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Escherichia coli gpt Gene Provides Dominant Selection for Vaccinia Virus Open Reading Frame Expression Vectors

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Mycophenolic acid, an inhibitor of purine metabolism, was shown to block the replication of vaccinia virus in normal cell lines. This observation led to the development of a dominant one-step plaque selection system, based on expression of the *Escherichia coli gpt* gene, for the isolation of recombinant vaccinia viruses. Synthesis of xanthine-guanine phosphoribosyltransferase enabled only the recombinant viruses to form large plaques in a selective medium containing mycophenolic acid, xanthine, and hypoxanthine. To utilize the selection system efficiently, we constructed a series of plasmids that contain the *E. coli gpt* gene and allow insertion of foreign genes into multiple unique restriction endonuclease sites in all three reading frames between the translation initiation codon of a strong late promoter and synthetic translation termination sequences. The selection-expression cassette is flanked by vaccinia virus DNA that directs homologous recombination into the virus genome. The new vectors allow high-level expression of complete or partial open reading frames and rapid construction of recombinant viruses by facilitating the cloning steps and by simplifying their isolation. The system was tested by cloning the *E. coli* β-galactosidase gene; in 24 h, this enzyme accounted for approximately 3.5% of the total infected-cell protein.

Vaccinia virus is a useful vector for gene expression in mammalian cells (for recent reviews, see references 12, 14, and 18). Advantages include the maintenance of infectivity, wide host range, large DNA capacity, and correct synthesis, processing, and transport of proteins. Because transcription of vaccinia virus genes is carried out by virus-encoded enzymes in the cytoplasm and splicing of RNA does not occur, there are requirements for vaccinia virus promoters and uninterrupted open reading frames. In addition, the large size and lack of infectivity of the vaccinia virus genome prohibit the construction of recombinants by standard in vitro cloning techniques. A two-step procedure has been developed to overcome these difficulties. In the first step, a plasmid is constructed that contains a foreign gene(s) controlled by a vaccinia virus promoter(s) and flanked by sequences derived from a nonessential site on the viral genome. In the second step, the foreign genetic material in the plasmid vector is inserted into the viral genome by homologous recombination in vivo.

There are several areas in which fundamental improvements in vaccinia virus vectors can be made. These relate to methods of recombinant virus selection, optional use of incomplete open reading frames, and levels of foreign gene expression. Recombinant vaccinia viruses have been identified primarily by plaque hybridization (20), thymidine kinase (*tk*)-negative selection (13), or β-galactosidase expression (5, 21). Of these, only insertional inactivation of the *tk* gene is a true selection step. Disadvantages of this method, however, include requirements for (i) inactivation of the viral *tk* gene which attenuates virus infectivity (4), (ii) use of special TK^- cell lines, and (iii) use of mutagenic selective agents, e.g., 5-bromodeoxyuridine. In addition, spontaneous *tk* mutants arise at a high frequency, necessitating additional steps to distinguish them from recombinants. Potential methods of using the neomycin resistance gene as a dominant selectable marker for isolating recombinant vaccinia viruses containing foreign genes of interest were suggested by Franke et al. (7). In this communication, we show that mycophenolic acid (MPA), an inhibitor of purine metabolism, reversibly blocks formation of vaccinia virus plaques on a variety of cell lines. This inhibition was overcome by incorporating the *Escherichia coli gpt* gene into vaccinia virus and adding xanthine and hypoxanthine to the medium. Using this information, we constructed a series of new vectors in which coexpression of the *gpt* gene provides efficient dominant selection of recombinants and overcomes all of the deficiencies of TK^- selection. At the same time, we incorporated into these vectors a strong late promoter that provides high levels of expression, translation initiation and termination codons, and multiple restriction sites in three different frames which permit expression of partial or complete foreign genes.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases and low-melting-point agarose were from Bethesda Research Laboratories, Inc. T4 polymerase was from Pharmacia, Inc. The enzymes were used as specified by the suppliers. MPA was from Calbiochem-Behring. Xanthine and hypoxanthine were from Sigma Chemical Co. MPA and xanthine were dissolved in 0.1 N NaOH, and hypoxanthine was dissolved in water and sterile filtered; the solutions were stored frozen as 10-mg/ml stocks.

Virus and cells. Vaccinia virus WR, originally from the American Type Culture Collection, was replicated in HeLa cells, and purified as reported previously (14). Human TK^- 143 cells (23) were grown in Eagle medium with 10% fetal bovine serum CVI and BSCI cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum.

Formation of *gpt*$^+$ recombinant virus. Recombinant viruses were prepared as described (14) with the following modifications. CVI cells (5×10^6; confluent monolayers) were infected with 0.2 PFU of vaccinia virus per cell. At 2 h after infection, 1 ml of a calcium DNA precipitate (consisting of 5 μg of supercoiled plasmid DNA, 1 μg of vaccinia virus

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DNA, and of 14 μg of sheared herring sperm DNA) was added to the cells. After 15 min of incubation at room temperature, 9 ml of medium (Dulbecco modified Eagle medium, 8% FBS, penicillin, streptomycin) was added. The medium was renewed after 4 h, and the incubation was continued for another 36 to 48 h. Virus stocks were prepared by resuspending the infected cells in 1 ml of medium and freezing and thawing them three times.

Selection of *gpt*$^+$ virus. For the isolation of *gpt*$^+$ recombinants, a plaque assay on BSCI cells was done as follows. Confluent BSCI cells were preincubated in the *gpt* selection medium (Dulbecco modified Eagle medium, 2.5% fetal bovine serum, 25 μg of MPA per ml, 250 μg of xanthine per ml, 15 μg of hypoxanthine per ml) for 14 to 24 h. The virus stock was digested with an equal volume of trypsin (0.25 mg/ml) for 30 min at 37°C and sonicated for 20 s on ice. Dilutions (10^{-3}, 10^{-4}, and 10^{-5}) of the trypsinized virus stock were used to infect the BSCI cells. After 1.5 h of incubation at 37°C, the cells were overlaid with the *gpt*-selective medium containing 1% of low-melting-point agarose. After 2 days of incubation, the cells were stained with neutral red (GIBCO Laboratories). The plaques were readily visible after overnight incubation.

Preparation and analysis of DNA. Recombinant plasmids were constructed and isolated as described (16). For the genomic analysis of recombinant viruses, 2.5×10^6 BSCI cells were infected with the material obtained from a single plaque and grown for 24 h in selective medium. Total cellular DNA was extracted (10), digested with *Hin*dIII, electrophoresed through a 1% agarose gel, and subjected to Southern blot analysis (16).

Construction of recombinant plasmids. (i) pTK61-gpt. *Hin*dIII linkers were added to the *Hin*dIII-*Hpa*I fragment of the *E. coli gpt* gene obtained from plasmid pSV2-gpt (19). Subsequently, the fragment was inserted into the unique *Hin*dIII site of pGS61 (14), resulting in the plasmid pTK61-gpt.

(ii) pP11. The *Hin*dIII and *Sst*I sites, flanking the promoter from the gene encoding the 11-kilodalton polypeptide (the 11K polypeptide) in plasmid pSC42, were converted into *Xho*I site by T4 polymerase treatment and ligation of *Xho*I linkers. The plasmid pSC42 has the *Cla*I-*Eco*RI fragment containing the promoter from the gene encoding the 11K polypeptide (1) cloned into the *Sph*I site of pUC19 (S. Chakrabarti, unpublished data).

(iii) pTKgpt-F1s and pTKgpt-oF1s. For details of the construction of pTKgpt-F1s and pTKgpt-oF1s, see Fig. 3.

(iv) pTKgpt-F2s and pTKgpt-F3s. The frameshift mutations were produced by oligonucleotide-directed mutagenesis (15). To obtain pTKgpt-F2s, we used a 30-mer oligonucleotide (5′-GACCTGCAGGAATTCCATTTATAGCATAG A-3′), and to obtain pTKgpt-F3s, we used another 30-mer (5′-ACCTGCAGGAATTCCCATTTATAGCATAGA-3′). The mutants were screened by plasmid sequencing (11) with the help of a 20-mer primer (5′-GCGATGCTACGCTAGT CACA-3′) derived from the upstream region of the promoter from the gene encoding the 11K polypeptide (nucleotides −96 to −76 upstream of the initiation codon). The primary structures around the promoter region of the gene encoding the 11K polypeptide and the unique cloning sites in all vectors were confirmed by plasmid sequencing.

(v) pTKgpt-F1sβ and pTKgpt-oF1sβ. The *Bam*HI fragment of pMC1871 (26) was cloned into the *Bam*HI sites of pTKgpt-F1s and pTKgpt-oF1s to form pTKgpt-F1sβ and pTKgpt-oF1sβ.

(vi) pTKgpt-F2sβ. The *Sma*I-*Sal*I fragment of pMC1871 was inserted into the *Hin*cII site of pTKgpt-F2s to form pTKgpt-F2sβ.

(vii) pTKgpt-F3sβ. The *Sal*I fragment of pMC1871 was cloned into the *Sal*I site of pTKgpt-F3s to form pTKgpt-F3sβ.

RESULTS

Inhibition of growth of vaccinia virus by MPA. The mycotoxin MPA inhibits the enzyme inosine monophosphate dehydrogenase and thereby prevents the formation of xanthine monophosphate. This results in the intracellular depletion of purine nucleotides and in an inhibition of cell growth (19). Treatment of host cells with MPA should therefore severely inhibit the growth of viruses. To prove this assumption, we assayed the effect of increasing amounts of MPA on plaque formation by vaccinia virus. We found that 25 μg of MPA per ml of medium resulted in a nearly complete inhibition of plaque formation in all cell lines tested so far (BSCI, CVI, and human TK$^-$ 143 cells). In BSCI and CVI cells, only a few tiny plaques could be observed on crystal violet-stained monolayers after 2 days of incubation (Fig. 1A). Replacement of the selective medium with normal medium resulted in plaque formation comparable to that of the control (Fig. 1B), indicating that the inhibition is reversible.

Plaque formation in the presence of MPA owing to *E. coli gpt* expression. The inhibition of the de novo synthesis of purines by MPA can be overcome by a cell that expresses the *E. coli gpt* gene, which codes for the enzyme xanthine-guanine phosphoribosyltransferase (XGPRT), in the presence of xanthine and hypoxanthine in the growth medium (19). To determine whether the block of purine synthesis by MPA can also be overcome by a recombinant virus expressing the bacterial XGPRT, we first constructed the plasmid

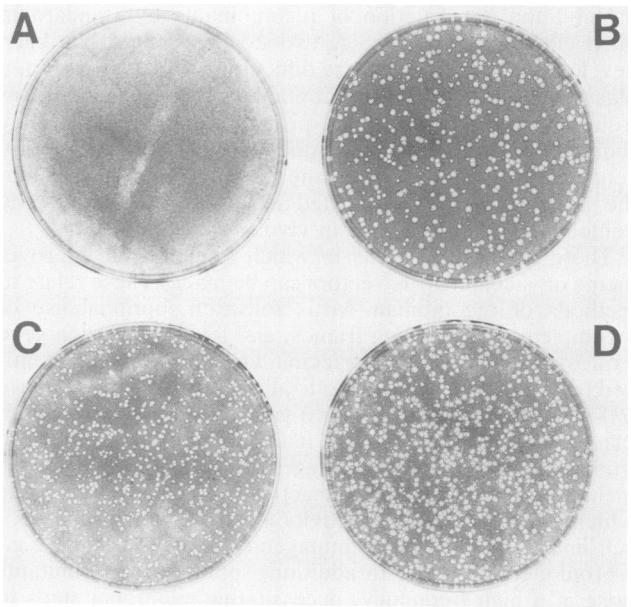

FIG. 1. Plaque formation of vaccinia virus in the presence and absence of MPA. Confluent BSCI cells were preincubated overnight in selective medium and subsequently infected with 1,000 PFU of wild-type vaccinia virus (A, B) or a recombinant virus that expresses the *E. coli gpt* gene (C, D). The cells were incubated for 2 days in the presence (A, C) or absence (B, D) or MPA, xanthine, and hypoxanthine and then stained with crystal violet.

pTK61-gpt. In this construct, the *gpt* gene is controlled by the promoter from the vaccinia virus gene encoding the 7.5K polypeptide and is flanked by viral *tk* sequences. This gene promoter was chosen because it is active early and late in infection (6) and might provide continuous production of the bacterial purine salvage enzyme. The plasmid was transfected into CVI cells that were infected with wild-type virus so that the *gpt* gene would be recombined into the viral *tk* locus. Putative recombinants were detected by a plaque assay on BSCI cells in the presence of MPA, xanthine, and hypoxanthine. Large plaques formed only when the *gpt* gene was used for transfection, suggesting that the desired recombinants behaved in the anticipated manner. One of the recombinants was plaque purified twice under selective conditions, and a small virus stock was grown. A plaque assay demonstrated that this recombinant, in contrast to the wild-type virus, formed plaques on BSCI cells in the presence of selective medium (Fig. 1C).

The genomic analysis of virus grown from six randomly picked plaques that formed during the first selection step is shown in Fig. 2. The *Hin*dIII fragments of all six genomes contain the anticipated 2.0-kilobase-pair (kbp) fragment that hybridizes with the *gpt* probe (Fig. 2A). After the labeled DNA was washed off, the same Southern filter was hybridized to a vaccinia virus *tk* gene-specific probe (Fig. 2B). The *tk* sequences can be detected as a large fragment (4.7 kbp) and a small one (1.0 kbp), indicating the integration of the *gpt* gene into the viral *tk* locus. Since all plaques picked after the first selection step have integrated the selective marker, no other screening procedures are necessary to identify a viral recombinant.

Construction of the insertion and expression vectors pTKgpt-F1s, pTKgpt-F2s, and pTKgpt-F3s. We constructed a series of plasmids that use the *gpt* gene as a selective marker and that allow the expression of foreign genes controlled by the promoter of the major late 11K polypeptide (Fig. 3). The 5′ regulatory region of the gene encoding the 11K polypeptide lies within a 30-kbp segment located immediately upstream of the ATG initiation codon (1, 27). Since the initiation codon forms part of a highly conserved TAAATG sequence within which the 5′ ends of late mRNAs map (1, 24), we chose not to alter this region. The fortuitous presence of an *Eco*RI site immediately downstream of the ATG facilitated the insertion of a polylinker with multiple unique cloning sites. Three vectors in which 0, 1, or 2 guanosine residues follow the ATG allow any coding sequences to be inserted in the correct reading frame. These vectors also provide all-frame stop codons at the end of the polylinker. The sequences downstream of the initiation codon of the gene encoding the 11K polypeptide for the three vectors (termed pTKgpt-F1s, pTKgpt-F2s, and pTKgpt-F3s) are shown in Fig. 4. The vector pTKgpt-oF1s (Fig. 3) is the orientational isomer to pTKgpt-F1s and therefore has the same sequence downstream of the ATG in the gene encoding the 11K polypeptide as pTKgpt-F1s.

Formation of recombinant vaccinia viruses that express β-galactosidase. To ensure the normal functioning of the vector constructs and to be able to easily quantitate the amounts of protein expressed, *E. coli lacZ* gene fragments that lack their own initiation codons (but still have their own termination codons) were inserted in frame into an appropriate restriction site of each of the four vectors. Four plasmids were obtained that were termed pTKgpt-F1sβ, pTKgpt-F2sβ, pTKgpt-F3sβ, and pTKgpt-oF1sβ. These plasmids were used to construct viral recombinants. In each case, all the plaques obtained under selective conditions were able to convert the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in an agar overlay into its blue hydrolysis product (5). This indicated the coexpression of the selected marker gene and the gene of interest and showed that the *lacZ* gene fragments were in the predicted reading frames.

To quantitate the amounts of β-galactosidase produced by the viral recombinants, two Gpt^+ $\beta\text{-}Gal^+$ viruses, derived from the vectors pTKgpt-F1s and pTKgpt-oF1s, were plaque purified three times and small stocks were grown in CVI cells. These stocks were used to infect CVI cells at a multiplicity of 7.5 PFU of the respective virus. For comparison, the same assay was done also with a virus (vtat) based on the vector pSC11 (5) that also expresses the *lacZ* gene driven by the promoter of the gene encoding the 11K polypeptide (but the β-galactosidase of which has a slightly different N terminus), and with a vaccinia virus-T7 RNA polymerase hybrid system that expresses the *lacZ* gene behind the bacteriophage T7 promoter after coinfection with a T7 polymerase-producing virus (8, 9). The results of this analysis are shown in Fig. 5. The viruses based on the vectors pTKgpt-F1s, pTKgpt-oF1s, and pSC11 express similar amounts of β-galactosidase, indicating that *lacZ* gene activity is relatively independent of the orientation and of the kind of neighboring sequences in the virus. The specific activity of pure β-galactosidase is 300,000 U/mg. On the basis of this number, the bacterial enzyme produced by

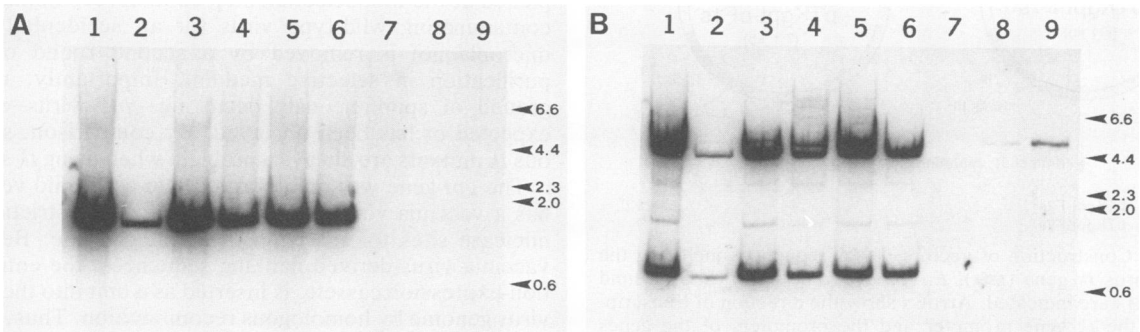

FIG. 2. Genomic analysis of viruses from six randomly picked Gpt^+ plaques. Southern blots were prepared from *Hin*dIII-digested DNA. On the right side, the fragment sizes (in kilobase pairs) of a phage lambda *Hin*dIII digest are indicated by arrowheads. (A) The Southern blot was hybridized with a [^{32}P]dCTP-labeled *gpt* gene-specific probe. Lanes 1 to 6, DNA of BSCI cells infected with virus clones 1 to 6; lane 7, DNA of uninfected BSCI cells; lanes 8 and 9, 10 and 100 ng of wild-type vaccinia virus DNA, respectively. (B) The same Southern blot hybridized with a vaccinia virus *tk* gene-specific probe is shown. In lanes 8 and 9, the vaccinia virus 5.1-kbp *Hin*dIII J fragment is visible; this fragment contains the *tk* gene.

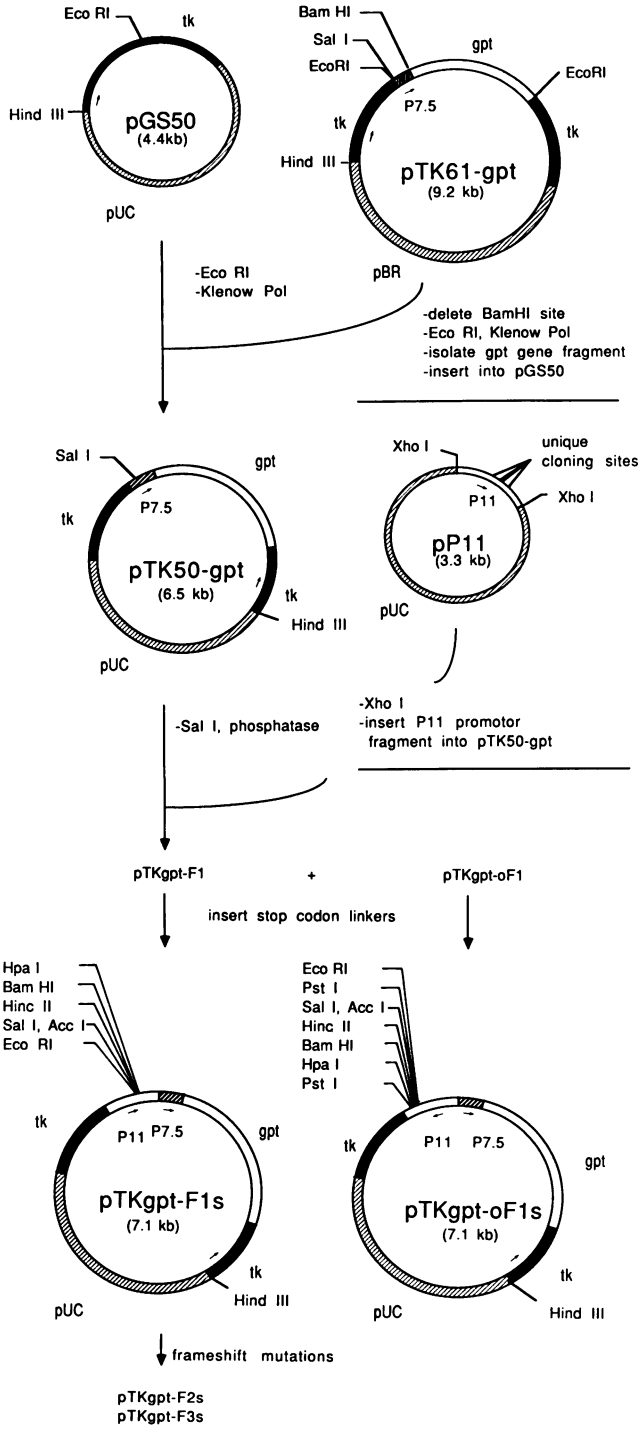

FIG. 3. Construction of vectors. DNA sequences including the vaccinia virus *tk* gene (■), *E. coli gpt* gene (▭), and plasmid vector (▨) are indicated. Arrows show the direction of transcription from the *tk* gene promoter and the promoters of the genes encoding the 11K polypeptide (P11) and the 7.5K polypeptide (P7.5). The pUC sequences contain the ampicillin resistance gene and the origin of bacterial replication. The unique cloning sites downstream of the initiation codon of the gene encoding the 11K polypeptide are indicated.

virus-infected cells is more than 3% of the total cellular protein, an amount that should be easily detectable in a Coomassie blue-stained gel. In fact, a strong band in the 100,000-kilodalton range was observed upon electrophoresis of proteins from cells infected with a pTKgpt-F1s-based virus, and not in the proteins of wild-type virus-infected cells (data not shown). The level of β-galactosidase expression with the promoter of the gene encoding the 11K polypeptide was about twofold higher than that achieved with the hybrid vaccinia virus-T7 RNA polymerase system under the infection conditions described in Fig. 5.

DISCUSSION

Selection for animal cells that express the *E. coli* gene coding for XGPRT was originally described by Mulligan and Berg (19) and is based on the fact that the corresponding enzyme of mammalian cells cannot efficiently use xanthine for GMP synthesis when de novo purine synthesis is blocked. Cells which stably expressed the *gpt* gene, however, could be selectively grown in the presence of MPA and xanthine. Our initial experiments indicated that MPA inhibited vaccinia virus replication. We were unsure, however, whether this system could be adapted to a one-step virus plaque selection procedure. The first question was whether cells that had been pretreated with MPA would still be competent to support virus replication if XGPRT were made. The second question was that if MPA blocked purine metabolism, vaccinia virus might be unable to synthesize mRNA necessary to produce XGPRT. According to this notion, even a delay in addition of MPA would not help, since multiple rounds of replication in neighboring cells are required to form a visible plaque, and this problem would arise in each newly infected cell. With this in mind, we placed the *gpt* gene under the control of both early and late vaccinia virus transcriptional regulatory signals to have immediate and continued formation of *gpt* mRNA. We hoped that on adding an appropriate concentration of MPA, a small amount of mRNA would be made initially and that translation of this mRNA would permit more optimal levels of purine metabolism to resume. Apparently these conditions are met, at least for the three cell lines tested, at a concentration of 25 μg of MPA per ml, since wild-type virus forms no plaques or a few microsize plaques, whereas *gpt*-recombinants form good-size plaques that can be readily picked. It is possible that the optimal MPA concentration will vary with other cell lines. In our experience, all plaques picked at the first selection step contain recombinants. Any contaminating wild-type virus (or an accidentally picked microplaque) is removed by a second round of plaque purification in selective medium. Importantly, no background of spontaneously occurring gpt^+ virus would be expected or has been observed. By comparison, spontaneous *tk* mutants are always a problem when using *tk* selection.

The *gpt* gene was incorporated into a plasmid vector that has a vaccinia virus promoter and unique restriction endonuclease sites for insertion of a foreign gene. Because of vaccinia virus-derived flanking sequences, the entire selection-expression cassette is inserted as a unit into the vaccinia virus genome by homologous recombination. Thus, all of the Gpt^+ recombinants analyzed also contained the foreign gene that had been inserted into the plasmid vector. For our convenience, the flanking sequences used in this study were derived from the vaccinia virus *tk* gene; however, since *tk* selection is no longer required, any nonessential site in the vaccinia virus genome should be suitable.

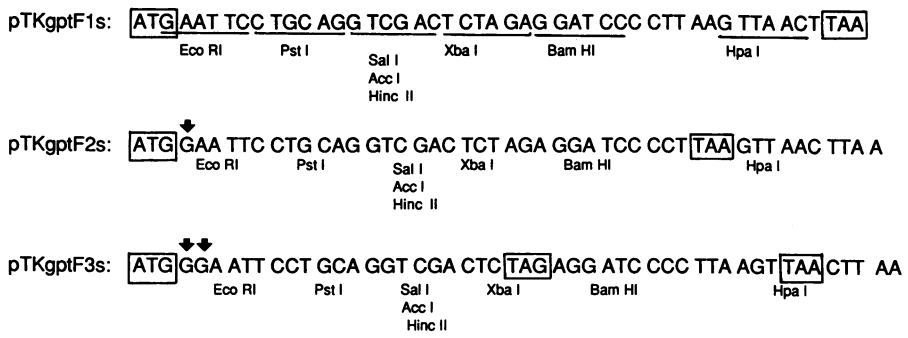

FIG. 4. Sequence of the multiple cloning sites downstream of the initiation codon of the gene encoding the 11K polypeptide. The frameshift mutations were induced by inserting additional G residues (arrows) downstream of the ATG codon. Note that only the restriction sites *Eco*RI, *Sal*I, *Hinc*II, *Acc*I, *Bam*HI, and *Hpa*I are unique. The initiation and termination codons are boxed.

To achieve high levels of expression, the promoter chosen for the vectors was derived from the major 11K structural protein (27). Although the mechanism of late transcription is still poorly understood and involves the attachment of a unique 5′ poly(a) leader (2, 25, 28) the important sequences are contained within a relatively small region starting about 30 bp upstream of the RNA start site and include the translation initiation codon which is a part of the conserved TAAATG sequence. For this reason, the insertion sites for foreign genes were placed just downstream of the ATG. Since the vectors may prove useful for expressing open reading frames, we engineered multiple cloning sites in all three frames as well as termination codons. The system was tested by expression of β-galactosidase; the yield of enzyme was estimated to be greater than 3% of the total cell protein. This expression level is higher than that obtained by using the more widely used promoter of the gene encoding the 7.5K polypeptide (12) and, under the present conditions, even exceeded that obtained with the recent bacteriophage T7-vaccinia virus hybrid system (8). Whether this will hold true for expression of other genes remains to be determined. The vectors described here may be suitable for direct cloning and expression of open reading frames in mammalian cells in a manner similar to that used with bacteriophage λ in *E. coli*.

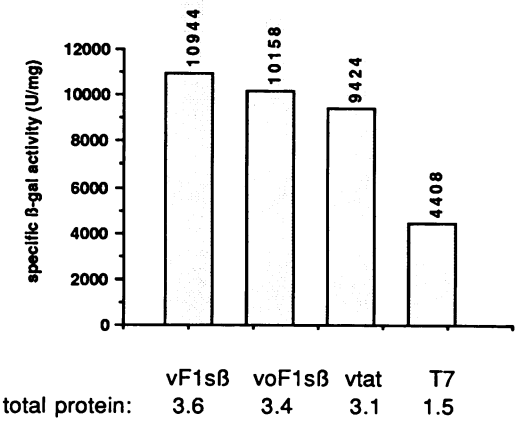

FIG. 5. β-Galactosidase expression in cells infected with the recombinant viruses. Confluent CVI cells (2.5×10^6) were infected with 7.5 PFU of the indicated virus per cell; after a 24-h incubation, cytoplasmic extracts were prepared and the protein content (3) and specific β-galactosidase activity (17) were determined. The virus vF1sβ was derived from the vector pTKgpt-F1s; the virus v0F1sβ is a derivative of pTKgpt-oF1s; and vtat was derived from pSC11 (2). T7 refers to the viruses vTF7-3 (this virus expresses the bacteriophage T7 RNA polymerase) and vTFgal2 (a virus expressing the *lacZ* gene behind the T7 promoter); cells were infected with 7.5 PFU of each of the two viruses per cell. Prior to infection, the titers of all viruses were redetermined. β-Galactosidase activities are mean values of two independent experiments.

The development of additional selection methods will further enhance the versatility of vaccinia virus vectors. Franke et al. (7) demonstrated the enrichment and plaque-forming ability of recombinant vaccinia viruses that express the neomycin resistance gene when grown in the presence of the antibiotic G418 and indicated their intention of developing a coexpression vector similar to that described here for *gpt*.

In summary, *gpt* selection provides a number of important advantages over previous procedures devised to isolate recombinant vaccinia viruses. These include one-step plaque isolation without the need for enrichment, application to a variety of cell lines, use of alternative insertion sites in the vaccinia virus genome, and absence of spontaneous selectable mutants. In addition, 5-bromodeoxyuridine, which is used for *tk* selection, is highly mutagenic, whereas MPA acid is nonmutagenic in the Ames test and in the related SOS test (22). Avoidance of mutagens should ensure virus stability. Finally, we would like to point out that *gpt* selection also might prove useful with different virus vectors, including other members of the poxvirus family, herpesviruses, adenoviruses, retroviruses, and baculoviruses.

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