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Biochemistry

A poxvirus-derived vector that directs high levels of expression of cloned genes in mammalian cells

(vaccinia virus/cowpox virus/A-type inclusion)

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ABSTRACT **High levels of expression of cloned genes have been obtained in mammalian cells by using poxvirus-derived insertion/expression vectors. These vectors employ the cis-acting element (CAE I) that directs the transcription of one of the most strongly expressed genes of cowpox virus. This gene (the 160K gene) encodes the 160-kDa protein that is the major component of the A-type cytoplasmic inclusions. Its counterpart in vaccinia virus (VV) is the 94K gene contained in the *Hin*dIII A fragment of the viral DNA. Two insertion vectors have been constructed; each is designed to allow cloned genes to be placed immediately downstream of a modified version of CAE I within a poxvirus genome. One vector, p1200, enables the CAE I–cloned-gene constructs to be inserted into the thymidine kinase gene of VV. This vector was used to create a VV recombinant that directed expression of the chloramphenicol acetyltransferase (CAT) gene. The other vector, p2101, enables the CAE I–cloned-gene constructs to be inserted into the VV 94K gene. The prototype of this vector was used to create a VV recombinant that directed expression of a hybrid CAT–*lacZ* gene. Infection of cultured human cells with these recombinants led to high levels of synthesis of either the CAT gene product or the CAT–*lacZ* gene product. Each of these proteins was produced in quantities that were easily detected by Coomassie blue staining of total cell proteins resolved by polyacrylamide gel electrophoresis. We estimate that these vectors are capable of directing the synthesis of milligram amounts of gene product per 10^9 mammalian cells.**

The poxvirus-derived vectors, whose development was pioneered by Panicali and Paoletti (1) and Mackett *et al.* (2), provide a simple and practical means of gaining the expression of cloned genes in cells of vertebrate animals. In addition, these vectors have considerable potential for development as live virus vaccines capable of serving a variety of medical and veterinary uses (reviewed in ref. 3).

We have constructed insertion/expression vectors that enable poxvirus-derived vectors to direct levels of gene expression that are higher than those currently obtained with vectors employing poxviral promoter elements. Previously, we showed that one of the most strongly expressed genes of cowpox virus is that encoding the major protein component of the A-type cytoplasmic inclusions (4). The major protein component of the A-type inclusions produced in cells infected with the Brighton red strain of cowpox virus (CPV) is a 160-kDa protein. At late times after infection, this protein may constitute up to 4% of the total protein in the cell (4). Therefore, this viral protein is far more abundant in the infected cell than most other viral proteins, including the structural proteins. The 160-kDa protein is the product of a late gene (designated the 160K gene) that is located in the *Kpn* I G fragment of the DNA of the Brighton red strain of CPV (5, 6).

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The CPV 160K gene is transcribed immediately after the onset of viral DNA replication. Its mRNAs are uniform in length, and most of them contain 5–21 adenylate residues upstream of the initiation codon (5). Remarkably, this 5′-poly(A) leader sequence is not complementary to the template strand of the viral DNA. Instead, the sequences of the mRNAs and the viral DNA diverge at the site of the three adenylate residues in the sequence 5′-TAAATG-3′ containing the gene's initiation codon. Late transcripts of several poxvirus genes have also been shown to contain similar 5′-terminal poly(A) sequences (7–10). Therefore this feature of late viral mRNAs may be important for optimal expression of late genes.

The mechanisms involved in the regulation of viral gene expression are not fully understood. In part, this regulation is effected by the control of the transcription of viral genes, and this involves interactions between virus-specific cis-acting signals, viral trans-acting factors, and viral enzymes (11–17). It is not yet clear how the late 5′- poly(A)-containing mRNAs are generated. However, cis-acting elements can be used to gain late transcription of genes cloned into poxvirus-derived vectors (2, 18). In this paper, we describe the construction of insertion/expression vectors employing the cis-acting element directing the transcription of the 160K gene. We show that recombinant vaccinia viruses (VVs) containing these constructs direct high levels of expression of cloned genes in cultured mammalian cells.

MATERIALS AND METHODS

Virus Strains and Cells. VV (Western Reserve strain) was cultured as described (5). The phage vector M13MK19 and the *Escherichia coli* strains TG1, HB2154, and HB2151 (19) were kindly provided by G. Winter (MRC Laboratory of Molecular Biology, Cambridge, U.K.).

Construction of the p1200 Insertion/Expression Vector. An insertion vector (p1133) that would mediate the insertion of foreign DNA into the VV thymidine kinase (tk) gene (2) was constructed by inserting the *Hin*dIII J fragment of VV DNA into the *Hin*dIII site of plasmid vector pUC19. The regulatory element (designated cis-acting element I or CAE I) that directed the transcription of the CPV 160K gene was obtained from a 6.4-kilobase (kb) *Cla* I fragment (Fig. 1*A*) of the *Kpn* I G fragment of the DNA of the Brighton red strain of CPV. The 886-base-pair (bp) *Eco*RI–*Acc* I fragment (Fig. 1*B*) containing the initiation codon of the 160K gene was inserted into the *Eco*RI site in the DNA of pUC9 to create plasmid p2030. Then, the oligonucleotide-directed mutagenesis procedure described by Carter *et al.* (19) was used to alter the nucleotide sequence of the cloned CAE I, as shown in Fig. 1 *C* and *D*. The 135-bp *Nco* I–*Hin*dIII fragment derived from

Abbreviations: CPV, cowpox virus; VV, vaccinia virus; CAE I, cis-acting element I; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase.

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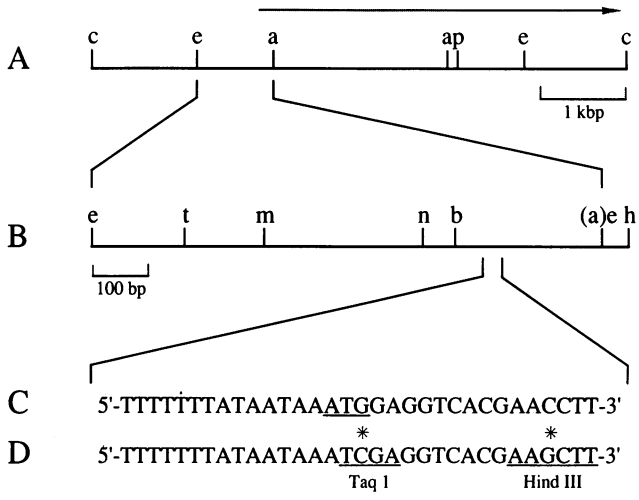

FIG. 1. Site-directed mutagenesis of the CAE I directing the transcription of the 160K gene. (*A*) Restriction map of the 6.4-kb *Cla* I fragment of CPV DNA containing the 160K gene (5). The arrow indicates both the direction of transcription of the 160K gene and the extent of the transcribed region. (*B*) Restriction map of the 886-bp *Eco*RI fragment contained in plasmid p2030. Restriction enzyme cleavage sites are abbreviated as follows: a, *Acc* I; b, *Bgl* II; c, *Cla* I; e, *Eco*RI; h, *Hin*dIII; m, *Msp* I; n, *Nco* I; p, *Pst* I; and t, *Taq* I. a(e) denotes that the *Acc* I site at this position was converted to an *Eco*RI site. The fragment (a)e–h corresponds to the polylinker region of the pUC9 vector DNA in p2030. (*C*) Nucleotide sequence of the coding strand of the viral DNA at the beginning of the coding region of the 160K gene. The initiation codon is underlined. (*D*) Nucleotide sequence of the same region after site-directed mutagenesis. Asterisks indicate the altered nucleotides.

this modified 886-bp *Eco*RI–*Acc* I fragment was excised and inserted between the *Nco* I and *Hin*dIII sites in plasmid p2030 (Fig. 1*B*) to produce plasmid p2046. A 533-bp *Taq* I fragment containing the sequence corresponding to that immediately upstream of the initiation codon of the 160K gene was inserted into the single *Cla* I site in the DNA of plasmid p1133. One of the plasmids (p1200) containing the modified CAE I sequence in the orientation shown in Fig. 2 was selected for use as an insertion/expression vector.

Insertion of the Chloramphenicol Acetyltransferase (CAT) Gene Downstream of the CAE I in the VV Genome. A plasmid derivative (p863) of pSV2-cat^S (20), containing the CAT gene, was provided by E. Linney (Duke University, Durham, NC). A 0.8-kb *Taq* I fragment containing the entire coding region of the CAT gene was inserted into the *Cla* I site of the p1200 DNA, generating a plasmid (p1245) that contained the CAT gene in the appropriate orientation relative to the CAE I. This CAE I–CAT gene hybrid was inserted into the DNA of VV according to the method of Mackett *et al.* (21). A recombinant virus (A394) containing the inserted CAE I–CAT gene was selected for further study.

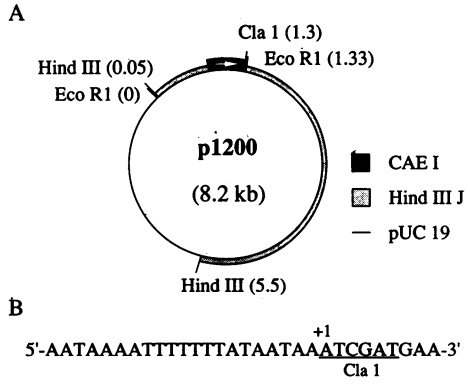

FIG. 2. (*A*) Restriction map of the p1200 insertion/expression vector. The arrow indicates the direction of transcription from CAE I. (*B*) Nucleotide sequence (coding strand) at the *Cla* I insertion site. The nucleotide designated +1 corresponds to the first nucleotide in the initiation codon of the 160K gene.

Measurement of Gene Expression. RNA analyses were done as described (5). Assays of CAT enzyme activity and PAGE analyses of proteins were done by standard methods (20, 22).

RESULTS

The Modified CAE I Directs Late Transcription. The exact nature of the cis-acting element required to promote efficient transcription of late genes has not yet been clearly defined. Analyses of putative late promoter elements of several VV genes have indicated that about 30 bp upstream of and including the conserved sequence 5′-TAAAT-3′ are required to effect late transcription (13–17). In our vector constructions, we used a 533-bp fragment of DNA taken from the region immediately upstream of the initiation codon of the 160K gene. This region was chosen because it contained both the entire upstream noncoding region and the unusual sequence consisting of 28 repeats of the triplet GAT. We reasoned that this region was likely to be large enough to contain all the sequence elements necessary to direct the transcription of the 160K gene. And, in this system, the use of a 533-bp fragment does not significantly reduce the cloning capacity of the poxvirus genome. Smith and Moss (23) have inserted 25 kb of phage λ DNA into the VV genome.

Analyses of RNA extracted from cells infected with VV recombinant A394 demonstrated that the 533-bp fragment acted in cis to direct late transcription. Therefore this fragment was designated cis-acting element I (CAE I). We have not yet identified the minimum components of CAE I that are necessary to effect wild-type levels of transcription of the 160K gene. Nuclease S1 protection experiments indicated that the major endpoints of complementarity between the CAE I–CAT gene and its mRNAs extended only 3–8 nucleotides upstream of the nucleotide (+1) corresponding to the first nucleotide of the initiation codon in the 160K gene. Whereas primer extension analyses indicated that most of these mRNAs contained 4–26 nucleotides upstream of the +1 nucleotide (data not shown). These results suggest that the mRNAs of the CAE I–CAT construct contained 5′-terminal poly(A) sequences similar to those at the 5′ ends of the 160K gene's mRNAs (5). The mRNAs of both of these genes appear to contain 4–26 adenylate residues immediately upstream of the +1 nucleotide (the third adenylate in the conserved 5′-TAAAT-3′ sequence). Therefore, the modified CAE I inserted into the tk gene of the VV recombinant appears to be functionally equivalent to the native CAE I in the CPV 160K gene.

CAE I Directs High Levels of Gene Expression. Expression of the CAE I–CAT gene in A394-infected human cells was examined in two ways. First, the CAT activity present in extracts of cells was measured at various times after infection (Fig. 3). CAT activity increased sharply after the onset of viral DNA synthesis (4 hr after infection) and reached high levels 24 hr after infection. Second, the proteins in the infected cells were analyzed by PAGE. The recombinant VV directed the production of CAT in quantities that were easily detected by staining with Coomassie brilliant blue (Fig. 4). Only two other viral proteins were detected in these gels (see Fig. 4, lanes B and C); one had an apparent molecular mass of 94 kDa, and the other had an apparent molecular mass of 32 kDa. The 94-kDa protein is antigenically related to the product of the CPV 160K gene (4). The identity of the 32-kDa protein is unknown.

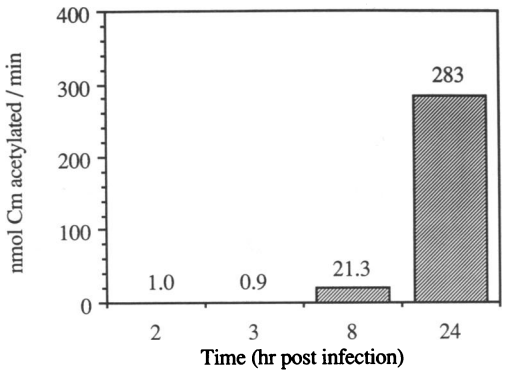

FIG. 3. Measurement of CAT gene expression. Monolayers of human 143 cells (3×10^6 cells per dish) were infected with either A394 or wild-type VV, at multiplicities of infection of 5 plaque-forming units per cell. At various times after infection, the cells were harvested and then disrupted by sonication (30 sec in a cup-horn sonicator). CAT activity in the soluble fraction was measured according to the method of Gorman *et al.* (20). CAT activity (per extract from 3×10^6 cells) is expressed as nmol of chloramphenicol (Cm) acetylated per min at 37°C.

Identification of the VV Gene Encoding the 94-kDa Protein. DNA of a 2-kb *Acc* I fragment derived from the coding region of the CPV 160K gene and contained in plasmid p2031 (5) was ^{32}P-labeled by nick-translation and then hybridized with various cloned fragments of VV DNA. This probe hybridized with a 3.5-kb *Eco*RI fragment corresponding to the *Eco*RI E fragment (24) of the *Hin*dIII A fragment of VV DNA (Fig. 5*A*). Comparison of the positions of the *Eco*RI, *Pst* I, and *Bam*HI sites in this fragment with those in the equivalent region of CPV DNA indicated a strong similarity between these regions. Further evidence of the similarity between these regions was provided by nucleotide sequence analysis. The 613-bp sequence extending from the right-hand-end *Eco*RI site (Fig. 5*B*) through the putative initiation codon of the 94K gene (the gene encoding the 94-kDa protein) is almost identical to the sequence of the corresponding region of CPV DNA containing both the CAE I and the beginning of the coding region of the 160K gene (5). The major difference between the sequences is that the VV DNA lacks 26 of the 28

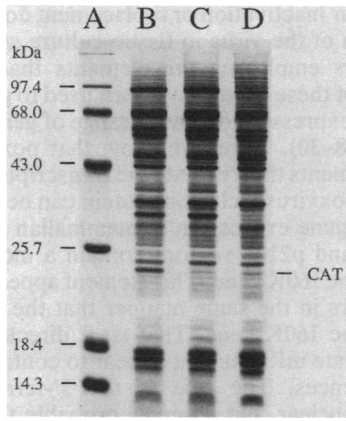

FIG. 4. Expression of the CAT gene in human 143 cells. Monolayers of human 143 cells (3×10^6 cells per dish) were infected with either A394 or wild-type VV at multiplicities of infection of 5 plaque-forming units per cell. Twenty-four hours after infection the cells were harvested. Their solubilized proteins were resolved by electrophoresis in a 15% polyacrylamide gel and then visualized by staining with Coomassie brilliant blue R-250 (Bio-Rad). Lanes: A, protein molecular mass standards; B, lysate of A394-infected cells containing the CAT protein; C, lysate of cells infected with wild-type VV; D, lysate of uninfected cells.

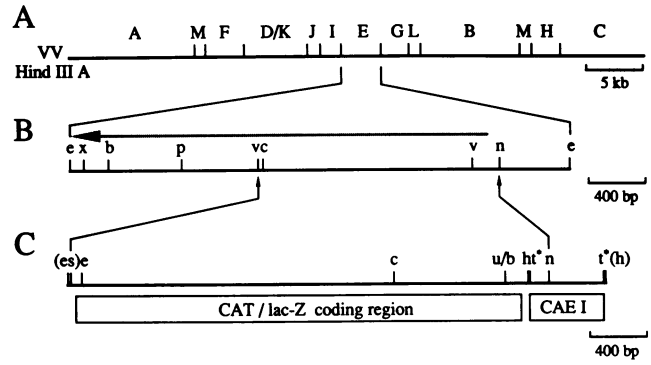

FIG. 5. Insertional mutagenesis of the 94K gene of VV. (*A*) An *Eco*RI map of the *Hin*dIII A fragment of VV DNA (24). (*B*) Restriction map of the cloned 3.5-kbp *Eco*RI fragment (contained in plasmid p1019) containing the 5′-terminal portion of the 94K gene. The tail of the arrow corresponds to the position of the beginning of the transcribed region of the 94K gene. Restriction sites are abbreviated as follows: c, *Cla* I; e, *Eco*RI; h, *Hin*dIII; n, *Nco* I; p, *Pst* I; s, *Sma* I; v, *Eco*RV; and x, *Xho* I. (*C*) Restriction map of the insert contained in plasmid p2080. This plasmid is a pUC19 derivative containing a CAT–*lacZ* hybrid gene downstream of the CAE I. The hybrid was constructed as follows. First, a 780-bp *Hin*dIII–*Bam*HI fragment containing the CAT gene was taken from pSV2-cat^S (20) and placed downstream of CAE I at the *Hin*dIII site shown in Fig. 1*D*. Next, a *Bam*HI-generated fragment containing the coding sequence of the *lacZ* gene, as derived from pMC1871 (25), was fused in-frame to the 5′ end of the CAT gene. The two coding regions were joined at the *Pvu* II cleavage site in codon 38 of the CAT gene and the *Bam*HI cleavage site (repaired) in codon 8 of the *lacZ* gene. "u/b" denotes the site of this junction. The CAE I–CAT–*lacZ* hybrid was then inserted between the *Pst* I and *Hin*cII sites in pUC19 to create p2080. Only the *Taq* I sites (t*) contained in CAE I are shown. The sites shown in parentheses are contained in the polylinker region of pUC19. The insertional mutagenesis vector (p1346) was constructed by replacing the N-terminal portion of the 94K gene (1.7-kb region between the *Nco* I and *Eco*RV sites in plasmid p1019) with the CAT–*lacZ* hybrid gene (3.4-kb *Nco* I–*Sma* I fragment from p2080).

consecutive GAT repeats present in the CPV DNA. In addition, there are 11 single-base differences between the open reading frame upstream of the 94K gene and that of the open reading frame upstream of the 160K gene. Also, there is one less nucleotide in the noncoding region upstream of the 94K gene's initiation codon (creating a run of six thymidylate residues instead of a run of seven thymidylate residues). In the first 324 bp of the coding regions of the 94K gene and the 160K gene there are only 2 base differences. These data demonstrate that the 94K gene is the VV counterpart of the CPV 160K gene.

Insertional Mutagenesis of the 94K Gene. The VV 94K gene appears to be expressed at a high level similar to that of the CPV 160K gene, even though the 94-kDa protein does not form typical A-type inclusions (4). The apparent conservation of "inclusion protein" genes in the genomes of a wide variety of poxviruses including many that do not produce A-type inclusions (5) suggested that these genes might have some role in addition to that of directing the production of the A-type inclusion. Therefore, it was of interest to determine if this gene was essential for viral replication and, if it were not, if replacement of the coding region of the 94K gene with that of a cloned gene would lead to greater levels of expression than those obtained with the p1200 vector. These questions were answered by replacing the N-terminal region of the 94K gene with a hybrid CAE I–CAT–*lacZ* gene. A CAT–*lacZ* hybrid gene was used for two reasons. First, its gene product has β-galactosidase activity, which provided a marker that was used to identify the recombinants. Second, the CAT gene sequences contained in the mRNAs facilitated comparisons of steady-state levels of CAT mRNAs transcribed from this

construction with those transcribed from the CAE I–CAT gene construct inserted into the tk gene. Recombinants that expressed the CAT–*lacZ* gene were generated by transfecting plasmid p1346 (Fig. 5) into cells infected with wild-type VV. One of these recombinants (designated A450) was plaque-purified and then studied further.

Fig. 6 (lane C) shows PAGE analysis of the proteins in cells infected with A450. This recombinant did not direct the production of the 94-kDa protein that was abundant in cells infected with wild-type VV (Fig. 6, lane B). Instead, A450 directed the synthesis of a protein (120 kDa) that was the product of the CAT–*lacZ* gene. These results show that, despite the abundance of the 94-kDa protein in cells infected with wild-type VV, this protein is not essential for virus replication, at least in human 143 cells.

The 120-kDa protein was easily visualized by staining with Coomassie blue, indicating that it was one of the most abundant virus-encoded proteins in the cell. RNA hybridization analyses, using a probe specific for the portion of the CAT gene contained in both A394 and A450, indicated that each of these recombinants generated similar steady-state amounts of CAE I-directed transcripts (data not shown). Therefore, sequences flanking the insertion site in the 94K gene do not appear to direct levels of gene expression that are higher than those directed by the modified CAE I inserted at the site of the tk gene. These results suggest that the 28 GAT repeats present in the CPV CAE I have little influence on transcription of the downstream sequences because only two of these repeats are present in the DNA of the A450 recombinant.

The 94K gene provides a second site in the VV genome that is suitable for gaining efficient expression of cloned genes. The A450 recombinant is a useful recipient for such insertions into the 94K gene because it already contains the CAT–*lacZ* marker gene in this locus. Subsequent insertions will replace this marker gene. A plasmid insertion/expression vector that may be used to replace the marker gene with other genes has been constructed. This plasmid vector, p2101, is depicted in Fig. 7.

DISCUSSION

The objective of this work was to obtain efficient expression of genes cloned into poxvirus-derived vectors. This has been achieved by constructing insertion/expression vectors employing the cis-acting elements directing the expression of the 160K gene, which is one of the most strongly-expressed genes of CPV.

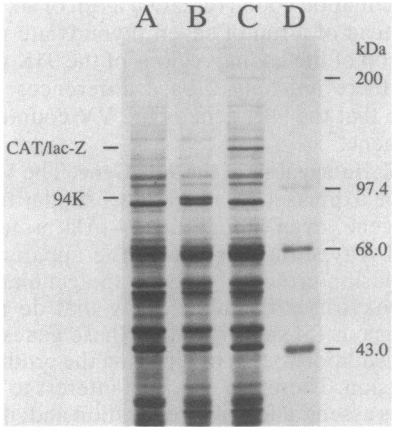

FIG. 6. Expression of the CAT–*lacZ* gene inserted into the 94K gene of VV. Monolayers of human 143 cells (3×10^6 cells per dish) were infected with either A450 or wild-type VV at multiplicities of infection of 5 plaque-forming units per cell. The cells were harvested 24 hr after infection. The proteins of the cells were solubilized and then electrophoresed in an 8% polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue. Lanes: A, lysate of uninfected human 143 cells; B, lysate of cells infected with wild-type VV; C, lysate of A450-infected cells; D, protein molecular mass standards.

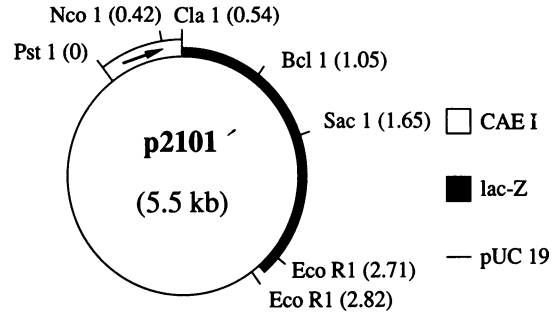

FIG. 7. Restriction map of the p2101 insertion/expression vector. This plasmid consists of CAE I (open bar), the 3′ terminal portion of the *lacZ* coding region (shaded bar), and pUC19 (single line). p2101 was constructed by replacing the 1.1-kb *Nco* I–*Cla* I fragment of p2080 (Fig. 5*C*) with the 120-bp *Nco* I–*Cla* I fragment of CAE I. The arrow indicates the direction of transcription from CAE I.

The CPV 160K gene encodes the major protein component of the A-type inclusion bodies. Its almost identical counterpart, the VV 94K gene, encodes a 94-kDa protein that accumulates in large amounts in the cytoplasm of VV-infected cells (4). Unlike the 160-kDa protein, the 94-kDa protein does not aggregate into typical A-type inclusions. Currently, neither its functions nor its effects on the host are known. The fact that the 94K gene can be inactivated by insertion of the CAT–*lacZ* gene suggests that this gene is not required for viral multiplication in the culture systems used in this study. It may simply be a derivative of a gene whose primary function was to produce inclusion bodies that enclosed the progeny virions.

Viruses of several types, notably the entomopoxviruses and the baculoviruses, produce proteinaceous inclusion bodies that facilitate the transmission of virus from one host to another (26). The genes encoding the major protein components of the baculovirus inclusion bodies have been extensively characterized (reviewed in ref. 27). These genes have some similarities to the 160K and 94K genes; for example, they appear to be the most strongly expressed of the viral genes, and their inactivation or replacement does not prevent the replication of the virus in tissue culture systems. Baculovirus vectors employing the elements that regulate the transcription of these genes have been used to gain extremely high levels of expression of a wide range of genes in cultured insect cells (28–30). Here, we show that poxvirus vectors employing elements that regulate the transcription of the gene encoding the poxvirus inclusion protein can be used to direct high levels of gene expression in mammalian cells.

The p1200 and p2101 vectors contain a modified CAE I derived from the 160K gene. This element appears to function in these vectors in the same manner that the native CAE I functions in the 160K gene. That is, it directs the efficient production of late mRNAs that appear to contain 5′-terminal poly(A) sequences. The role of the 5′-terminal poly(A) sequences is unclear, but it seems probable that they have some important functions because many other late viral mRNAs have now been found to possess such 5′-termini (7–10). It has been suggested that these 5′-terminal poly(A) sequences can affect viral gene expression, either by increasing the translational efficiency of the mRNA or by increasing the stability of the mRNA during the later stages of the viral replication cycle (5).

The levels of expression of the CAT–*lacZ* gene obtained after insertion into the VV 94K gene were similar to those obtained with the CAE I–CAT gene inserted into the tk gene.

This suggests that the modified CAE I contains the major elements required to effect efficient expression of the cloned gene. However, because there are differences both between the two genes and between their transcripts, we are not ruling out the possibility that other elements are involved. Previously, we found that the mRNAs of the 160K gene are unlike those of other characterized late viral mRNAs in that they contain defined 3′ ends (5). We have shown that the production of the defined 3′ ends is directed by a cis-acting element designated CAE II located downstream of the coding region of the 160K gene. When the CAE II is placed downstream of genes inserted into the p1200 vector, it directs the production of mRNAs that contain defined 3′ ends (unpublished data). The structure of the 3′ ends of eukaryotic mRNAs appears to play an important role in determining the stability of the RNA (31, 32). Therefore, the CAE II may affect the levels of gene expression by influencing the nature of the 3′ ends of the viral mRNAs and consequently their stability.

The p1200 and p2101 vectors are each capable of directing the synthesis of gene products in amounts that can easily be detected by Coomassie blue staining of PAGE-resolved total cell proteins (Figs. 4 and 6). This vector system has also been used to direct similarly high levels of expression of the S1 gene of reovirus (33). These levels of gene expression compare well with those obtained using other poxvirus-derived vectors. Under roughly equivalent conditions, the A394 recombinant directs the synthesis of 4–6 times as much CAT as that directed by a widely used VV vector employing the early/late promoter of the 7.5K gene (21). In addition, this recombinant appears to be capable of directing levels of expression that are similar to, if not greater than, those obtained with either the binary poxvirus vectors that employ the RNA polymerase of phage T7 to transcribe cloned genes (34) or the VV vectors that employ the promoter of the gene encoding the major 11-kDa virion protein (35).

The VV vectors provide a simple method of obtaining high levels of expression of genes in the wide range of mammalian and avian cells that are capable of supporting the replication of this virus. Currently available data suggest that the mechanisms involved in the transcription of viral genes are similar in poxviruses of all types (36, 37). Therefore, by inserting CAE I–cloned-gene constructs into the genomes of other poxviruses, it should be possible to gain efficient gene expression in a variety of host animals and host cells.

Finally, these vectors have excellent potential for use in the construction of live poxvirus-derived vaccines. One potential problem with such expression-vector vaccines is that successive inoculations are likely to lead to the production of progressively smaller amounts of antigen because of immune responses that inhibit the replication of the virus (38). Therefore, the ability to direct the synthesis of large amounts of protein may be especially valuable if multiple successive vaccinations are needed, either to present additional immunogens or to maintain an immunity induced by a primary vaccination.

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