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Genetics

Vaccinia virus: A selectable eukaryotic cloning and expression vector

(transfection/homologous recombination/herpesvirus thymidine kinase/transcriptional regulation)

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ABSTRACT **Foreign DNA was inserted into two nonessential regions of the vaccinia virus genome by homologous recombination in cells infected with virus and transfected with plasmids containing the foreign DNA elements flanked by vaccinia virus DNA. Thymidine kinase-negative (TK^-) recombinants were selected after inserting foreign DNA into the coding region of the *TK* gene of wild-type vaccinia virus; TK^+ recombinants were selected after inserting the herpesvirus *TK* gene into TK^- mutants of vaccinia virus. For TK^+ expression, it was necessary to insert a 275-base-pair DNA fragment containing the initiation site and sequences upstream of an early vaccinia virus transcript next to the coding sequences of the herpesvirus gene. The unique ability of the herpesvirus TK to phosphorylate ^{125}I-labeled deoxycytidine provided independent confirmation of gene expression. These studies demonstrate the use of vaccinia virus as a selectable cloning and expression vector, confirm the map location of the vaccinia virus *TK* gene, and provide initial information regarding the location of vaccinia virus transcriptional regulatory sequences.**

Several virus groups, including the papovaviruses (1–3), papillomaviruses (4), adenoviruses (5, 6), and retroviruses (7, 8), have been employed as eukaryotic cloning and expression vectors. The relatively small sizes of these virus genomes have facilitated the *in vitro* construction of recombinant DNA molecules. Although genetic engineering of larger viruses is more difficult, such vectors have the advantage of greater capacity and potential of retaining complete infectivity in a wide range of host cells. For vaccinia virus, there is an added incentive of creating recombinants that may have value as live virus vaccines.

In considering the development of vaccinia virus as an expression vector, the following biological characteristics of this unique agent must be taken into account (9, 10): a large [180-kilobase (kb)] genome, a lack of infectivity of isolated viral DNA, the packaging of viral enzymes necessary for transcription within the infectious particle, the probability that vaccinia virus has evolved its own transcriptional regulatory sequences, and the cytoplasmic site of virus transcription and replication. Initially, the major technical problems involved insertion of DNA into the large genome, efficient expression of heterologous genes, and selection of recombinant virus.

Insertion of DNA into the vaccinia virus genome can be accomplished by homologous recombination *in vivo*. Because vaccinia virus DNA by itself is not infectious, intact virus and calcium phosphate-precipitated viral DNA (11, 12) or plasmids containing viral sequences (13) are added in succession. By using plasmids, it is possible to perform the majority of manipulations *in vitro* except for the final step of transfection.

Presumably, efficient expression of foreign genes will depend on the use of vaccinia virus promoters. Although vaccinia virus transcriptional signals have not been defined, the region upstream of one early gene was found to be extremely rich in adenine and thymine residues and differed substantially from prokaryotic or eukaryotic consensus sequences (14). We have now tested the possibility that this DNA segment contains vaccinia virus-specific transcriptional signals by inserting it next to the coding sequences of a foreign gene.

For selection of recombinant virus, advantage was taken of the recent localization of the vaccinia virus thymidine kinase (TK; EC 2.7.1.21) gene (13). Our plan was to construct plasmids containing foreign DNA inserted within the vaccinia virus *TK* gene and then use 5-bromodeoxyuridine to select *in vivo* recombinants on the basis of the resulting TK^- phenotype. The success of this approach would also confirm the map location of this gene. As an alternative method of selection, the herpesvirus *TK* gene fused to a putative vaccinia promoter segment was added to cells infected with a TK^- vaccinia virus mutant. TK^+ recombinants were selected by using amethopterin to inhibit thymidylate synthesis (15).

The studies described in this communication demonstrate the use of vaccinia virus as a selectable eukaryotic cloning and expression vector and provide information regarding the location of vaccinia virus transcriptional signals.

MATERIALS AND METHODS

Preparation of DNA. Recombinant plasmids were prepared from pBR328 (16) or pUC7 (a gift of J. Viera and J. Messing) and purified as described by Birnboim and Doly (17). DNA fragments were isolated from agarose gels by electrophoresis onto DEAE-paper (18) or by binding to powdered glass (19).

Marker Rescue. Two hours after infection of TK^- 143 cells (20) with TK^+ or TK^- vaccinia virus WR (0.01–0.05 plaque-forming unit/cell), calcium phosphate-precipitated plasmid DNA was added (13). For isolation of TK^+ recombinants, amethopterin-containing selective medium was added at 6 hr and cells were harvested at 48 hr after infection (13). For isolation of TK^- mutants, selection with bromodeoxyuridine at 25 μg/ml was used only during the subsequent plaque assay.

Blot Hybridizations. For dot-blot hybridizations, monolayers in 16-mm-diameter wells were harvested 48 hr after infection, lysed by three freeze–thaw cycles, treated with trypsin at 0.125 mg/ml for 30 min at 37°C, and collected on nitrocellulose sheets by filtration using a microsample manifold (Schleicher & Schuell). The filter was washed with 100 mM NaCl/50 mM Tris·HCl, pH 7.5; blotted three times on successive Whatman 3 MM papers saturated with (*i*) 0.5 M NaOH, (*ii*) 1 M Tris·HCl, pH 7.5, and (*iii*) 2× NaCl/Cit (NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate); baked at 80°C for 2 hr; and then in-

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Abbreviations: kb, kilobase(s); bp, base pair(s); TK, thymidine kinase; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate.

cubated with 5× Denhardt's solution (21) supplemented with 0.1 mg of denatured salmon sperm DNA per ml in 4× NaCl/Cit at 65°C for 4 hr. DNA labeled with ^{32}P by nick-translation (22) and sodium dodecyl sulfate at a final concentration of 0.1% were added and hybridization was continued for 12 hr. The filter was washed twice for 15 min at 65°C with 2× NaCl/Cit containing 0.1% sodium dodecyl sulfate and then with 0.2× NaCl/Cit containing 0.1% sodium dodecyl sulfate.

Transfer of DNA restriction fragments from agarose gels was performed by a modification (23) of the Southern (24) blotting technique. The filters were hybridized to ^{32}P-labeled DNA and washed as described above.

Herpesvirus Pyrimidine Kinase Assay. A plaque autoradiography procedure involving ^{125}I-labeled deoxycytidine (^{125}I-deoxycytidine) (New England Nuclear) as a specific substrate for the herpesvirus pyrimidine kinase (25, 26) was used. Monolayers of TK^- 143 cells were incubated with ^{125}I-deoxycytidine at 1 μCi/ml (1 Ci = 3.7×10^{10} becquerels) and tetrahydrouridine (Sigma) at 20 μg/ml to inhibit cytidine deaminase (27) between 14 and 48 hr after infection.

RESULTS

Insertion of Foreign DNA into the Vaccinia Virus *TK* Gene and Selection of Recombinants. Initial experiments were designed to develop a general method of inserting foreign DNA into a nonessential region of the vaccinia virus genome and selecting recombinants. The vaccinia virus *TK* gene seemed an ideal target because its inactivation would allow selection for the TK^- phenotype. The *TK* gene was recently mapped, by marker rescue and by cell-free translation of hybrid-selected mRNA, within the *Hin*dIII J fragment of vaccinia virus (13). Additional data suggested that a unique *Eco*RI site was located within the body of the *TK* gene (28). To facilitate genetic manipulations, the *Hin*dIII J fragment was transferred to a derivative of pBR328 with an *Eco*RI site that had been eliminated by nuclease S1 digestion. A convenient 2.4-kb *Eco*RI E fragment of adenovirus type 18 DNA was inserted into the unique *Eco*RI site within the *TK* gene and the new plasmid containing vaccinia virus and adenovirus sequences was called pVJAd.

The next step involved the use of homologous recombination to transfer the adenovirus DNA flanked by vaccinia sequences into the vaccinia virus genome. TK^- 143 cells were infected with wild-type TK^+ vaccinia virus and then transfected with calcium phosphate-precipitated pVJAd. At this stage, no selection was used. The yield of TK^- virus, determined by plaque assay in the presence of bromodeoxyuridine, was approximately 3×10^5 TK^- plaques per μg of pVJAd added. This value was 5–20 times higher than the yield of spontaneous TK^- mutants but about ⅓ to ⅕ the number obtained upon parallel transfection with a plasmid containing DNA from a previously isolated TK^- vaccinia virus nonsense mutant (28).

The increase in number of TK^- plaques after transfection with pVJAd suggested that insertion of adenovirus DNA into the vaccinia *TK* gene had occurred. To confirm this, and distinguish recombinants from spontaneous TK^- mutants, a rapid dot-blot hybridization procedure was used. Of 40 plaque isolates screened, 70% clearly hybridized to an adenovirus probe and 100% hybridized to a vaccinia virus probe.

The site of integration of foreign DNA into the vaccinia virus genome was determined by analysis of restriction endonuclease fragments. Cleavage of pVJAd with *Eco*RI produced two fragments of about 2.4 and 10 kb. The smaller piece contained the entire adenovirus DNA insert, whereas the larger one contained both vaccinia and plasmid sequences. When the blot was probed with ^{32}P-labeled adenovirus DNA, we detected the 2.4-kb fragment from both pVJAd and the TK^- recombinant virus but not from the wild-type virus control (Fig. 1*A*). Cleavage of pVJAd with *Hin*dIII resulted in the formation of three fragments, two of which (5.7 and 1.8 kb) contained adenovirus sequences. Fragments of these sizes from pVJAd and the TK^- recombinant were detected by autoradiography (Fig. 1*A*). When DNA from the recombinant virus was not cleaved, the adenovirus DNA sequences were associated only with the high molecular weight virion DNA (Fig. 1*A*).

The above experiments demonstrated that the entire adenovirus DNA segment was inserted into the vaccinia virus genome. To identify the site of integration, restriction fragment blots were probed with ^{32}P-labeled *Hin*dIII J fragment (Fig. 1*B*) and ^{32}P-labeled total vaccinia virus DNA (Fig. 1*C*). It is evident that the 5.1-kb *Hin*dIII fragment of wild-type virus is absent from the TK^- recombinant (Fig. 1 *B* and *C*). This fragment is replaced by a larger *Hin*dIII fragment of 5.7 kb and a smaller one of 1.8 kb containing both vaccinia virus and adenovirus DNA. Because of the small amount of vaccinia DNA in the 1.8-kb *Hin*dIII fragment, it was clearly visible only upon longer exposure. The identical patterns obtained when *Eco*RI fragments of wild-type and recombinant virus are probed with vaccinia DNA (Fig. 1 *B* and *C*) indicate that no additional genomic changes have occurred.

Collectively, these data demonstrate the site-specific insertion of a 2.4-kb adenovirus DNA segment into the *Hin*dIII J

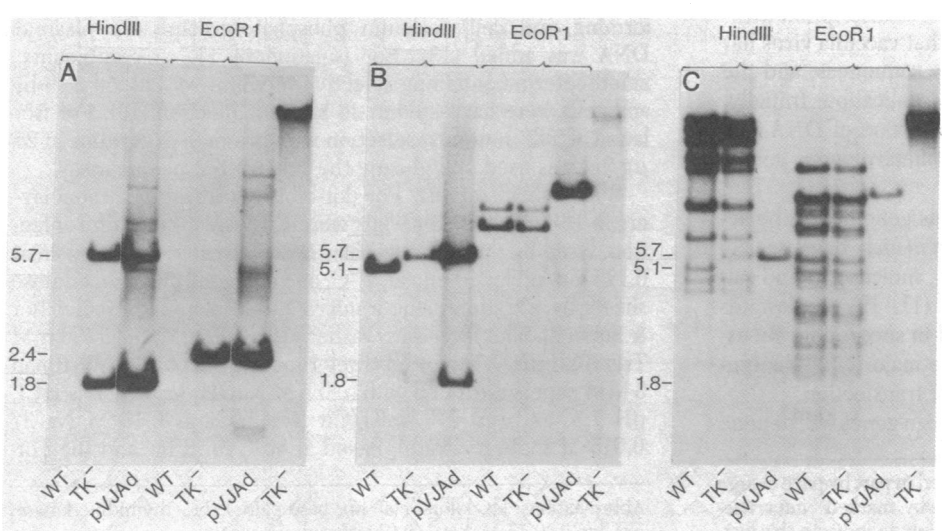

FIG. 1. Evidence for site-specific insertion of foreign DNA into the vaccinia virus genome. A TK^- recombinant virus shown to contain adenovirus DNA by dot-blot hybridization was grown in HeLa cells and purified by sucrose gradient sedimentation. DNA from the recombinant (TK^-), purified wild-type virus (WT), and pVJAd was digested with *Hin*dIII or *Eco*RI, subjected to electrophoresis on a 1% agarose gel, blotted to nitrocellulose, and probed with a ^{32}P-labeled 2.4-kb *Eco*RI E fragment of adenovirus type 18 (*A*), a 5.1-kb *Hin*dIII J fragment of vaccinia virus (*B*), and total vaccinia virus DNA (*C*). The track at the extreme right of each panel contains DNA that was not digested with restriction endonuclease. An autoradiograph is shown. Fragment sizes are given in kb.

fragment of the vaccinia virus genome. Moreover, the TK^- phenotype of these recombinants indicates that the *TK* gene has been inactivated by insertion of foreign DNA.

Insertion and Expression of the Herpesvirus *TK* Gene Within the Vaccinia Virus Genome. As a second selection method, we inserted the herpesvirus *TK* gene into TK^- vaccinia virus mutants and isolated TK^+ recombinants. Previous studies indicated that a 9-kb segment of the vaccinia virus genome, located proximal to the left inverted terminal repetition, was not essential for infectivity (29, 30). This seemed to be a suitable region for the insertion of foreign DNA. Accordingly, a 3-kb *Eco*RI/*Ava* I segment from the nonessential region was cloned in pBR328 and the plasmid was called pMH5/1. A *Bam*HI fragment containing the entire herpesvirus *TK* gene (31) was then inserted between the *Bgl* II and *Bam*HI sites of pMH5/1 (Fig. 2). The resulting recombinant is called pVHTK1.

It was anticipated that vaccinia virus regulatory sequences would be needed to obtain efficient expression of heterologous DNA. Inspection of the nucleotide sequence of an early vaccinia virus gene encoding a 7.5-kilodalton polypeptide revealed a *Rsa* I site betwen the transcriptional and translational initiation sites (14). A *Hinc*II/*Rsa* I fragment of approximately 275 bp containing the transcriptional initiation site and upstream sequences was blunt-end ligated to *Hinc*II-cleaved pUC7 (Fig.

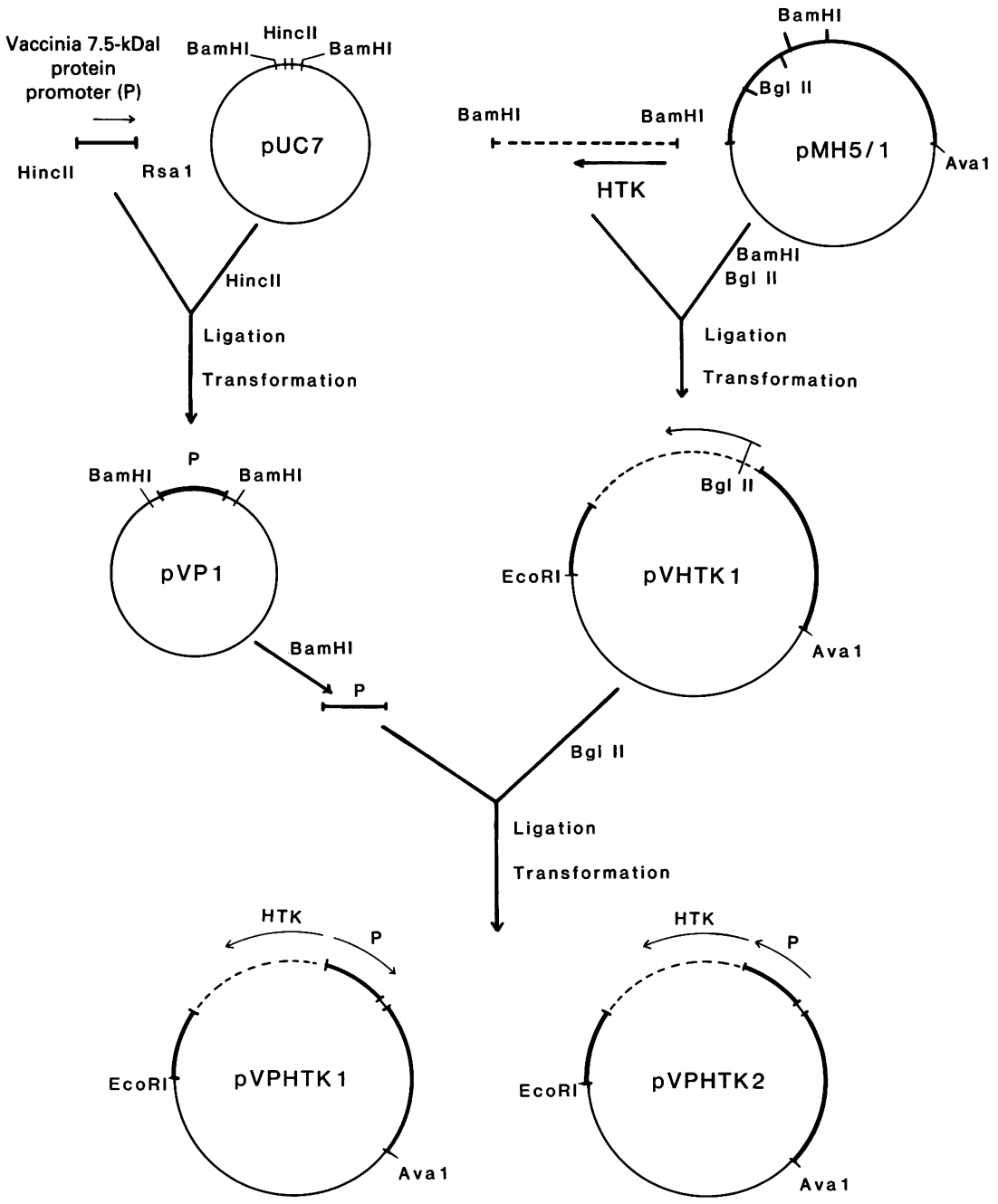

FIG. 2. Construction of plasmids containing the herpesvirus *TK* gene fused to a vaccinia virus promoter. Plasmid sequences are represented as a fine solid line, vaccinia sequences as a heavy solid line, and herpesvirus sequences as an interrupted line. Three plasmids, pVHTK1, pVPHTK1, and pVPHTK2, contain a herpesvirus DNA segment including the *TK* gene flanked by the same vaccinia virus DNA sequences. In pVPHTK1 and pVPHTK2, a 275-base-pair (bp) fragment containing a putative vaccinia virus promoter (P) for the gene encoding a 7.5-kilodalton polypeptide has been inserted in incorrect and correct orientations, respectively, adjacent to the herpesvirus TK (HTK) coding sequences.

2). By then excising the inserted vaccinia virus DNA with *Bam*HI, we effectively added restriction endonuclease linkers to the segment. The *Bam*HI fragment was then inserted at the unique *Bgl* II site of pVHTK1 (Fig. 2). Because the *Bgl* II site is located between the transcriptional and translational initiation sites of the herpesvirus *TK* gene (32, 33), this placed putative vaccinia regulatory sequences adjacent to herpesvirus TK coding sequences. In pVPHTK2, the regulatory and coding sequences are in proper orientation, whereas in pVPHTK1 they are opposite.

Transfection experiments were carried out by adding calcium phosphate-precipitated pVHTK1, pVPHTK1, or pVPHTK2 to TK^- cells infected with a TK^- mutant of vaccinia virus. The yield of TK^+ virus was determined by plaque assay in selective medium (28). At the lowest dilution tested (1:100), no TK^+ plaques were detected when the plasmid used for transfection contained the uninterrupted herpesvirus *TK* gene or the *TK* gene with vaccinia regulatory sequences in opposite orientation. However, when the two sequences were in correct orientation, 5,200 plaque-forming units per μg of plasmid was obtained. Of 23 TK^+ plaque isolates tested, all hybridized to herpesvirus TK DNA (Fig. 3*A*). For subsequent experiments, virus was used that had been plaque purified twice in selective media and then amplified by successive passages in selective and nonselective media.

Site-specific integration of the herpesvirus *TK* gene into vaccinia virus DNA was demonstrated by blot hybridization of restriction fragments. Inspection of Fig. 2 reveals that homologous recombination of pVHTK2 with vaccinia virus DNA should lead to the deletion of vaccinia sequences between the *Bgl* II and *Bam*HI sites and insertion of herpesvirus DNA. Because the restriction endonuclease sites are not regenerated when *Bgl* II and *Bam*HI fragments are ligated, it was not possible to neatly excise the integrated herpesvirus DNA. The recombinant vaccinia DNA was therefore digested with *Hin*dIII or *Xho* I, both of which cut outside of the herpesvirus DNA segment. Hybridization of ^{32}P-labeled herpesvirus TK DNA to blots of electrophoretically separated restriction digests revealed bands of the predicted size (Fig. 3*B*). No hybridization of the probe to wild-type vaccinia virus DNA was detected.

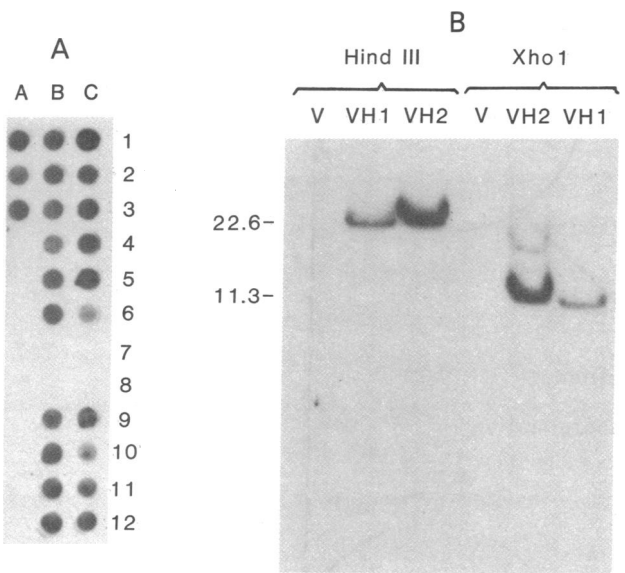

FIG. 3. Demonstration of herpesvirus DNA sequences in vaccinia virus recombinants. (*A*) Screening of plaque isolates by dot-blot hybridization. The filter was hybridized with ^{32}P-labeled herpesvirus *TK Bam*HI fragment. Samples A 1–3, B 1–6, B 9–12, C 1–6, and C 9–12 contain DNA from cells infected with independent TK^+ plaque isolates. Samples B 7 and 8 contain DNA from cells infected with wild-type virus and samples C 7 and 8 are from mock-infected cells. An autoradiograph is shown. (*B*) Analysis of vaccinia virus recombinants by restriction endonuclease analysis. DNA was extracted from cells infected with wild-type vaccinia (V) and with independent TK^+ recombinants (VH1 and VH2) and digested with *Hin*dIII or *Xho* I. After agarose gel electrophoresis, the DNA fragments were transferred to a nitrocellulose sheet and hybridized with ^{32}P-labeled herpesvirus TK DNA. An autoradiograph is shown.

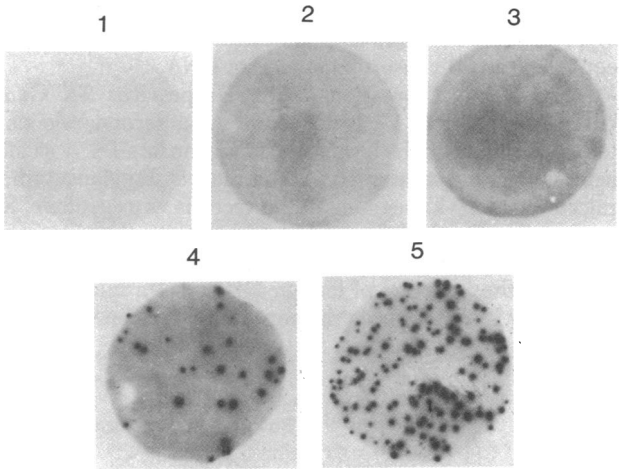

FIG. 4. Plaque autoradiograph demonstrating expression of herpesvirus TK. Monolayers of TK^- 143 cells mock-infected (*1*) or infected with TK^- vaccinia virus mutant (*2*), TK^+ wild-type vaccinia virus (*3*), and vaccinia virus/herpesvirus TK recombinants at 10-fold different dilutions (*4*) and (*5*). Except for *2*, medium containing thymidine and amethopterin was added 2 hr after infection. At 14 hr after infection, cells were labeled for 20 hr with ^{125}I-deoxycytidine. After washing and fixation of the cell monolayers, autoradiographs were made.

A specific assay based on the ability of the herpesvirus TK enzyme to phosphorylate ^{125}I-deoxycytidine (25, 26) was used as a second measure of expression. As shown by autoradiography, recombinant vaccinia virus plaques incorporated the halogenated pyrimidine, whereas no incorporation was detected in visible plaques formed by wild-type TK^+ vaccinia virus or by uninfected monolayers (Fig. 4). The number of recombinant plaques that formed in the presence of amethopterin correlated precisely with the number and position of the autoradiographic spots at two virus dilutions. Expression of the herpesvirus *TK* gene, as judged by growth in selective medium and by ^{125}I-deoxycytidine incorporation, was still obtained after six successive plaque purifications of the vaccinia virus recombinant. The absence of ^{125}I incorporation by wild-type vaccinia virus apparently reflects the stringent substrate specificity of the latter TK.

DISCUSSION

The directed insertion of foreign DNA into two nonessential regions of the vaccinia virus genome has been described. Selection was achieved either by interrupting the endogenous *TK* gene of wild-type vaccinia virus or by adding the herpesvirus *TK* gene to TK^- mutants. In the latter case, expression depended upon placement of a 275-bp fragment of known sequence (14) containing the transcriptional initiation site and upstream sequences of an early vaccinia virus gene next to herpesvirus TK coding sequences. The salutary effect of the correctly oriented vaccinia sequence implies that it contains transcriptional regulatory signals and was necessary for efficient early expression of the *TK* gene. Presumably, the same 275-bp vaccinia virus DNA fragment could be used to initiate transcription of prokaryotic and eukaryotic as well as other virus genes. Selection could be obtained by inserting those genes of

interest in tandem with the herpesvirus *TK* gene or by insertion into and inactivation of the vaccinia virus *TK* gene.

Despite careful analysis of more than a dozen early vaccinia virus transcripts, no evidence of splicing has been obtained (14, 34–37). If, as seems likely, vaccinia virus lacks splicing enzymes, there may be a constraint on the use of the vector for expression of some eukaryotic genes. However, such difficulties may be avoided by the insertion of cDNA clones into the vaccinia virus genome.

Although the foreign DNA fragments inserted into the vaccinia virus genome were a few thousand nucleotides long, we suspect that the potential capacity is significantly greater. Vaccinia virus mutants containing many tandem repetitions of a 1,650-bp segment have been identified, indicating that even larger genomes can be packaged (38). Moreover, the capacity could be enhanced even further by using vaccinia virus (29, 30) or closely related rabbitpox virus (39) mutants with deletions of up to 30 kb as vectors. This should make it possible for a single recombinant virus to express many different genes.

In summary, we anticipate that the methods described here of inserting foreign genes into vaccinia virus and of obtaining expression and selection will be generally applicable. The successful use of vaccinia virus for immunization and eradication of smallpox raises the exciting possibility of employing vaccinia virus recombinants expressing antigens of pathogenic organisms to prevent and eliminate currently important infectious diseases of man and animals.

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