Regulated Expression of Nuclear Genes by T3 RNA Polymerase and *lac* Repressor, Using Recombinant Vaccinia Virus Vectors

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Recombinant vaccinia viruses that express the bacteriophage T3 RNA polymerase (VV-T3pol) or the *Escherichia coli lac* repressor (VV-lacI) under control of the early-late vaccinia promoter $P_{7.5}$ were constructed. To determine whether phage polymerase and *lac* repressor can function in the nucleus of mammalian cells, the bacterial chloramphenicol acetyltransferase (CAT) gene was cloned downstream of a T3 promoter (P_{T3} -CAT) or downstream of a T3 promoter-*lac* operator fusion element ($P_{T3}O_{lac}$ -CAT), and these reporter gene cassettes were introduced stably into NIH 3T3 or *Ltk*⁻ cells. Infection of 3T3/ P_{T3} -CAT or *Ltk*⁻/ P_{T3} -CAT cells by VV-T3pol led to rapid expression of CAT (>20 ng of CAT protein per 10⁶ cells). The presence of hydroxyurea (which blocks virus DNA replication) did not prevent CAT production. When 3T3/ $P_{T3}O_{lac}$ -CAT cells were infected with both VV-T3pol and VV-lacI (multiplicities of infection of 2.5 and 10, respectively), >30-fold repression of CAT gene activity by *lac* repressor was observed. This could be reversed to unrepressed levels by the presence of 10 mM *o*-nitrophenyl- β -D-galactoside (IPTG) in the medium. Regulated expression of the target gene was observed with cell lines that had been maintained for over 1 year (>50 passages in culture), and Southern blot analysis revealed the presence of the CAT gene only in the nuclear fraction in these cells, demonstrating the stability of the target gene. These results indicate that vaccinia virus-encoded proteins can function in the mammalian nucleus and provide the basis for a genetic system in which essential vaccinia virus genes, placed in the chromosome of a cell, can be used to complement defective virus particles. This approach may prove useful for other virus systems.

Recombinant vaccinia virus particles that carry the T7 RNA polymerase gene have proven useful for overexpressing proteins in a variety of mammalian cell lines, demonstrating that the T7 RNA polymerase can function in the cytoplasm of these cells and that the vaccinia virus capping and methylating functions, which act in *trans*, can activate the T7 transcripts to a translatable form (8, 10, 11). It has also been shown that the T7 RNA polymerase can be transiently expressed in mammalian cells and that it can be targeted to the nucleus by using a nuclear localization signal (6); however, no demonstration has been made that the T7 RNA polymerase can function in the nucleus of mammalian cells. In other work, we have reported the establishment of stable mammalian cell lines that express the T3 RNA polymerase (T3pol) in both the cytoplasm and the nucleus, and we have demonstrated that T3pol can transcribe DNA molecules that are transiently introduced into these cells (5). Furthermore, these T3 transcripts may be translated independently of vaccinia virus infection by the use of a non-translated leader sequence from encephalomyocarditis virus (22). Transcription from T3 and T7 promoters by the respective RNA polymerases may be regulated in both procaryotic and eucaryotic cells through the use of bacteriophage promoter-*lac* operator fusions in the presence of *lac* repressor (5, 9). Thus, all of the elements for a regulated nuclear transcription system utilizing the procaryotic phage RNA polymerase and *lac* repressor molecules have been anticipated.

In this work, we demonstrate that nuclear genes can be transcribed by T3pol and that the *lac* repressor can be used to regulate the transcription of the nuclear target genes. This system will be useful for expressing target genes in stable cell lines. In addition, these studies demonstrate that vaccinia virus can be used to produce proteins that are able to function in the mammalian nucleus. Among the applications of these results is the development of a genetic system in which essential vaccinia virus proteins can be placed in the cell nucleus and expressed by T3pol during virus infection, thus allowing complementation of defective vaccinia virus particles. This approach may prove useful in other virus systems. Finally, the system described here has allowed us to test the function of T3pol and *lac* repressor in the mammalian nucleus, with the intention of establishing a stable cell line that expresses both of these proteins and gives rise to functional mRNA in the absence of vaccinia virus functions.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 and *Ltk*⁻ cells (20) were grown in Dulbecco modified Eagle medium containing 10% calf serum and newborn calf serum, respectively. Cells were cultivated at 37°C with 5% CO₂. Vaccinia virus (strain WR) was grown in African green monkey kidney cells (BSC-40) as previously described (4).

Recombinant vaccinia viruses. The vaccinia virus insertion vector pSC11 (2) was linearized with the restriction enzyme *Sma*I and ligated with a 1.8-kilobase (kb) blunt-end DNA fragment that contains the coding sequence for the bacteriophage T3pol, with a 1.85-kb blunt-end DNA fragment that contains the T3pol gene having a simian virus 40 (SV40) nuclear targeting signal, or with a 1.2-kb blunt-end DNA

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fragment that contains the coding sequence of the *Escherichia coli lac* repressor (see below). All of these genes were thus inserted downstream of the vaccinia virus $P_{7.5}$ promoter (2). After ligation and transformation into *E. coli* JM83, positive clones were identified with ^{32}P nick-translated specific probes. The recombinant plasmids were then introduced into the *tk* region of vaccinia virus by previously described methods (2, 19). Briefly, BSC-40 cells grown in 60-mm dishes were infected with vaccinia virus (0.01 PFU per cell); 4 h later, cells were transfected with 10 μg of plasmid DNA, using the calcium phosphate precipitation method (19). Cell cultures were harvested at 48 h postinfection (p.i.), and recombinant viruses were isolated by plaque assay after addition of 5-bromo-4-chloro-3-indole- β -D-galactosidase to the agar overlay (2). Blue plaques were picked and plaque purified three times, and stocks of recombinant viruses were prepared in BSC-40 cells (21).

Plasmid construction. Plasmid pTG100 contains the T3pol gene, modified to remove all upstream ATG codons and to place the remaining ATG codon into the optimal sequence context for eucaryotic translational initiation (T. J. Giordano, Ph.D. thesis, Rutgers University, New Brunswick, N.J., 1989). The T3pol gene was excised from pTG100 as a *Bgl*II-to-*Sma*I fragment, the ends of the fragment were polished with the Klenow fragment of DNA polymerase, and the fragment was ligated into the *Sma*I site of the vaccinia virus insertion vector pSC11 to give pSCT3pol. A modified version of the T3pol gene (T3pol_{nts}) that contains the nuclear targeting signal derived from the SV40 large-T protein was excised from pYZ28 (22) and inserted into pSC11 in the same fashion. The *lacI* repressor gene was excised from pRSV-I (14) as a *Hind*III-*Sal*I fragment, polished with Klenow fragment, and cloned into the *Sma*I site of the vaccinia virus insertion vector.

Plasmid pYZ31 carries the chloramphenicol acetyltransferase (CAT) gene from pBS-ECAT (15), excised as a *Bst*XI-to-*Pst*I fragment and cloned downstream of the T3 promoter in pBS-KS⁺ (Stratagene) (Y. Zhou, Ph.D. thesis, State University of New York, Brooklyn, N.Y., 1990). pYZ35 contains the same CAT sequences cloned into the *Bam*HI-to-*Hind*III sites of pTG127 (12), under control of the T3 promoter-*lac* operator fusion element $P_{T3/0+14}$.

Construction of cell lines. To establish stable cell lines that contain the CAT gene under control of a T3 promoter, NIH 3T3 and *Ltk*⁻ cells were transfected with the recombinant plasmid pYZ31 or pYZ35 (10 μg per dish) by the calcium phosphate DNA precipitation technique, using pSV2neo (50 ng per dish) as a selectable marker (3). After 48 h, the cells were trypsinized and split 1:10 into 100-mm plates with medium containing a 400- $\mu\text{g}/\text{ml}$ concentration of the antibiotic G418 (GIBCO Laboratories). Three weeks later, neomycin-resistant (Neo^r) colonies were subcloned and maintained in selective medium. Neo^r clones were also isolated from *Ltk*⁻ cells by using transfection protocols similar to those used for NIH 3T3 cells. For isolation of *tk*⁺ clones, cotransfection was carried out with 200 ng of pTK2, followed by selection in hypoxanthine-aminopterin-thymidine medium (19). Individual *Ltk*⁻ clones were expanded and maintained in selective medium as described above.

CAT assays. Confluent monolayers (60-mm dishes) of cells were infected with the vaccinia virus-T3pol (VV-T3pol) construct at 2.5 PFU per cell unless otherwise indicated. Cells were harvested at different times after infection, washed with phosphate-buffered saline (PBS), and suspended in 200 μl of 0.25 M Tris hydrochloride (pH 7.5). Cell extracts were prepared by three cycles of freeze-thawing

(dry ice-methanol, 37°C), and CAT assays were carried out with about 20 μg of protein of cell extract. The percent conversion of [^{14}C]chloramphenicol to acetylated product was determined by scintillation counting of the radioactive spots from thin-layer chromatograms (localized by autoradiography) (13).

To quantitate the amount of CAT protein produced by stable cell lines or in cells in which the CAT gene was transiently introduced, known amounts of purified CAT protein (a gift from Newell Bascomb, Enimont America, Princeton, N.J.) were run in 15% sodium dodecyl sulfate-polyacrylamide gels in parallel with cell extracts. The gels were stained with Coomassie blue or subjected to Western blot (immunoblot) analysis using rabbit anti-CAT serum (a gift from Newell Bascomb). Antibody reactivity was developed by immunoperoxidase staining, and the amount of CAT protein in cell extracts was estimated by comparison with the extent of antibody reactivity seen with the purified protein samples.

Immunofluorescence microscopy. Cells grown on coverslips were infected with vaccinia virus recombinants (5 PFU per cell); at 6 h p.i., cells were washed with PBS and fixed in 2% formaldehyde-0.2% Triton X-100 in PBS for 10 min at 4°C. The cells were made permeable by treatment for 5 min with cold acetone and then incubated for 30 min with blocking solution (20% fetal bovine serum-0.5% Tween 20 in PBS). Antibodies (rabbit anti-T3pol serum or rabbit anti-*lacI* serum) were added at 1:250 dilution in blocking solution, followed by incubation for 2 h at room temperature. Specimens were washed with PBS and then treated with secondary antibody (goat anti-rabbit immunoglobulin G conjugated with rhodamine; 1:500 dilution) in blocking solution. Coverslips were washed with PBS, mounted in glycerol-PBS (1:1, vol/vol), and examined by fluorescence microscopy.

RESULTS

Construction of recombinant vaccinia virus particles that express T3pol. The T3pol gene, modified to remove all upstream start codons and to place the remaining start codon in the optimal sequence context for translational initiation in eucaryotic cells (22; Giordano, Ph.D. thesis, 1989), was cloned into the *Sma*I site of the vaccinia virus insertional vector pSC11 (2) under control of the vaccinia virus early-late promoter $P_{7.5}$ to give pSCT3pol. The modified T3pol gene was then introduced by homologous recombination into the *tk* region of the vaccinia virus genome (2, 21) to give VV-T3pol. A recombinant vaccinia virus that expresses T3pol with a nuclear targeting signal (VV-T3pol_{nts}) was constructed in the same way. The recombinant vaccinia virus particle VV-*lacI* contains the entire *lac* repressor gene (*lacI*) under control of the $P_{7.5}$ promoter.

Infection of BSC-40 cells with VV-T3pol and VV-T3pol_{nts} led to production of T3pol similar in size to the purified enzyme (Fig. 1). The level of RNA polymerase made in VV-T3pol-infected cells by 16 h p.i. was 0.06 to 1.0% of total cell protein, corresponding to about 100 ng of T3pol per 10⁶ cells.

The cellular location of T3pol in the infected cells was determined by immunofluorescence. In VV-T3pol-infected cells, diffuse staining of the cell was observed, indicating that the polymerase was distributed in both cytoplasmic and nuclear compartments (Fig. 2A and B). In the case of VV-T3pol_{nts} infection, discrete staining of the nuclear region was observed (Fig. 2C and D). This result is consistent with previous findings demonstrating that the SV40 nuclear tar-

FIG. 1. Expression of bacteriophage T3pol by vaccinia virus recombinants. BSC-40 cells were infected (2 PFU per cell) with VV-T3pol or VV/T3pol_{nts}. Extracts were prepared at 24 h p.i. and tested for expression of T3pol by Western blot analysis using anti-T3pol serum. (A) Size comparison of T3pol produced in cells infected with vaccinia recombinants versus purified T3pol enzyme. (B) Antibody reactivity with different amounts (in nanograms) of purified T3pol in comparison with the antibody reactivity observed in extracts of VV-T3pol-infected cells (equivalent to 5×10^4 cells).

geting signal can bring about nuclear targeting of the T7 and T3 RNA polymerases (6, 22).

In the case of VV-lacI infection (as in the case of VV-T3pol infection), both nuclear and cytoplasmic localizations of *lac* repressor were observed (Fig. 2E and F). This result is in contrast to previous reports that in uninfected cells the *lac* repressor is largely found in the cytoplasm (14). We do not know the explanation for this discrepancy; it may reflect a consequence of virus infection or a variation among cell lines.

Construction of cell lines expressing CAT under T3 promoter control. Plasmid pYZ31 contains the bacterial CAT gene cloned downstream of the T3 promoter in the Bluescript vector pBS-KS⁺ (Stratagene). The transcription unit in this vector consists of a T3 consensus promoter, the CAT gene, the splice junction and polyadenylation sequence from the SV40 t region, and tandem termination signals for the T7 RNA polymerase (Fig. 3, top). This transcription unit is referred to as P_{T3} -CAT. Plasmid pYZ35 is similar to pYZ31 but contains a synthetic *lac* operator sequence (O_{lac}) centered 14 base pairs downstream of the start site of a T3 promoter ($P_{T3/0+14}$; 12). This transcription unit is referred to as $P_{T3}O_{lac}$ -CAT.

3T3/ P_{T3} -CAT and 3T3/ $P_{T3}O_{lac}$ -CAT cell lines were generated by introducing pYZ31 and pYZ35, respectively, into cultured NIH 3T3 cells (along with plasmid pSV2neo), using standard $CaPO_4$ -mediated DNA transfection methods. Clones resistant to the antibiotic G418 (Neo^r) were selected

FIG. 2. Subcellular localization of T3pol and *lacI* in cells infected with vaccinia virus recombinants. NIH 3T3 cells grown on cover slips were infected with different vaccinia virus recombinants; at 6 h p.i., cells were fixed, permeabilized, and probed with anti-T3pol serum (A to D) or anti-*lacI* serum (E and F). Phase-contrast (A, C, and E) and immunofluorescence (B, D, and F) photographs were taken. (A and B) Infected with VV-T3pol; (C and D) infected with VV-T3pol_{nts}; (E and F) infected with VV-lacI.

FIG. 3. (Top) Structure of the CAT reporter gene. The transcription cassette in pYZ31 consists of a T₃ promoter (P_{T3}), the CAT gene (□), the splice donor and acceptor sequences (—) and polyadenylation signal (A_n) from the SV40 t region, and tandem T7 termination signals (T₇). The transcription cassette in pYZ35 is similar but contains a synthetic lac operator sequence (O_{lac}; ■) centered 14 base pairs downstream of the start site for RNA synthesis (→). The figure is not drawn to scale. (A to C). Response of the CAT gene in clones of NIH 3T3 and Ltk⁻ cells. Individual cell lines containing the transcription unit P_{T3}-CAT were infected with 2.5 PFU of VV-T3pol per cell. At 24 h p.i., cell extracts (≈10⁶ cells) were prepared and tested for CAT activity. (A) CAT activity in 3T3/P_{T3}-CAT clones selected for Neo^r; (B) CAT activity in Ltk⁻/P_{T3}-CAT clones selected for Neo^r (clones 1 to 6) or for the tk⁺ phenotype (clones 7 to 13); (C) control experiment showing that CAT activity is dependent on the presence of P_{T3}-CAT in the chromosome and on expression of T3pol by VV-T3pol: lane 1, 3T3/P_{T3}-CAT cells infected with VV-T3pol; lane 2, 3T3/P_{T3}-CAT cells infected with wild-type vaccinia virus; lane 3, 3T3 cells infected with VV-T3pol.

and expanded into cell lines (3). Ltk⁻/P_{T3}-CAT cell lines were constructed in a similar manner except that selection was performed either by cotransfection with pSV2neo and selection for Neo^r colonies or by cotransfection with the herpesvirus tk⁺ gene (in pTK2) and selection in hypoxanthine-aminopterin-thymidine medium (19).

Individual expanded cell lines were tested for the presence of a responsive T3 transcriptional unit by infection with

FIG. 4. Southern blot analyses of nuclear and cytoplasmic fractions of the stable cell line 3T3/P_{T3}O_{lac}-CAT. Cells maintained continuously in culture for over 1 year were fractionated into nuclear and cytoplasmic components by 1% Nonidet P-40 treatment, and the nucleic acids were extracted with phenol-chloroform as previously described (19). The DNA was digested with the restriction enzymes *Dra*I and *Eco*RI, and 10 μg of nuclear DNA and 50 μg of cytoplasmic nucleic acids were analyzed by Southern blot analysis. A 0.6-kb DNA fragment (*Dra*I-*Sca*I) from the CAT gene in pYZ31 was ³²P-labeled and used as a probe. Lanes: P, plasmid pYZ31; C, cytoplasmic fraction; N, nuclear fraction.

VV-T3pol at 2.5 PFU per cell (Fig. 3A). Sixteen of eighteen 3T3 Neo^r clones tested exhibited significant CAT activity after infection with VV-T3pol. There appeared to be substantial variation in CAT activity from clone to clone. This variation may reflect the position of the target gene in the chromosome or the number of copies of the target gene in the cell.

The Ltk⁻ cell line (Fig. 3B) gave 10 of 13 responders, (5 of 7 when selected for the Neo^r phenotype and 5 of 6 when selected for the tk⁺ phenotype). As with the 3T3 cell lines, there was variation in CAT activity from clone to clone. The most responsive Ltk⁻/P_{T3}-CAT cell line was not as responsive as the best 3T3/P_{T3}-CAT line (compare Fig. 3A with Fig. 3B). In both cases, synthesis of CAT in the cells was absolutely dependent on the presence of P_{T3}-CAT in the cell chromosome and the presence of the T3pol gene in the vaccinia virus vector (Fig. 3C).

The nuclear target genes appear to be quite stable in these cell lines. The 3T3/P_{T3}O_{lac}-CAT cell line has now been carried under selection with G418 (400 μg/ml) for over 1 year (>50 passages) with no loss of responsiveness when infected with VV-T3pol. Southern blot analysis of nuclear and cytoplasmic fractions demonstrated the presence of CAT DNA sequences only in the nuclear fraction (Fig. 4).

Kinetics of CAT synthesis in the VV-T3pol system. The optimal conditions for expression of the P_{T3}-CAT construct in 3T3/P_{T3}-CAT after infection by VV-T3pol was determined by examining three parameters: time after virus infection, multiplicity of virus infection (MOI), and the role of late virus transcription.

CAT activity in clones of 3T3/P_{T3}-CAT cells first became detectable 4 h after virus infection and increased with time to 24 h (Fig. 5). The response in clones of Ltk⁻/P_{T3}-CAT cells began with a slight delay, at 6 h p.i., and increased with time (Fig. 5). The optimal response was observed at MOIs of between 1 and 5 PFU per cell for 3T3/P_{T3}-CAT and 5 to 10 PFU per cell for Ltk⁻/P_{T3}-CAT (Fig. 6). The presence of hydroxyurea (which blocks viral DNA replication but not early virus gene expression) in the medium did not prevent production of CAT, indicating that the phage RNA polymerase is expressed as an early virus function by the early-late promoter P_{7.5} and that late virus functions are not required

FIG. 5. Time course of CAT activity in 3T3/ P_{T3} -CAT and Ltk^{-}/P_{T3} -CAT clones after virus infection with VV-T3pol. Cells were infected (2.5 PFU per cell) with VV-T3pol, and extracts prepared at various times after virus infection were tested for CAT activity (10^6 cells). Two representative clones from each cell line are shown.

for expression of the nuclear target gene (Fig. 6). The presence of the nuclear targeting signal in T3pol had little effect on CAT expression, either from a transiently introduced target gene or from the stable nuclear target gene in 3T3/ $P_{T3}O_{lac}$ -CAT cells (Fig. 7A).

The levels of target gene expression from a stable cell line maintained for over 50 passages was determined by quantitative Western blot analysis with anti-CAT serum (Fig. 7B). The expression of CAT protein in 3T3/ $P_{T3}O_{lac}$ -CAT cells at 6 h after infection with VV-T3pol was similar in the presence or absence of hydroxyurea. The amount of CAT produced increased with time after infection to levels of about 20 ng per 10^6 cells.

Regulation of nuclear expression by *lac* repressor. It has

FIG. 6. CAT activity in response to different virus MOIs. 3T3/ P_{T3} -CAT (clone 15) and Ltk^{-}/P_{T3} -CAT (clone 1) were infected with VV-T3pol at different virus MOIs. Where indicated, cells were treated with hydroxyurea (HU; 5 mM), after 1 h of virus adsorption, to inhibit viral DNA replication but not early viral gene expression (7). Cell extracts (equivalent to 10^6 cells) were prepared at 6 h after virus infection and tested for CAT activity.

FIG. 7. Levels of target gene expression. (A) CAT activity measured after transient uptake of reporter genes or in a stable cell line after infection with vaccinia virus recombinants. For transient expression, 3T3 cells were infected (2.5 PFU per cell) with the recombinant virus VV-T3pol or VV-T3pol_{INTS} or with the wild-type vaccinia virus (VV). At 3 h p.i., plasmid pYZ31 (10 μ g per plate) was added; cell extracts were collected at 24 h p.i. and tested for CAT activity. For expression in stable cell lines, 3T3/ $P_{T3}O_{lac}$ -CAT cells were infected and processed as for 3T3 cells. (B) Absolute amounts of CAT in stable cell lines. 3T3/ $P_{T3}O_{lac}$ -CAT cells were infected with VV-T3pol at different MOIs (indicated as PFU per cell), and extracts were prepared for Western blot analysis at 6 h p.i. The reactivities of the anti-CAT serum with known amounts of purified CAT protein are indicated. Note that anti-CAT serum cross-reacted with a cellular protein of about 22 kilodaltons.

FIG. 8. Regulation of CAT synthesis in 3T3/ $P_{T3}O_{lac}$ -CAT by VV-lacI-encoded repressor. Cells (at 45 passages) were infected with VV-T3pol (2.5 PFU per cell) or with a mixture of VV-T3pol (2.5 PFU per cell) and VV-lacI (10 PFU per cell). Where indicated, IPTG (10 mM) was added immediately after virus adsorption. Cell extracts (equivalent to 10^6 cells) were prepared at various times after virus infection and tested for CAT activity (A). The radioactive spots shown in the autoradiogram were excised from the chromatogram and counted in scintillation fluid (B). The percent conversion of [^{14}C]chloramphenicol to acetylated product is presented.

been shown by using $P_{T3}O_{lac}$ fusions that T3pol activity may be regulated by *lac* repressor both in *E. coli* and in mammalian cells, in which these proteins are active in the cytoplasm (5, 12). To determine whether these proteins allow regulated transcription of nuclear target genes, the 3T3/ $P_{T3}O_{lac}$ -CAT cell line was infected with VV-T3pol in combination with VV-lacI, and the response of the CAT gene to the inducer isopropyl- β -D-thiogalactoside (IPTG) was determined. At a ratio of VV-lacI to VV-T3pol of 4, tight repression of the CAT target gene was observed, and this repression could be reversed to control values by 10 mM IPTG (Fig. 8A). (We have observed that NIH 3T3 cell lines easily tolerate IPTG concentrations as high as 30 mM.) The time course of CAT synthesis in derepressed cells (in the presence of IPTG) appeared to be similar to that observed after infection by VV-T3pol alone (Fig. 8B). Under optimal conditions (VV-

T3pol at 2.5 PFU per cell and VV-lacI at 10 PFU per cell, 16 h p.i.) repression, measured as activity in the presence of IPTG versus activity in the absence of IPTG, was about 30-fold. Complete repression (>95%) was observed between 6 to 8 h p.i. Repression of CAT expression was also observed after mixed infection with VV-T3pol_{nts} and VV-lacI (data not shown).

DISCUSSION

In this work, we have demonstrated that target genes in the cell chromosome may be transcribed by T3pol that is encoded by recombinant vaccinia virus vectors. The activity of T3pol can be regulated by the use of a $P_{T3}O_{lac}$ control element and *lac* repressor (also delivered by a recombinant vaccinia virus vector) over a 30-fold range. These findings suggest that further development of the T3pol-*lac* repressor expression system in stable cell lines may have widespread utility.

Vaccinia virus is regarded as a cytoplasmic virus in which all of the virus functions are believed to occur in this cell compartment (17). The demonstration in this report that foreign genes encoded by recombinant vaccinia virus vectors are able to function in the nucleus opens the question as to whether vaccinia virus functions might have a nuclear role during infection.

Although it is known that RNA polymerase II-mediated transcription of nuclear genes is inhibited after vaccinia virus infection (17), we have demonstrated that the nuclear DNA template may still be utilized by T3pol. Thus, this system has considerable promise for the expression of nuclear genes by vaccinia virus vectors. We anticipate that this system will have practical applications, for example in the construction of cell lines that can complement vaccinia virus particles that are defective in essential genes. This will greatly aid in the development of vaccinia virus genetic systems.

Recombinant vaccinia vectors have proven extremely attractive as the basis of high-level mammalian expression systems. However, the requirement to generate recombinant vaccinia particles that carry the target genes requires extra steps that may render the system less attractive to some laboratories. The introduction of target genes into the host chromosome by standard transfection methods may be technically more familiar to many groups, and this feature of the system described here may facilitate the use of vaccinia virus-based expression systems in these laboratories.

We do not know whether the variation in responsiveness of the P_{T3} -CAT genes in the various cell lines reflects the copy number of the target genes or is a position effect. It is known that the phage polymerases will not transcribe chromatinized DNA (16), and efficient expression from the target genes may require that the genes be located in an optimal context. We are currently exploring the effect of viral enhancer sequences that are placed immediately upstream of the phage promoter to see what effect this has on transcriptional efficiency.

The nuclear target genes appear to be quite stable. One cell line, 3T3/ $P_{T3}O_{lac}$ -CAT, has been maintained under selection for over a year (>50 passages) with no change in levels of CAT activity with passage number. This cell line produces about 20 ng of CAT protein per 10^6 cells after infection with VV-T3pol. This level of expression is only fivefold lower than the level of T3pol produced in cells infected with VV-T3pol.

In some cell lines, such as HeLa and MDCK, vaccinia virus infection leads to little (MDCK) or delayed (HeLa)

inhibition of host protein synthesis (unpublished results). In these cells, activation of nuclear target genes by the VV-T3pol vector described here may allow studies of the effects of these genes on normal cellular function. For example, the effects of overexpression of nuclear transcription factors on gene expression might be studied in such a system. In addition, the opportunity to synthesize nuclear RNA independently of any host RNA polymerase should prove useful in studies of RNA splicing, polyadenylation, capping, or other posttranscriptional processes. Because vaccinia virus can be maintained in persistent infections (18), it might also be possible to develop stable cell cultures that produce high levels of various gene products. The system that we describe here could also be exploited to study the role of genes from other animal viruses on processes such as transcription, translation, and cell fusion.

In cells that were infected with VV-lacI, CAT expression from $P_{T3}O_{lac}$ -CAT was ≈ 30 -fold repressed but was fully inducible by the addition of IPTG. This result is in contrast to previous reports in which expression from a vaccinia virus promoter-lac operator fusion element was 200-fold repressed but inducible to only 20% of the control level (9). It is likely that the degree of repression and derepression in these systems reflects the stoichiometries of the repressor, the operator, and the polymerases involved and that optimization of this regulatory system will require manipulation of the relative abundance of each of these elements.

After submission of this manuscript, Benton et al. (1) reported that a nuclear transport signal was required to compartmentalize T7 RNA polymerase to the nucleus of the yeast *Saccharomyces cerevisiae* and to obtain transcription of nuclear target genes. As we have observed here, T3pol lacking a nuclear transport signal can transcribe a nuclear target gene in mammalian cells even under conditions in which the amount of enzyme is restricted to that produced early in virus infection. Differences in target gene expression by T7 RNA polymerase in yeast cells and by T3pol in animal cells may simply reflect the infectious process in the latter system, i.e., changes in the permeability of the nuclear membrane, changes in nucleosome structure, etc. Clearly, the two systems behave differently, as shown by the inability of target genes to be translated in yeast cells whereas target gene proteins are faithfully expressed in the VV-T3pol system.

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LITERATURE CITED

- Benton, B. M., W. K. Ena, J. Dunn, W. F. Studier, R. Sternglanz, and P. Fisher. 1990. Signal-mediated import of bacteriophage T7 RNA polymerase into the *Saccharomyces cerevisiae* nucleus and specific transcription of target genes. *Mol. Cell. Biol.* 10:353-360.
- Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vectors: coexpression of β -galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* 5:3403-3409.
- Colbere-Garapin, F., F. Horodniceanu, P. Kourilsky, and A. Garapin. 1981. A new dominant hybrid selective marker for higher eukaryotic cells. *J. Mol. Biol.* 150:1-14.
- Dallo, S., and M. Esteban. 1987. Isolation and characterization of attenuated mutants of vaccinia virus. *Virology* 159:408-422.
- Deuschle, U., R. Pepperkok, F. Wang, T. J. Giordano, W. T. McAllister, W. Ansorge, and H. Bujard. 1989. Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and lac repressor. *Proc. Natl. Acad. Sci. USA* 86:5400-5404.
- Dunn, J. J., B. Kripl, K. E. Bernstein, H. Westphal, and F. W. Studier. 1988. Targeting bacteriophage T7 RNA polymerase to the mammalian cell nucleus. *Gene* 68:259-266.
- Esteban, M., and J. A. Holowczak. 1978. Replication of vaccinia DNA in mouse L cells. IV. Protein synthesis and viral DNA replication. *Virology* 86:376-390.
- Fuerst, T. R., P. L. Earl, and B. Moss. 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. *Mol. Cell. Biol.* 7:2538-2544.
- Fuerst, T. R., M. P. Fernandez, and B. Moss. 1989. Transfer of the inducible lac repressor/operator system from *Escherichia coli* to a vaccinia virus expression vector. *Proc. Natl. Acad. Sci. USA* 86:2549-2553.
- Fuerst, T. R., and B. Moss. 1989. Structure and stability of mRNA synthesized by vaccinia virus-encoded bacteriophage T7 RNA polymerase in mammalian cells: importance of the 5' untranslated leader. *J. Mol. Biol.* 206:333-348.
- Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83:8122-8126.
- Giordano, T. J., U. Deuschle, H. Bujard, and W. T. McAllister. 1989. Regulation of coliphage T3 and T7 RNA polymerases by the lac repressor-operator system. *Gene* 84:209-219.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
- Hu, M. C., and N. Davidson. 1987. The inducible lac operator-repressor system is functional in mammalian cells. *Cell* 48:555-566.
- Jang, S. K., H. G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer. 1988. A segment of the 5' non-translated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.* 62:2636-2643.
- Lorch, Y., J. W. LaPointe, and R. D. Kornberg. 1987. Nucleosomes inhibit the initiation of transcription but allow chain elongation with displacement of histones. *Cell* 49:203-210.
- Moss, B. 1985. Replication of poxviruses, p. 685-703. In B. Fields (ed.), *Virology*. Raven Press, New York.
- Paez, E., S. Dallo, and M. Esteban. 1985. In vivo generation of a dominant 8Md deletion at the left terminus of vaccinia DNA. *Proc. Natl. Acad. Sci. USA* 82:3365-3369.
- Pellicer, A., and M. Esteban. 1982. Gene-transfer, stability and biochemical properties of animal cells transformed with vaccinia DNA. *Virology* 122:363-380.
- Perucho, M., and M. Esteban. 1985. Inhibitory effect of interferon on the genetic and oncogenic transformation by viral and cellular genes. *J. Virol.* 54:229-232.
- Rodriguez, J. F., D. Rodriguez, J. R. Rodriguez, E. McGowan, and M. Esteban. 1988. Expression of the firefly luciferase gene in vaccinia virus: a highly sensitive gene marker to follow virus dissemination in tissues of infected animals. *Proc. Natl. Acad. Sci. USA* 85:1667-1671.