessed by the ability of the recombinant plasmids (pFB-TK and pFBdel-TK) to rescue TK+ virus from TK- virus. The data (Table 1) indicate that the wild-type promoter sequence in pFB-TK was able to rescue TK+ virus whereas pFB (without the inserted HSV TK) was not able to rescue TK+ virus. Deletion of the AccI–BamHI fragment (pFBdel-TK) abolished promoter function as assessed by this marker rescue assay. Therefore, the PF promoter function is probably contained within the AccI–BamHI fragment and sequences upstream of the AccI site were not able to provide promoter function for the HSV TK gene inserted at the BamHI site.

In order to determine the 3' limit of sequences required for PF promoter function and to remove potential initiation codons upstream of the BamHI site in pFB, a series of deletions was generated from the BamHI site by Bal31 nuclease digestion. Two of these mutants, FB2 and FB27 from which bases 187 to 207 (inclusive) and 174 to 207 were deleted, respectively (Fig. 2) are shown schematically in Fig. 4. An additional mutant, FBter, in which bases 204 and 205
were transposed by mutagenesis to introduce a stop codon before the BamHI site, was also constructed. These altered promoter fragments were ligated to the HSV TK gene and promoter function was assessed by marker rescue as described above. With the exception of pFB27-TK the mutations had little or no effect on the recovery of TK+ virus (Table 1). For pFB27-TK, in which 31 bp were deleted, no TK+ virus was detected (Table 1). The plasmid pXI, which contains the HSV TK gene with its homologous promoter, was unable to rescue TK− VV (Table 1).
Table 2. TK activity of recombinant HSV TK-VV

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* Infection at 37 °C with 20 p.f.u. of virus per cell in the presence of araC.
† Activity defined as pmol dT phosphorylated/10⁶ cells; mock-infected control values deducted.
‡ Percent VV-FB-TK activity.

Expression of HSV TK activity by recombinant vaccinia viruses compared with TK expression by VV-WR

Recombinant viruses carrying the HSV TK gene were plaque-purified from marker rescue recombination reactions with pFB-TK, pFB2-TK and pFBter-TK and designated VV-FB-TK, VV-FB2-TK and VV-FBter-TK respectively. Specific insertion into the HindIII F fragment was confirmed by digestion of purified DNA from recombinant viruses with HindIII and EcoRI. The expected fragment sizes and hybridization patterns were obtained when digested DNA was transferred to GeneScreen Plus and probed with radiolabelled HSV TK DNA (data not shown).

The time course of expression of TK activity in TK-143B cells infected with VV-FB-TK or VV-WR was compared in the presence or absence of the DNA replication inhibitor araC. In the absence of araC HSV TK activity at 4 h post-infection was significantly elevated over that in VV-WR-TK- or mock-infected control cells and peaked around 8 h post-infection (Fig. 5a). In the presence of araC, TK activity continued to increase and reached a plateau at 12 h indicating that the inserted gene was expressed early in infection, before DNA replication. The time course of expression of HSV TK from the PF promoter was similar to that for TK expressed by the wild-type VV-WR (Fig. 5b). However the level of TK enzyme activity for the latter was approximately 10-fold higher.

VV-FB2-TK carries a deletion which removes the major start site for early transcription of the PF promoter. The levels of HSV TK activity from cells infected with VV-FB-TK or VV-WR were compared in the presence or absence of the DNA replication inhibitor araC. In the absence of araC HSV TK activity at 4 h post-infection was significantly elevated over that in VV-WR-TK- or mock-infected control cells and peaked around 8 h post-infection (Fig. 5a). In the presence of araC, TK activity continued to increase and reached a plateau at 12 h indicating that the inserted gene was expressed early in infection, before DNA replication. The time course of expression of HSV TK from the PF promoter was similar to that for TK expressed by the wild-type VV-WR (Fig. 5b). However the level of TK enzyme activity for the latter was approximately 10-fold higher.

VV-FB2-TK carries a deletion which removes the major start site for early transcription of the PF promoter. The levels of HSV TK activity from cells infected with VV-FB-TK or VV-FB2-TK for 6 to 8 h in the presence of araC were compared to assess the effect of this deletion on TK expression. Deletion of the major site for initiation of early RNA transcription in VV-FB2-TK (and the concomitant removal of potential initiation codons from the transcript) resulted in a small but reproducible increase in the level of HSV TK activity expressed in infected cells (Table 2). In the presence of araC (five experiments) VV-FB2-TK had, on average, 29% more TK activity than VV-FB-TK. In a number of experiments (e.g. Table 2, expt. 5) with VV-FBter-TK, in which a termination codon was substituted for an initiation codon (Fig. 4) there was no significant effect on the level of TK enzyme expressed compared with VV-FB-TK.

5' End mapping of the HSV TK transcripts in recombinant VV

The 5' end of the mRNA transcribed at early times from the modified promoter in VV-FB2-TK was determined and compared with the equivalent species from VV-FB-TK. The 5' end of the mRNA from VV-FB-TK mapped 86 nucleotides from the end of the primer (Fig. 6), as expected from the sequence. This corresponds to the major early start site for PF. The 5' end of VV-FB2-TK mRNA species mapped at approximately 68 bases (Fig. 6). Considering the 18 base deletion in the F2 promoter region, transcription in this mutant nevertheless appears to have begun at a position equivalent to the usual start site in the gene, despite the altered nucleotide sequence.
Fig. 5. Time course of infection and TK activity in lysates from infected 143B cells. (a) TK activity in lysates from VV-FB-TK-infected cells in the absence (○) or presence (●) of araC or from VV-WR-TK-infected cells (□). (b) TK activity in lysates from VV-WR-infected cells in the absence (△) or presence of araC (▲). Assays were carried out on lysates from duplicate infections and mock-infected control values were deducted. TK activity is defined as pmol dT phosphorylated in 15 min at 37 °C per 10⁶ cells.

Fig. 6. Mapping the 5' end of the PF-HSV hybrid mRNA produced by VV-FB-TK and VV-FB2-TK. A synthetic oligonucleotide complementary to bases −1 to +16 of the coding portion of the HSV TK gene was extended with reverse transcriptase using as template early RNA isolated from cells infected with VV-FB-TK (lane 1) or VV-FB2-TK (lane 2). DNA fragments from Sau3A-cut pBR322, end-filled with [α-³²P]dATP were used as markers (lane M).

DISCUSSION

Promoter activity in the DNA sequence adjacent to a BamHI site in the HindIII F fragment of VV has been demonstrated by the expression of several genes inserted in the appropriate orientation at that site either in its normal location (Panicali & Paoletti, 1982; Panicali et al., 1983; Paoletti et al., 1984) or after translocation to the VV TK gene (Boyle et al., 1985; Coupar et al., 1986). This promoter region has been further defined in this study using an assay requiring its function. Only plasmids carrying an active promoter spliced to the HSV TK gene allowed the rescue of recombinant TK⁺ viruses in the presence of methotrexate in TK⁻ 143B cells infected
with VV-WR-TK⁻ (Table 1). A similar strategy has been employed by Vassef et al. (1985) to isolate and compare randomly cleaved VV sequences for promoter function.

Several mutations were made in the PF promoter region to probe its extremities. Removal of 122 bp between the AccI and BamHI sites (Fig. 2) destroyed promoter activity, as assessed by ability to rescue TK⁺ recombinant vaccinia virus (Table 1). Thus, some or all of these sequences contribute to the PF promoter; AT-rich sequences further upstream of the AccI site may be necessary but are not sufficient for function. Deletion of 18 bp from the 3' end (FB2) had no detrimental effect on promoter function and did not alter the position of the major initiation site, relative to the putative promoter sequence, for early transcription (Fig. 6), despite the change in nucleotide sequence. However, removal of 31 bp (FB27) eliminated promoter activity (Table 1). Thus, the 3' boundary of the promoter region appears to lie between bases 174 and 186 (Fig. 2).

By analogy with deletion studies performed with the 7-5K, 11K and 28K mol. wt. polypeptide promoters (Cochran et al., 1985; Bertholet et al., 1985; Weir & Moss, 1987), which showed that these promoters are contained within 230 (and possibly 100) bp, 100 bp and 70 bp respectively, PF might therefore lie wholly within the AccI-BamHI fragment.

Since the 5' terminal mapping data indicated that PF functions primarily at early times during infection the PF sequences were inspected for homology with the early promoter consensus sequence TATA ... (20 to 24 bases) ... AATAA (Plucienniczak et al., 1985). Limited homology was observed between bases 140 to 143 (three of four matches) and 166 to 170 (four of five matches) (Fig. 2), for example, but other matches could also be found. Considering the sequence variability in promoter regions (Plucienniczak et al., 1985) and the relative inefficiency of the PF promoter (Coupar et al., 1986) it may not be surprising that homology with the consensus sequence is limited.

Consistent with the apparent inefficiency of PF (Coupar et al., 1986), the level of PF transcript detected in VV-WR-infected 143B cells was low in comparison with the level of VV-WR TK transcript (B. E. H. Coupar, unpublished observations); the level of TK activity was similarly 10-fold lower. Although plaque size and number were generally reduced, recombinant virus growth was not completely inhibited by bromodeoxyuridine (data not shown), suggesting a low level of phosphorylation. The apparent loss of TK activity at longer infection times (Fig. 5) was perhaps due to leakage of the enzyme into the culture or wash media, which were not collected, or to cytopathic effects of viral infection which appeared less marked in the presence of araC. A similar effect has been observed previously with β-galactosidase expression in recombinant VV (Chakrabarti et al., 1985).

The nucleotide sequence of the 5' end of the PF transcript revealed that the insertion of foreign genes at the BamHI site placed one, or possibly two, ATG codons upstream of the initiation codon for the exogenous gene (Boyle et al., 1985). The location of the PF transcription start site now indicates that only the codon at bases 204 to 206 (Fig. 2) is present in the mRNA, except when minor transcription initiation sites are used. According to the consensus sequence proposed by Kozak (1984, 1986), this codon would be expected to initiate translation fairly efficiently and reduce translation from the initiation codon of the exogenous gene, particularly if it was in a different reading frame (Kozak, 1984; Liu et al., 1984). Its removal might enhance expression of foreign genes inserted at the BamHI site. In mutant FB2, this initiation codon was removed and a small increase in the level of TK activity was consistently observed (Table 2) but this did not necessarily occur at the level of translation. In fact, in another mutant, FBter, where this initiation codon was converted to a stop codon so that the initiation codon of the HSV TK gene became the first one of the open reading frame, the level of HSV TK expression was not significantly enhanced compared with the wild-type (FB), in several experiments, one of which is shown in Table 2. This suggests that expression from the PF promoter may be attenuated primarily at the level of transcription.

This study has partially defined the boundaries of the PF promoter which is weak compared with those of other VV genes (Coupar et al., 1986). However, sufficient protein expression occurs from PF to induce antibody and cell-mediated immune responses and to sensitize target cells for lysis by cytotoxic T cells (Coupar et al., 1986). Influenza virus haemagglutinin expressed using PF induced antibody levels comparable to those induced using either the 7-5K or 11K
promoters (Coupal et al., 1986). Although the level of exogenous gene products expressed by recombinant VV used as vaccines may influence the nature and extent of immune responses to the expressed products, other factors e.g. the temporal regulation of expression, have been shown to be of equal importance (Coupal et al., 1986). In the absence of other characterized promoters, PF may still be useful in constructing viruses carrying multiple foreign genes.

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