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Eukaryotic transient expression system dependent on transcription factors and regulatory DNA sequences of vaccinia virus

(transfection/chimeric gene/chloramphenicol acetyltransferase)

MARK A. COCHRAN*, MICHAEL MACKETT†, AND BERNARD MOSS‡

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205

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ABSTRACT **A transient expression system in which chimeric genes are expressed in cells infected with vaccinia virus was developed. Recombinant plasmids containing the promoter regions of vaccinia virus genes ligated to the coding segment of the prokaryotic chloramphenicol acetyltransferase (CAT) gene were constructed. When the plasmids were introduced into vaccinia virus-infected cells by transfection, the chimeric gene was expressed and significant levels of CAT accumulated. CAT activity was not detected when the same recombinant plasmid was introduced into uninfected cells, nor was activity detected when the vaccinia virus promoter was absent from the plasmid or was replaced by simian virus 40 or Rous sarcoma virus promoters. This specificity indicated that expression is dependent on a *cis*-acting vaccinia virus promoter region within the recombinant plasmid and diffusible *trans*-acting transcription factors produced during virus infection. The lack of effect of a simian virus 40 enhancer element inserted upstream of the vaccinia virus promoter region also distinguished this system from systems dependent on RNA polymerase II. Although replication of the recombinant plasmid could not be detected in either uninfected or vaccinia virus-infected cells, an inhibitor of DNA synthesis significantly reduced CAT expression. This result, as well as the kinetics of CAT synthesis, suggests that replication of viral DNA templates can enhance transcription of chimeric genes in recombinant plasmids.**

Recombinant DNA that is introduced into eukaryotic cells by transfection or microinjection may be transiently expressed in an unintegrated state (1–11). In such systems, transcription occurs via the RNA polymerase II system within the cell nucleus and is dependent on appropriate *cis*-regulatory sequences associated with the transfected genes. The transcripts must then be properly processed and transported to the cytoplasm for translation to occur. We considered that an analogous cytoplasmic transient expression system might be developed with vaccinia virus-infected cells. Poxviruses, of which vaccinia virus is the prototype, have large DNA genomes encoding biosynthetic enzymes that enable them to use the cytoplasm as a site of transcription and replication (reviewed in ref. 12). Promoter regions upstream of vaccinia virus genes have been identified by both *in vivo* (13–15) and *in vitro* (16) experiments. These regions are extremely rich in adenine and thymine residues and deviate significantly from established eukaryotic consensus sequences (14, 17–19). In this communication, we demonstrate that a heterologous gene ligated to a vaccinia virus transcriptional regulatory sequence is expressed at high levels after transfection of recombinant plasmids. Expression is dependent on the cells also being infected with vaccinia virus, evidently to provide *trans*-acting transcription factors. This transient expression system complements the previously described use of vaccinia virus as a vector for the expression of inserted genes (13, 15, 20).

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MATERIALS AND METHODS

Recombinant Plasmids. Recombinant plasmids were prepared from pBR328 (21), pUC7, or pUC9 (22) and purified as described by Birnboim and Doly (23). DNA fragments were isolated from agarose gels by electrophoresis onto DEAE-paper (24). Plasmids were constructed as indicated in the text, using standard procedures.

Infection, Transfection, and Chloramphenicol Acetyltransferase (CAT) Assays. CV-1 monkey kidney cells were grown to 70% confluency in 25-cm^2 flasks (approximately 2.5×10^6 cells per flask) and infected with wild-type vaccinia virus strain WR at multiplicities stated in the text or incubated with an equivalent volume of medium. After 30 min at 37°C, calcium phosphate-precipitated DNA was added (25, 26). The DNA precipitates contained recombinant plasmid and calf thymus DNA such that the total was 20 μg per ml of transfection mixture. After 30 min at room temperature, fresh medium prewarmed to 37°C was added. When indicated, the medium was supplemented with cytosine arabinonucleoside (araC) at 40 μg/ml. Cells were harvested at various times after infection and suspended in 0.2 ml of Tris·HCl, pH 7.5. After freezing and thawing three times, the disrupted cells were dispersed by sonication and the suspension was assayed for CAT activity as described (15).

RESULTS

Construction of a Chimeric Gene. Gorman *et al.* (1, 27) demonstrated the utility of the prokaryotic *CAT* gene for transient expression studies. The enzyme assay is rapid, sensitive, and quantitative and there is no detectable background CAT activity in eukaryotic cells. Therefore, we wished to construct a chimeric *CAT* gene containing a vaccinia virus promoter region. The entire *CAT* coding segment, without its endogenous promoter, was previously (15) excised from pBR328 (21) with restriction endonuclease *Taq* I and inserted into the *Acc* I site of pUC7 (22). The resulting plasmid, designated pCAT, contains *Bam*HI sites flanking the CAT gene (Fig. 1). A DNA fragment, extending from about 240 base pairs (bp) before and 35 bp beyond the RNA start site of a vaccinia virus gene encoding an M_r 7500 (7.5-kDa) polypeptide, was excised with restriction enzymes *Hin*cII and *Rsa* I (17) and inserted into *Hin*cII-cleaved pUC9 (22). The *CAT* gene was then introduced into the *Bam*HI site of the latter plasmid and the resulting recombinant was called pCP1 (Fig. 1). Since the first ATG downstream of the

Abbreviations: araC, cytosine arabinonucleoside; bp, base pair(s); CAT, chloramphenicol acetyltransferase; pfu, plaque-forming unit; SV40, simian virus 40.

*Present address: Microgenesys, West Haven, CT 06516.
†Present address: Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K.
‡To whom reprint requests should be addressed.

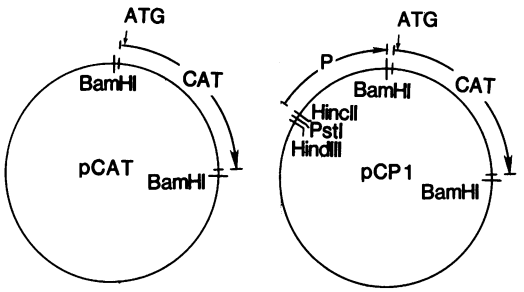

FIG. 1. Structures of recombinant plasmids. The plasmids pCAT and pCP1 contain the entire coding sequence of the *CAT* gene. In pCP1, a DNA fragment including the promoter region of a vaccinia virus gene encoding a 7.5-kDa polypeptide was placed immediately before the *CAT* gene. The arrows indicate the polarity of promoters and coding sequences. The ATG corresponding to a translational initiation codon and relevant restriction endonuclease sites are shown.

viral RNA start site represents the authentic translational initiation signal of the *CAT* gene, expression should result in the formation of active enzyme.

Transient Expression of CAT. To test for expression of the *CAT* gene, pCAT and pCP1 were added as calcium phosphate precipitates to CV-1 monkey cells that were infected or mock-infected with vaccinia virus. The cells were harvested at 24 hr after infection and extracts were tested for CAT activity. As seen in Fig. 2*A*, high levels of CAT were detected in extracts of infected cells transfected with pCP1 but not in extracts of uninfected cells transfected with the same plasmid. Similar results also were obtained with BSC-1 monkey cells and primary chicken embryo fibroblasts (not shown). In contrast, CAT activity was not detected after transfection of infected or uninfected cells with pCAT (Fig. 2*A*). Thus, transient expression appeared to depend on a *cis*-acting vaccinia virus promoter region adjacent to the *CAT* gene and *trans*-acting factors produced during vaccinia virus infection.

The level of transient expression in the vaccinia system was compared to that of a more conventional uninfected cell system. For the latter we used pSV2cat, which contains the simian virus 40 (SV40) enhancer element and early promoter, and pRSVcat, which contains the enhancer and promoter of the long terminal repeat of Rous sarcoma virus (27). We confirmed previous findings of Gorman *et al.* (27) regarding the relative proportions of CAT activity obtained with these two plasmids in uninfected CV-1 cells (Fig. 2*B*). Interestingly, no activity could be measured in extracts from infected cells that had been transfected with pSV2cat or pRSVcat, implying inhibition at some stage needed for expression. Nevertheless, considerable amounts of CAT were made in infected cells that were transfected with pCP1. At 24 hr, the amount of CAT in extracts from infected cells that were transfected with pCP1 was about 40 times higher than that in extracts from uninfected cells that were transfected with pRSVcat.

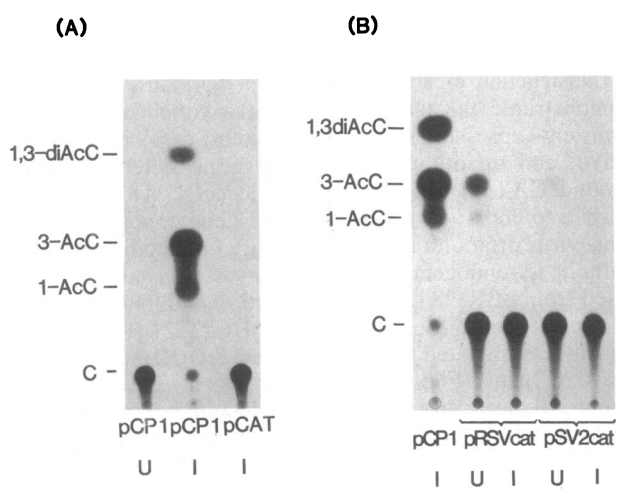

FIG. 2. Transient expression of CAT. (*A*) Uninfected or vaccinia virus-infected CV-1 cells were transfected with 20 μg of pCAT or pCP1 and cell extracts were prepared 24 hr later. CAT assays were performed and analyzed by chromatography on silica gel thin-layer plates. An autoradiograph is shown. (*B*) Uninfected or vaccinia virus-infected CV-1 cells were transfected with 10 μg of pCP1, pRSVcat, or pSV2cat. After 24 hr, extracts were tested for CAT activity as above. U, uninfected; I, infected; C, chloramphenicol; 1-AcC, 3-AcC, and 1,3-diAcC refer to the 1-acetate, 3-acetate and 1,3-diacetate derivatives of chloramphenicol. Autoradiographs are shown.

Since the mechanism of action of eukaryotic transcriptional enhancer elements has not yet been defined, it was of some interest to determine whether such sequences would influence expression of the *CAT* gene under control of a vaccinia virus promoter. The SV40 72-bp repeats have been shown to enhance transcription of CAT and other genes under transient assay conditions (2, 28). Therefore, a fragment containing SV40 DNA (map position 74 to 271) was excised from recombinant plasmid dl74 (29) with restriction enzymes *Sph* I and *Hin*cII and blunt end ligated into the *Sma* I site of pMM23 (19) to form a new plasmid called pMM24. This placed the enhancer-containing SV40 DNA segment in the same orientation upstream of the promoter region of the vaccinia virus 7.5-kDa polypeptide gene as it was relative to the original SV40 gene. Both pMM23 and pMM24 contain the *CAT* coding sequence downstream of the vaccinia virus promoter and are identical except for the presence of the SV40 enhancer in the latter. When vaccinia virus-infected cells were transfected with pMM23 and pMM24, similar levels of CAT expression were obtained, indicating the absence of a detectable enhancer effect.

Optimization of Transient Expression. CAT activity increased in proportion to the amount of helper vaccinia virus added (Fig. 3). This trend leveled off at high multiplicities partly because of increased cell lysis during harvesting, which resulted in measurable amounts of CAT in the medium. Routinely, a multiplicity of 30 pfu per cell was used.

CAT activity also was proportional to the amount of pCP1 used in transfection (Fig. 3). Concentrations of 10–40 μg/ml

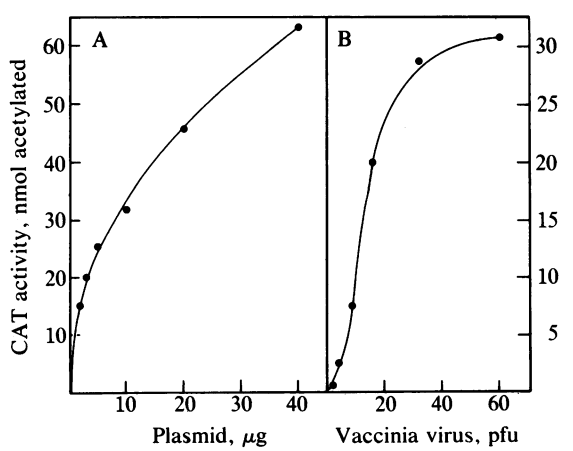

FIG. 3. Dependence of CAT activity on amount of transfected DNA and virus multiplicity. (*A*) CV-1 cells were infected at a multiplicity of 30 plaque-forming units (pfu) per cell and transfected with the indicated amounts of pCP1. (*B*) CV-1 cells were infected with the indicated virus multiplicity and transfected with 10 μg of pCP1. In each case, cells were harvested and analyzed 12 hr after infection. CAT activity is expressed as nmol of chloramphenicol acetylated per 2.5×10^6 cells.

were used for most experiments. The form of the plasmid was critical, since linearization of pCP1 by cleavage at the *Sma* I site, which lies distal to the *CAT* gene (Fig. 1), reduced activity by at least 90%.

The time course of CAT synthesis in cells infected with wild-type vaccinia virus and transfected with pCP1 is shown in Fig. 4. These results are compared to those obtained in cells infected with recombinant vaccinia virus vC24 (15), which contains the *CAT* gene under control of the same promoter used for pCP1. To keep conditions as uniform as possible, cells that were infected with vC24 were transfected with the pUC9 vector, which contains no vaccinia virus sequences. (Calcium phosphate transfection procedures significantly reduced CAT expression by recombinant virus.) CAT was detected within 2 hr after vC24 infection but only 4–6 hr after transfection with pCP1. This lag in transient expression was also observed when the cells were transfected 24 hr before infection with wild-type virus. The levels of CAT synthesized by recombinant virus and in the transient system increased linearly for at least 24 hr. However, the amount of CAT made under the latter conditions was about 30% of that expressed from the recombinant virus.

araC, an inhibitor of DNA replication, typically prevents the expression of late genes of vaccinia virus. However, this drug reduced CAT synthesis in cells infected with vC24 only by about 50% (Fig. 4), in agreement with previous results (15). This partial effect is related to the presence of separately regulated early and late RNA start sites within the promoter region (unpublished data). In the transient system, araC inhibited CAT expression by about 75% (Fig. 4) because of predominant use of the late RNA start site (unpublished data).

The effect of cytosine arabinoside on transient expression could be due to inhibition of virus or pCP1 DNA replication. Two independent methods were used to determine whether replication of pCP1 actually occurs in cells infected with vaccinia virus. DNA was extracted from cells that were uninfected or infected with vaccinia virus in the presence or absence of araC, at various times after transfection with pCP1. Autoradiographs, prepared after immobilization of the DNA on nitrocellulose and hybridization to ^{32}P-labeled pUC9 DNA, revealed no evidence of plasmid replication. The sensitivity of this experiment was increased by using restriction enzymes to distinguish between the input methylated pCP1 grown in bacteria and any unmethylated pCP1 produced by replication in eukaryotic cells (30, 31). DNA was isolated by the Hirt (32) procedure and then digested with *Bam*HI and either *Dpn* I or *Mbo* I. The latter two enzymes recognize the same four-base G-A-T-C sequence, but *Dpn* I cleaves only when the A residue is methylated at the 6-position and *Mbo* II cleaves when it is unmethylated. Since there is a single *Bam*HI site in pCP1 but many G-A-T-C sequences, unmethylated DNA would be linearized by the combination of *Bam*HI and *Dpn* I and digested into many small fragments by the combination of *Bam*HI and *Mbo* I. Conversely, methylated DNA would be linearized by the combination of *Bam*HI and *Mbo* I and digested into small fragments by *Bam*HI and *Dpn* I. Inspection of the overexposed autoradiograph in Fig. 5 reveals no detectable unmethylated pCP1 formed. Since there appears to be no replication of this plasmid under the conditions of transient infection, the effects of araC may be attributed to inhibition of virus DNA replication.

Use of Additional Vaccinia Virus Promoters. Promoter regions from several vaccinia virus genes have been sequenced (14, 17–19, 34), and three of these have been used to express genes inserted into vaccinia virus (13, 15, 19, 35). Use of the promoter region from the 7.5-kDa polypeptide gene has already been described. An additional promoter region was derived from an early gene encoding thymidine kinase and another from a late gene encoding a 28-kDa polypeptide. Recombinant plasmids containing these promoters ligated to the *CAT* gene were tested in a transient assay. As shown in Table 1, CAT expression occurred with the other promoters,

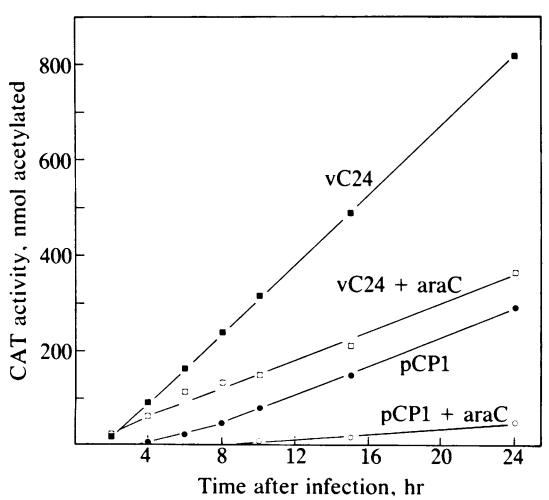

FIG. 4. Time course of CAT synthesis. Replicate cultures of CV-1 cells were infected with wild-type vaccinia virus at 30 pfu per cell and transfected with 20 μg of pCP1 or infected with recombinant virus vC24 at 30 pfu per cell and transfected with pUC9. Cultures were incubated in the absence (filled symbols) or presence (unfilled symbols) of araC at 40 μg/ml. At the indicated times after infection, cells were harvested and assayed for CAT. Activity is expressed as nmol of chloramphenicol acetylated per 2.5 × 10^6 cells.

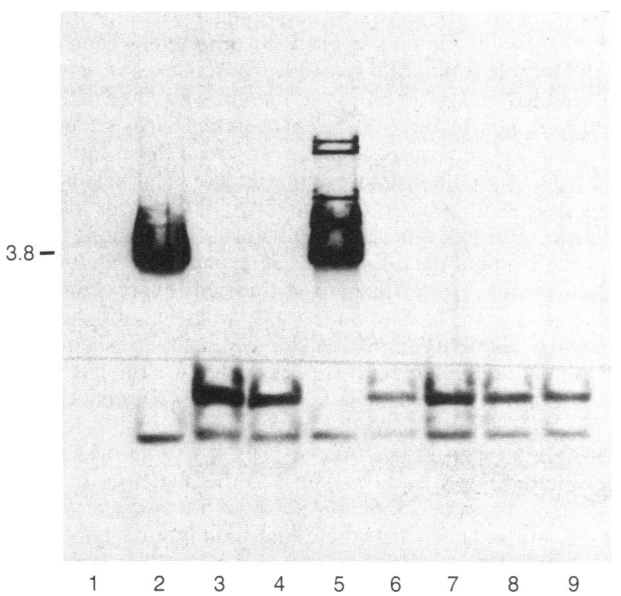

FIG. 5. Absence of pCP1 replication. Vaccinia virus-infected and uninfected CV-1 cells were transfected with 10 μg of pCP1 and maintained in the presence or absence of araC. DNA was prepared 5 and 24 hr after transfection and digested with *Bam*HI and *Dpn* I or *Bam*HI and *Mbo* I. DNA purified from about 5 × 10^5 cells was analyzed by electrophoresis on a 1.5% agarose gel, transferred to nitrocellulose (33), and hybridized to ^{32}P-labeled pCP1 DNA. An autoradiograph is shown. Migration of the linearized 3.8-kilobase plasmid is indicated. Lane 1, vaccinia virus DNA (100 ng) digested with *Bam*HI and *Dpn* I; lane 2, pCP1 DNA (100 ng grown in a *dam*$^+$ *Escherichia coli* strain) digested with *Bam*HI; lane 3, pCP1 DNA (100 ng grown in a *dam*$^+$ *E. coli* strain) digested with *Bam*HI and *Dpn* I; lane 4, infected, 24 hr, *Bam*HI and *Dpn* I; lane 5, infected, 24 hr, *Bam*HI and *Mbo* I; lane 6, infected, +araC, 24 hr, *Bam*HI and *Dpn* I; lane 7, infected, 5 hr, *Bam*HI and *Dpn* I; lane 8, infected, +araC, 5 hr, *Bam*HI and *Dpn* I; lane 9, uninfected, 24 hr, *Bam*HI and *Dpn* I.

Table 1. Transient activity with different vaccinia virus promoters

Promoter	Plasmid	CAT activity, nmol chloramphenicol acetylated per 2.5×10^6 cells	
		No drug	With araC
7.5-kDa	pCP1	380	100
Thymidine kinase	pMM19	27	15
28-kDa	pLCAT1	67	14
—	pCAT	<0.01	<0.01

Recombinant plasmids contained the promoter region from the gene encoding 7.5- or 28-kDa polypeptides or thymidine kinase ligated to the *CAT* gene. CV-1 cell monolayers were infected at a multiplicity of 30 pfu per cell and transfected with 20 μg of the indicated plasmid, and cell extracts were prepared 24 hr later.

although at a lower level than with the 7.5-kDa promoter. The relative efficiencies of the 7.5-kDa and thymidine kinase promoters in the transient assay (Table 1) corresponded to those obtained with the same constructs inserted into the vaccinia virus genome (15). In addition, araC inhibited transient expression of the late promoter most and the early promoter least.

DISCUSSION

We have developed a rapid way of expressing chimeric genes in primary and continuous cell lines. The method involves the use of recombinant plasmids containing vaccinia virus promoter regions as *cis*-acting regulatory elements. After insertion of the coding segment of a heterologous gene, such as *CAT*, standard transfection procedures were used to introduce the plasmid into vaccinia virus-infected cells. Promoter regions of three different vaccinia virus genes were used successfully in this system, whereas those from SV40 and Rous sarcoma virus were ineffective. This selectivity is consistent with nucleotide sequence differences between vaccinia virus and other eukaryotic promoter regions (14, 17) as well as functional differences determined by *in vitro* transcription (16). The efficiency of transient expression in the vaccinia system compared favorably with that obtained with the more standard uninfected cell system even when strong promoters and enhancers were provided for the latter. The cytoplasmic site of vaccinia virus transcription may contribute to the high efficiency, since Loyter *et al.* (36) reported that only a small proportion of cells that take up calcium-precipitated DNA also transfer it to the nucleus.

Expression of recombinant plasmids in vaccinia virus-infected cells indicates that the transfected DNA is not excluded from the sites of virus transcription or that viral RNA polymerase and other factors are diffusible. The requirement for diffusible viral proteins may explain the 6-hr delay before significant expression is detected. The delay in CAT synthesis is not due to slow DNA uptake, because it was observed even when the plasmid was added 24 hr before infection. Nor is it due to the nature of the promoter used, since expression occurs within 2 hr when the same chimeric gene is inserted into vaccinia virus. Under the latter conditions, rapid expression evidently occurs because the viral enzymes and DNA are packaged within the core of the virus particle.

The physical nature of the recombinant DNA was important, since expression dropped precipitously when the plasmid was linearized. This is probably not due to lack of DNA uptake, because linearized DNA works well for vaccinia virus marker rescue experiments. A requirement for supercoiled DNA also has been found for other transient expression systems (5). It is important to point out that recombination is unlikely to play a significant role in the vaccinia virus transient expression system for several reasons. First, the promoter fragment used was only a few hundred base pairs long, and much smaller ones serve equally well (unpublished data). Second, transient expression is unaffected by flanking the chimeric gene with vaccinia virus DNA sequences that promote homologous recombination. Third, the recombination frequency even under the latter conditions is less than 0.5%, whereas the rate of transient expression may be as much as 30% of that obtained by infection with recombinant virus.

The reduction in transient expression caused by araC, an inhibitor of DNA replication, was noteworthy. Since replication of the recombinant plasmid could not be detected, the requirement appears to be for viral DNA synthesis. The effect of araC was greatest when a late promoter was used to form the chimeric *CAT* gene and least when an early promoter was used, suggesting that the requirement is for regulatory factors that control late transcription. The most extensive analysis was done with a promoter region that contains early and late RNA start sites. Nuclease S1 mapping studies indicated that both RNA start sites were used for transient expression but that the late one predominated (unpublished data).

This transient expression system has proven to be extremely useful in our laboratory for studying the regulatory signals of vaccinia virus promoter regions. For example, it provides a rapid way of monitoring the effects of *in vitro* mutagenesis. Genes other than *CAT* also have been expressed in this system. When the influenza hemagglutinin was used, the protein was processed correctly and inserted into the plasma membrane, as determined by immunofluorescence. This result indicates that the transient system also may provide a useful way of analyzing effects of mutations on protein function. In many respects the transient system complements the use of vaccinia virus as a vector for insertion of foreign genes (13, 15, 20, 35).

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