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Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells

(cap-independent translation/encephalomyocarditis virus untranslated leader/vaccinia virus)

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ABSTRACT **A mouse cell line that constitutively synthesizes the bacteriophage T7 RNA polymerase was constructed. Fluorescence microscopy indicated that the T7 RNA polymerase was present in the cytoplasmic compartment. The system provided, therefore, a unique opportunity to study structural elements of mRNA that affect stability and translation. The *in vivo* activity of the bacteriophage polymerase was demonstrated by transfection of a plasmid containing the chloramphenicol acetyltransferase (CAT) gene flanked by T7 promoter and termination signals. Synthesis of CAT was dependent on the presence of a cDNA copy of the untranslated region of encephalomyocarditis virus (ECMV) RNA downstream of the T7 promoter, consistent with the absence of RNA-capping activity in the cytoplasm. CAT expression from a plasmid, pT7EMCAT, containing the T7 and EMCV regulatory elements was detected within 4 hr after transfection and increased during the next 20 hr, exceeding that obtained by transfection of a plasmid with the CAT gene attached to a retrovirus promoter and enhancer. Nevertheless, the presumably cap-independent transient expression of CAT from pT7EMCAT was increased more than 500-fold when the transfected cells also were infected with wild-type vaccinia virus. A protocol for high-level expression involved the infection of the T7 RNA polymerase cell line with a single recombinant vaccinia virus containing the target gene regulated by a T7 promoter and EMCV untranslated region.**

The high transcriptase activity, stringent promoter specificity, and single-subunit structure of the RNA polymerases from T7 and related bacteriophages are features that have contributed to their successful use for prokaryotic gene expression (1). Despite the differences in the structure, mode of synthesis, processing, and modification of prokaryotic and eukaryotic mRNAs, attempts also have been made to exploit bacteriophage RNA polymerases for expression of RNA or proteins in eukaryotic cells (2–9). In one approach (2–5), the anticipated difficulties associated with synthesis, processing, and transport of prokaryotic-like RNAs within the nuclear compartment were avoided by introducing the T7 RNA polymerase gene into vaccinia virus, a DNA virus that replicates in the cytoplasm (10). It was expected that the virus-encoded cytoplasmic enzymes for capping and methylation of vaccinia virus mRNA also would act on the T7 RNA polymerase transcripts. Although the amounts of RNA made by the vaccinia virus-encoded T7 polymerase were extremely high, comprising about 30% of the total steady-state RNA in the cytoplasm, only moderate amounts of protein were made (4). The limited translation was correlated with the low efficiency of capping of transcripts made by the bacteriophage T7 RNA polymerase. Presumably, the physical association of capping enzyme with the vaccinia RNA polymerase (11) facilitated capping of vaccinia transcripts. Therefore, rather than try to increase the extent of capping of mRNA made by the T7 RNA polymerase, it seemed preferable to make the translation of such mRNAs cap-independent. Fortunately, this technical problem had already been solved by the picornaviruses, a family of positive-stranded RNA viruses that replicate in the cytoplasm of eukaryotic cells.

Picornavirus mRNAs have a long untranslated leader region (UTR) that facilitates cap-independent ribosome binding (12). By placing a DNA segment corresponding to the UTR of encephalomyocarditis virus (EMCV) RNA (13) just downstream of a bacteriophage T7 promoter, expression in the bacteriophage T7/vaccinia virus hybrid system was enhanced 5- to 10-fold (5). Two protocols were developed for use of the vaccinia virus/T7/EMCV hybrid expression system. For analytical experiments, mammalian cells were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase and then transfected with a plasmid containing the T7 promoter/EMCV UTR-regulated target gene. For large-scale production, in which the amount of protein expressed equalled 10% of the total cell protein, cells were coinfected with two recombinant vaccinia viruses—one expressing the bacteriophage T7 RNA polymerase and another the target gene under control of the T7 promoter/EMCV UTR. The latter protocol, involving two separate viruses, was required because recombinant vaccinia viruses containing both genetic elements of the bacteriophage T7 transcription system were found to be unstable (2).

Here we describe another step in the exploitation of the bacteriophage T7 RNA polymerase, the construction of a stable mouse cell line that constitutively synthesizes bacteriophage T7 RNA polymerase and can be used for expression of target genes that have a T7 promoter. The target genes can be introduced by DNA transfection, thereby avoiding the use of vaccinia virus entirely. Under such conditions, however, the EMCV UTR was crucial. The cytoplasmic location of the bacteriophage T7 RNA polymerase in the transformed cell line provides a unique system for studying features, in addition to the cap structure, that affect the stability and translation of eukaryotic mRNAs. Alternatively, for large-scale expression of recombinant proteins, the cell line may be infected with a recombinant vaccinia virus containing the target gene regulated by the T7 promoter and EMCV UTR.

MATERIALS AND METHODS

Plasmid Construction and Cell Transformation. The T7 RNA polymerase gene was excised as a 2.6-kilobase-pair (kbp) *Bam*HI fragment from plasmid pTF7Gene-1, a derivative of pAR1173 (14) that was kindly provided by T. R. Fuerst (Molecular Vaccines, Gaithersburg, MD), and the

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMCV, encephalomyocarditis virus; UTR, untranslated region; AraC, 1-β-D-arabinofuranosylcytosine.

ends were filled in with the Klenow fragment of DNA polymerase. The blunt-ended fragment was then ligated to the 4.3-kbp *Hin*dIII–*Bgl* II fragment of pSV2-dhfr (15), after similar filling-in of its ends, and the new plasmid was called pOSV-T7RP. Mouse L(A9) cells were cotransfected with supercoiled plasmids pOSV-T7RP and pSV2-neo (16) in a molar ratio of 10:1, respectively. Transfection and selection for colonies resistant to G418 sulfate (Geneticin, GIBCO) were performed as described (17). G418-resistant clones were screened by Northern blot analysis as follows: total RNA was isolated from 10^6 cells of each clone (18), and 10 μg of RNA was fractionated in a formaldehyde/agarose gel (19), transferred to a nitrocellulose membrane, and hybridized to ^{32}P-labeled 2.6-kbp fragment from pAR1173 as described (20).

Bacteriophage T7 RNA Polymerase Assay. A pellet of 10^6 cells was resuspended in 0.2 ml of 10 mM Tris·HCl, pH 7.6/10 mM NaCl/1.5 mM $MgCl_2$ and freeze–thawed three times. After centrifugation, 3 μl of cytoplasmic supernatant was used for *in vitro* transcription as recommended by Promega for T7 RNA polymerase, with 0.6 μg of linear pT7EMCAT (5) as a template in a reaction volume of 25 μl. Purified T7 RNA polymerase (Promega) was used as a positive control. The transcription mixture was incubated for 1 hr at 37°C and the products were fractionated in a 4% polyacrylamide/urea gel.

Immunofluorescence. Cells were washed three times with phosphate-buffered saline (PBS), fixed with 10% formaldehyde in PBS (10 min at room temperature), permeabilized by a 5-min incubation on ice with methanol/acetone (1:1), washed three times with PBS, and incubated for 30 min at room temperature with 1% normal goat serum (Calbiochem) in PBS. The cells were then incubated with rabbit antiserum directed against bacteriophage T7 RNA polymerase (a generous gift from W. Studier, Brookhaven National Laboratory, Upton, NY) diluted in PBS containing 1% goat serum. After a 30-min incubation at room temperature, the cells were washed three times with PBS and incubated for 30 min at room temperature with fluorescein isothiocyanate-labeled goat antiserum against rabbit IgG (Calbiochem) diluted in PBS containing 1% goat serum. Cells were then washed three times with PBS, covered with 20% glycerol in PBS, and visualized by fluorescence microscopy.

Liposome-Mediated Transfection. To 1 ml of Dulbecco's modified Eagle's medium (DMEM) without serum, 15 μl of Vortex-mixed "TransfectAce" liposome formulation (L. Buonocore and J. K. Rose, Yale School of Medicine) was added followed by the addition of 5 μg of supercoiled plasmid. The mixture was then added to a PBS-washed subconfluent monolayer of 10^6 cells. Following 3 hr at 37°C in a CO_2 incubator, 1 ml of DMEM containing 20% fetal bovine serum was added. For pRSV-cat transfection, medium containing 20% serum was added after 5 hr, as recommended for nuclear expression (21).

Virus Infection and Chloramphenicol Acetyltransferase (CAT) Assay. Procedures were similar to those described previously (2).

RESULTS

Constitutive Expression of Cytoplasmic Bacteriophage T7 RNA Polymerase by Mouse L Cells. Separate plasmids containing the T7 RNA polymerase gene or the neomycin-resistance gene, under control of simian virus 40 early promoter and enhancer sequences, were used to cotransfect mouse L cells. Thirty-five independent G418-resistant colonies were screened for transcription of the T7 RNA polymerase gene by Northern blot analysis. The presence of active T7 RNA polymerase in cytoplasmic extracts was then determined using pT7EMCAT (5) as a template. Two clones were identified that contained full-size T7 RNA polymerase mRNA as well as an active cytoplasmic T7 RNA polymerase enzyme. The clone with the higher T7 polymerase activity was maintained and will be referred to as the OST7-1 cell line. Immunofluorescence analysis revealed that most or all of these cells expressed T7 RNA polymerase and that the enzyme was present in the cytoplasm (Fig. 1).

Expression of Genes with a T7 Promoter in OST7-1 Cells. Transfection experiments were carried out with OST7-1 cells to confirm the activity of the T7 RNA polymerase *in vivo* and determine the translation requirements of the T7 transcripts. Plasmids pT7EMCAT or pT7CAT (5), containing the CAT gene regulated by the T7 promoter with or without the EMCV UTR, respectively, were tested. At various times after transfection, the cells were lysed and the levels of CAT were measured (Fig. 2). No CAT activity was detected in cells that had been transfected with pT7CAT, whereas significant activity was obtained with pT7EMCAT, indicating a requirement for the EMCV UTR. CAT expression was not detected when the untransformed parental L-cell line was transfected with pT7EMCAT, confirming the requirement for T7 RNA polymerase. In OST7-1 cells transfected with pT7EMCAT, CAT activity was detected within 4 hr and increased continuously for at least 24 hr. Moreover, the activity at 4 hr was greater than that obtained at 24 hr from plasmid pRSV-cat (22), which has the efficient Rous sarcoma virus long terminal repeat regulating CAT expression.

Effect of Vaccinia Virus Infection on Expression. We wished to compare CAT expression in OST7-1 cells, which constitutively synthesize T7 RNA polymerase, with that in L cells that had been infected with a recombinant vaccinia virus, vTF7-3 (2), which expresses T7 RNA polymerase. Much greater CAT activity was detected when pT7EMCAT was used to transfect L cells that were infected with vTF7-3 than when the same plasmid was used to transfect uninfected OST7-1 cells (data not shown). One possible explanation for the difference in expression of CAT involved the level of T7 RNA polymerase in L cells that have been infected with recombinant vaccinia virus vTF7-3, which is higher than that in uninfected OST7-1 cells (data not shown). Alternatively, the T7 RNA polymerase might not be limiting and the vaccinia virus infection itself might enhance expression. To distinguish between the hypotheses, we transfected pT7EMCAT into OST7-1 cells that had been infected with wild-type vaccinia virus and measured CAT activity 24 hr later. The CAT activity after a 1:500 dilution of the lysate (Fig. 3, − AraC) was greater than that of undiluted lysate of uninfected OST7-1 cells that were transfected with pT7EMCAT (Fig. 2). Thus, wild-type vaccinia infection increased the expression level 500- to 1000-fold,

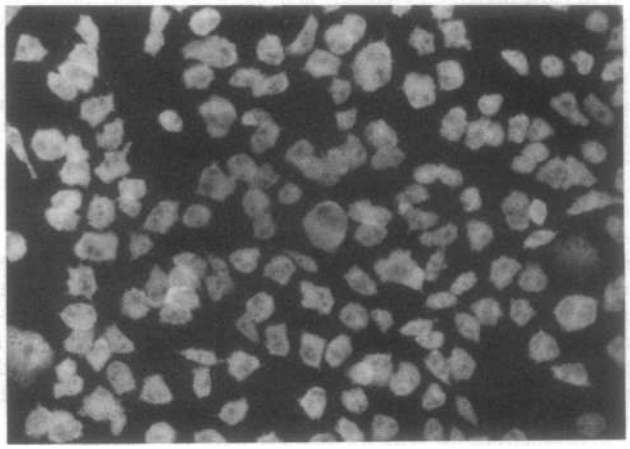

FIG. 1. Detection of T7 RNA polymerase in OST7-1 cells by immunofluorescence. OST7-1 cells were fixed and incubated first with rabbit antibodies to T7 RNA polymerase and then with fluorescein-labeled goat antibodies to rabbit IgG.

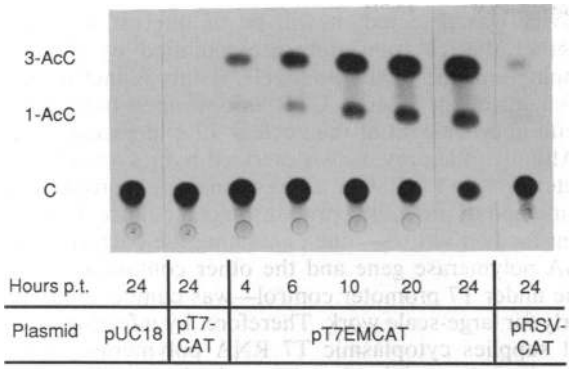

FIG. 2. CAT activity in vector-transfected OST7-1 cells. Subconfluent monolayers of 10^6 OST7-1 cells were transfected by a liposome-mediated transfection method with 5 μg of supercoiled pUC18, pT7CAT, pT7EMCAT, or pRSV-cat as indicated. At various times posttransfection (hours p.t.) the cells were collected and were lysed in 100 μl of 0.25 M Tris·HCl (pH 7.5), and without further dilution 40 μl of lysate was assayed for CAT activity. Autoradiogram shows chloramphenicol (C) and acetylated forms of chloramphenicol (AcC) separated by thin-layer chromatography.

indicating that differences in T7 RNA polymerase levels were not critical. A similar degree of enhancement was obtained when the order of infection and transfection of OST7-1 cells was reversed. Using a plasmid with a T7 promoter and EMCV UTR upstream of the *Escherichia coli lacZ* gene, we found that vaccinia virus infection increased the number of cells that stained blue with 5-bromo-4-chloro-3-indolyl β-D-galactoside from 1% to 100% (data not shown). Since the T7 transcripts have the EMCV UTR, this effect of vaccinia virus was probably not due to an effect of capping on translation. Vaccinia virus infection has been reported to induce nonspecific replication of transfected plasmids (23, 24), raising the possibility that template amplification might account for the enhancement of CAT expression. However, addition of the DNA-synthesis inhibitor AraC (25) had only a modest effect on the enhancement of CAT expression (Fig. 3). Control experiments verified the inhibition of plasmid replication by this treatment (data not shown). Thus, vaccinia virus appears to enhance cytoplasmic expression by an unknown mechanism.

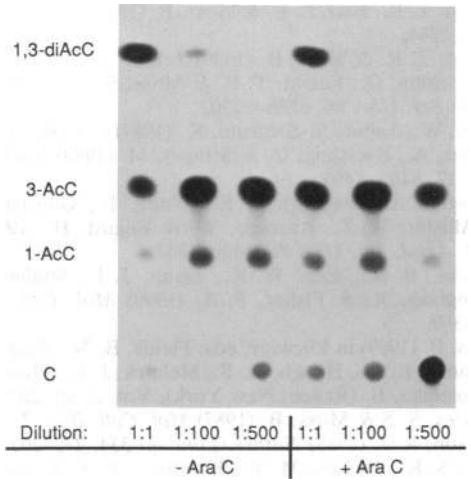

FIG. 3. Effect of vaccinia virus infection on CAT activity in vector-transfected OST7-1 cells. Subconfluent monolayers of 10^6 OST7-1 cells were infected with 10 plaque-forming units of wild-type vaccinia virus (strain vWR) per cell. After 30 min, the virus inoculum was removed and the cells were transfected with 5 μg of pT7EMCAT DNA by the liposome-mediated transfection method. At 24 hr after transfection, cells were collected, lysed in 100 μl of 0.25 M Tris·HCl (pH 7.5), and further diluted as indicated, and 40-μl samples were assayed for CAT activity. 1-β-D-Arabinofuranosylcytosine (AraC) was used at a final concentration of 0.04 mg/ml and was present where indicated at 2 hr prior to infection and during all the subsequent experimental steps.

Use of Recombinant Vaccinia Virus to Provide the Target Gene. The previous vaccinia virus/bacteriophage T7 hybrid expression system has been used for large-scale protein synthesis by coinfecting cells with two separate recombinant viruses, one to provide the T7 RNA polymerase and the other the target gene (3, 26). In principle, OST7-1 cells should make it unnecessary to use a virus that expresses T7 RNA polymerase, so that a single-virus infection protocol could be used. Therefore, we wished to compare the synthesis of CAT protein in L cells coinfected with vTF7-3 (which provides the T7 RNA polymerase) and vT7EMCAT to the CAT expression level in OST7-1 cells infected only with vT7EMCAT. CAT accumulation was monitored at 24 hr after infection by Coomassie brilliant blue staining of $NaDodSO_4$/polyacrylamide gels (Fig. 4 *Upper*) as well as by assaying CAT activity (Fig. 4 *Lower*). CAT expression in ordinary L cells infected with vT7EMCAT was insignificant as measured by either assay. However, the level of CAT expressed in OST7-1 cells infected with vT7EMCAT alone approached that in L cells

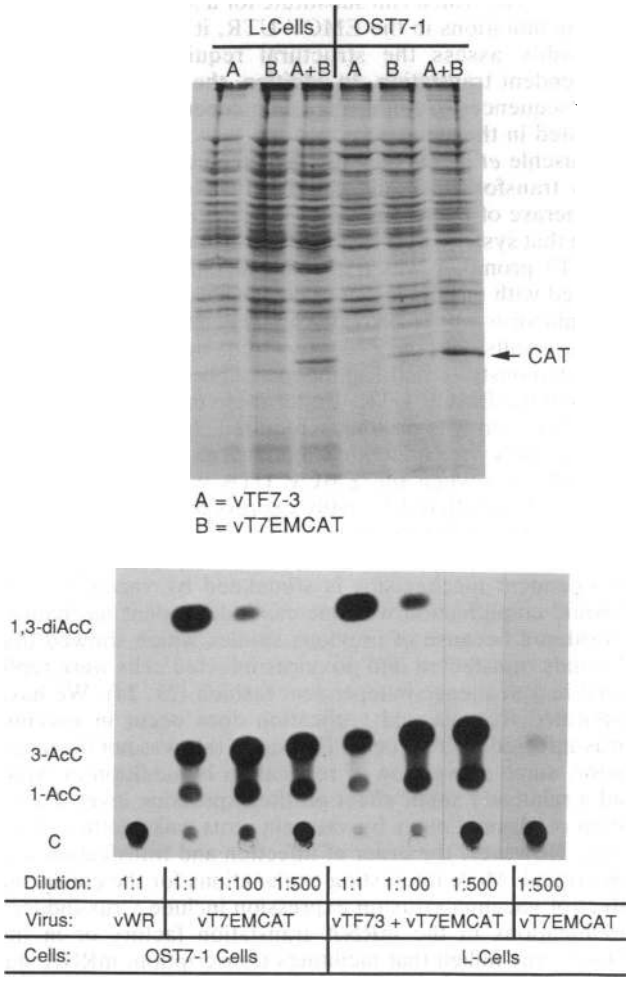

FIG. 4. Expression of CAT by recombinant vaccinia virus-infected cells. Approximately 10^6 mouse L cells or OST7-1 cells were infected with vT7EMCAT or wild-type vaccinia virus (vWR) at 10 plaque-forming units per cell or were coinfected with vTF7-3 and vT7EMCAT, each at 10 plaque-forming units per cell. At 24 hr after infection, cell lysates were prepared. (*Upper*) Twenty micrograms of lysate protein was loaded in each lane of a $NaDodSO_4$/10% polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie brilliant blue. (*Lower*) CAT activity was determined on the cell lysates as described in the legend to Fig. 3.

coinfected simultaneously with vT7EMCAT and vTF7-3. The combination of OST7-1 cells and vTF7-3, however, increased the yield of CAT (Fig. 4 *Upper*), suggesting that there might be some advantage in isolating transformed cell lines with higher levels of T7 RNA polymerase.

DISCUSSION

We have stably integrated the bacteriophage T7 RNA polymerase gene, under control of a simian virus 40 promoter, into mouse L cells. The cell line, OST7-1, constitutively expresses T7 RNA polymerase. Immunofluorescence analysis indicated that the enzyme was present in the cytoplasmic compartment, in agreement with the absence of a nuclear localization signal as shown by previous T7 RNA polymerase microinjection studies (27). Transfection experiments confirmed that the enzyme was transcriptionally active in OST7-1 cells. Since the bacteriophage T7 RNA polymerase has strict promoter and terminator specificities and makes unprocessed RNAs, the cell line can be used to assess the requirements for stability and translation of mRNA *in vivo*. In this report, we demonstrated that expression of a transfected target gene was dependent on the presence of the EMCV UTR, which can substitute for a 5′ cap structure. By making mutations in the EMCV UTR, it should be possible to readily assess the structural requirements for cap-independent translation. In addition, the suggested roles of other sequences in conferring cap independence (28–31) can be tested in the same way.

Deuschle *et al.* (8) recently described experiments with a stably transformed rabbit cell line that expresses the RNA polymerase of bacteriophage T3, which is similar to that of T7. In that system, expression of a transfected gene regulated by a T3 promoter was not detected unless the cells were infected with vaccinia virus. They suggested that the role of vaccinia virus was to provide capping enzyme. We obtained similar results with the T7 system (data not shown) but went on to demonstrate that cap independence could be achieved by using the EMCV UTR. Under these conditions, vaccinia virus infection was no longer required. Nevertheless, wild-type vaccinia virus infection stimulated expression more than 500-fold even when the EMCV UTR was used. Both the number of cells that detectably express the transfected plasmid and the level of expression were increased. We believe, therefore, that expression by both cap-dependent and -independent mechanisms is stimulated by vaccinia virus. Plasmid amplification was one cap-independent mechanism considered because of previous studies which showed that plasmids transfected into poxvirus-infected cells were replicated in a sequence-independent fashion (23, 24). We have confirmed that plasmid replication does occur in vaccinia virus-infected OST7-1 cells. However, this was not the major factor, since prevention of replication by addition of AraC had a relatively small effect on the expression level. Facilitation of plasmid entry by vaccinia virus was another possibility. However, the order of infection and transfection was not critical. More interesting explanations for the enhancing effect of vaccinia virus on expression include virus-induced modifications in the mRNA translation factors or in the cytoplasmic milieu that facilitates transcription, mRNA stability, or translation. The mechanism of enhancement appears to be specific for vaccinia virus, since no stimulation of pT7EMCAT expression occurred when the OST7-1 cells were infected with RNA viruses such as reovirus, influenza virus, or vesicular stomatitis virus or with a nuclear DNA virus such as herpes simplex virus type 1 (data not shown).

Lieber *et al.* (7) have taken the approach of establishing cell lines that express a modified T7 RNA polymerase that is targeted to the nucleus. This system was shown to transcribe the CAT gene under control of a T7 promoter. Since CAT activity was detected, it will be of interest to determine whether the T7 transcripts are modified by the nuclear capping enzyme of the host cell. If this is inefficient, employment of the EMCV UTR as described here may be a useful improvement of the nuclear T7 expression system.

Although the previously described hybrid vaccinia virus/bacteriophage T7/EMCV expression system provided a useful method of producing proteins in cell culture, the requirement for two viruses—one containing the bacteriophage T7 RNA polymerase gene and the other containing the target gene under T7 promoter control—was cumbersome, particularly for large-scale work. Therefore, by infecting a cell line that supplies cytoplasmic T7 RNA polymerase, only one recombinant vaccinia virus is required, providing a significant simplification of the expression system for mass production of proteins. Evidently, the T7 RNA polymerase is stable in the OST7-1 cytoplasmic environment, as the anticipated shut-off of the host protein synthesis by vaccinia virus did not abolish its activity. The system described here is not a continuous one, since the cells are eventually killed by vaccinia virus. The generation of stable mammalian cell lines that continuously express high levels of target genes, however, is much more time-consuming than the construction of recombinant vaccinia viruses. Moreover, potential problems associated with toxicity and negative selection are avoided since the desired gene product is not expressed until the T7 cell line is infected by the recombinant vaccinia virus. Thus, we anticipate that cell lines which express T7 RNA polymerase in the cytoplasm will find a variety of uses.

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