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In Vitro Mutagenesis of the Promoter Region for a Vaccinia Virus Gene: Evidence for Tandem Early and Late Regulatory Signals

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A vaccinia virus gene that is expressed throughout the reproductive cycle was found to have two sets of RNA start sites approximately 55 nucleotides apart. The site nearest to the coding segment is used early in infection and the one further upstream is used after DNA replication. A series of 5′ to 3′ deletions were made in the promoter region, and the truncated DNA segments were then ligated to the coding portion of the procaryotic chloramphenicol acetyltransferase gene to measure expression. The effects of these mutations on chloramphenicol acetyltransferase synthesis were determined in a vaccinia virus helper-dependent transient expression system and by forming infectious vaccinia virus recombinants that contain the chimeric genes. Deletions extending up to 3l nucleotides before the late RNA start site had no effect on either early or late expression. Removal of an additional 15 nucleotides produced a dramatic decrease in late expression but had no effect on early expression. The latter was not diminished until the deletion was extended from 31 to 24 nucleotides before the early RNA start site. These results were confirmed by transcriptional analyses. We concluded that this vaccinia virus gene has two promoters and that the regulatory signals for each are located within 31 nucleotides of their sites of transcription.

Poxviruses are distinguished by their large size, complex morphology, long double-stranded DNA, and cytoplasmic site of replication (B. Moss, *in* B. N. Fields, R. Chanock, R. Shope, B. Roizman, ed., *Human Viral Diseases*, in press). The 187-kilobase-pair genome contains more than 100 genes that are regulated in a temporal fashion. Immediately after infection, the early class of RNA is sythesized, capped, and polyadenylated by enzymes packaged within the virus core. Consequently, transcription of early genes is not prevented by inhibitors of either protein or DNA synthesis. In contrast, late genes are not expressed until after DNA replication has begun. Expression of most early genes then diminishes, although some continue to be active at late times.

Many early and late genes have been physically mapped and a few (21, 22, 26, 27) have been sequenced. In each case examined, a region immediately upstream of the mRNA start site was found to be extremely rich in adenylate and thymidylate residues and the sequences did not appear to contain highly conserved eucaryotic regulatory signals. Moreover, evidence for functional differences between vaccinia virus and cellular promoter regions has been obtained by in vitro transcription (16) and in vivo transient expression studies (M. A. Cochran, M. Mackett, and B. Moss, Proc. Natl. Acad. Sci. U.S.A., in press). Notably, vaccinia virus regulatory regions were active only in virus-infected cells or extracts derived from them, whereas RNA polymerase II-dependent promoters were active only in uninfected cells or extracts.

Vaccinia virus recombinants have been constructed which contain chimeric genes composed of vaccinia virus early or late promoter regions linked to foreign protein-coding segments (11, 12, 27). In each case, the promoter region used included the RNA start site and more than 200 base-pairs (bp) of upstream DNA. In vitro mutagenesis of the sequences preceding the RNA start site is necessary for further definition of vaccinia virus transcriptional regulatory signals. For such studies, we chose the promoter region of a gene encoding a 7,500-dalton polypeptide (referred to as the 7.5-kD gene) because it was the first to be sequenced (21) and has been used in recombinant vaccinia virus expression vectors (11, 12, 18, 19), transient expression assays (Cochran et al., in press), and in vitro transcription systems (16).

The 7.5-kD polypeptide gene, which maps within the inverted terminal repetition, was originally classified as immediate early since it was expressed in vitro by permeabilized virions and in vivo in the presence of inhibitors of protein synthesis (7, 23). The in vitro transcriptional start sites of the 7.5-kD gene, determined by labeling with [β-^{32}P]GTP, coincide with the 5′ ends of the mature capped mRNAs from infected cells that were arrested at the early stage of viral expression by treatment with cycloheximide (23). Unexpectedly, Mackett et al. (12) found that chloramphenicol acetyltransferase (CAT) synthesis directed by the 7.5-kD gene promoter in vaccinia virus recombinants was inhibited by approximately 50% when DNA synthesis was prevented, whereas CAT synthesis directed by the thymidine kinase (TK) promoter was unaffected as would be expected for a stringently regulated early gene. These results suggested that the 7.5-kD gene promoter has early and late regulatory signals. In the present study, we demonstrate independent early and late RNA start sites within the promoter region of the 7.5-kD gene and define the regulatory signals by determining the effects of in vitro mutagenesis on expression of recombinant plasmids and viruses.

MATERIALS AND METHODS

Transient expression assays. Transient expression was assayed as described by Cochran et al. (in press). Monolayers of 5×10^6 CV-1 cells were infected with 1.5×10^8 PFU of vaccinia virus (strain WR) and transfected with 10 μg of plasmid DNA. The cells were harvested at 24-h post-infection and suspended in 0.2 ml of 0.25 M Tris-hydrochloride

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(pH 7.5). After freezing and thawing three times, the disrupted cells were dispersed by sonication, and the suspension was assayed for CAT activity (12).

RNA:DNA hybridization and nuclease S1 digestion. Cytoplasmic RNA, obtained by Dounce homogenization of HeLa cells infected 6 h earlier with 5 PFU of recombinant virus per cell in the absence or presence of 100 μg of cycloheximide per ml, was purified by CsCl centrifugation as described previously (6). The RNA, together with 5′ ^{32}P end-labeled DNA, was dissolved in 10 μl of 80% formamide–40 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.4)–400 mM NaCl–1 mM EDTA and heated to 68°C for 10 min and then at 42°C for 3 h. Nuclease S1 digestion was carried out in 0.1 ml of S1 buffer (280 mM NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$ [pH 4.4]) with 480 U of S1 nuclease per ml at 20°C for 1 h. The relatively low temperature was chosen because of the A:T-rich composition of vaccinia virus DNA.

Construction of plasmids. Plasmid pMC4 which was derived from pCP1 (Cochran et al., in press) contained the following structural features: a 265-bp promoter-containing segment which was isolated as a *Hin*cII-*Rsa*I fragment from the upstream region of the 7.5-kD gene (12) and inserted into the *Hin*cII site of pUC9 (14), a *Bam*HI fragment which contained the CAT coding segment without its endogenous promoter, and a 400-bp *Pst*I lambda DNA fragment which was cloned into the *Pst*I site for use as a spacer in the construction of deletion mutants. A 50-μg sample of pMC4 was linearized at the *Hin*cII site and incubated with 2 U of endonuclease Bal31 in 20 mM Tris-hydrochloride (pH 8.0)–12 mM MgCl–200 mM NaCl–1 mM EDTA and incubated at 30°C for 10 min to remove 125 to 300 bp from each *Hin*cII terminus. The reaction was stopped by addition of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid], and the DNA was digested with *Pst*I to remove the remaining portion of the spacer. The 3′ overhang of the *Pst*I terminus was filled in with T4 DNA polymerase and 2 μg of plasmid per ml was recircularized by overnight incubation with T4 DNA ligase. The plasmid was then used to transform *Escherichia coli* HB101. Plasmids pMC4Δ−106, pMC4Δ−30, pMC4Δ−24, pMCΔ+8, and pMC4Δ+17 were prepared (3) and then analyzed by polyacrylamide gel electrophoresis after treatment with *Hin*dIII and *Bam*HI to release the Bal31-shortened promoter region. A second series of deletions were constructed from pMC4Δ−106 by exonuclease III digestion, followed by nuclease S1 digestion (20). A 50-μg sample of pMC4Δ−106 was linearized at the *Hin*dIII site and digested with 100 U of exonuclease III in 6.6 mM Tris-hydrochloride (pH 7.4)–6.6 mM $MgCl_2$–6.6 mM 2-mercaptoethanol–50 mM NaCl. After 2 min of incubation at 18°C, equal volumes of 2× concentrated S1 buffer and 480 U of nuclease S1 were added, and the DNA was incubated for 120 min at 18°C. Under these conditions, 20 to 60 bp were removed from each *Hin*dIII terminus. After treatment with the Klenow fragment of DNA polymerase I to ensure that the ends were flush, *Hin*dIII linkers were blunt-end ligated to the DNA. After digestion with an excess of *Hin*dIII, the plasmid was recircularized as described above. These deletion mutants were designated the pMC24Δ series.

The deletion endpoints of each selected mutant from both the pMC4Δ and pMC24Δ series were deduced from the electrophoretic mobilities of the *Hin*dIII-*Bam*HI fragments relative to sequence ladders generated by the base-specific chemical cleavage reaction of Maxam and Gilbert (13). Account was taken of the size of the linker, and corrections of 1.5 nucleotides were made because of the differences in termini generated by restriction endonuclease cleavage and sequence reactions (25). Mutants pMC24Δ−86, pMC24Δ−71, pMC24Δ−63, pMC24Δ−46, pMCΔ−31, pMCΔ−24, were sequenced around the point of deletion with base-specific chemical cleavage reactions.

Construction of vaccinia virus recombinants. Plasmids containing truncated 7.5-kD gene promoter fragments were digested with *Hin*dIII and treated with the Klenow fragment of DNA polymerase I to fill in the staggered ends. After digestion with *Bam*HI, the fragments were isolated by polyacrylamide gel electrophoresis, electroeluted onto DEAE paper (28), and ligated to insertion vector pMM22 (27) that had been digested with *Sma*I and *Bam*HI. Plasmid recombinants were isolated from transformed *E. coli* HB101 and screened by restriction endonuclease analysis and polyacrylamide gel electrophoresis.

Recombinant viruses were prepared by infecting cells with wild-type vaccinia virus and transfecting them with calcium phosphate-precipitated plasmids containing truncated 7.5-kD gene promoter sequences as described previously (12). The cells were harvested and TK$^-$ recombinants were isolated by plaque assay on TK$^-$ cells in the presence of *Bud*R, and the virus was screened for the presence of CAT DNA by dot-blot hybridization (11, 12). Virus containing CAT DNA was then plaque purified once more in TK$^-$ cells with *Bud*R selection, and large stocks were prepared under nonselective conditions in HeLa S-3 cells.

To confirm the predicted structures, 10 μg of DNA from some of the recombinants was hybridized with 10 ng of the *Hin*dIII-*Pvu*II fragment of pMC4 DNA that was end-labeled at the *Pvu*II termini. The conditions were similar to those described above except that there was only 50% formamide in the hybridization mix. After nuclease S1 digestion, the size of the protected hybrids was determined by electrophoresis in denaturing polyacrylamide gels alongside the base-specific cleavage products of the full-length probe.

RESULTS

Transcription from the promoter region of the 7.5-kD gene. To learn more about the regulation of expression of the natural 7.5-kD gene, the 5′ ends of the early and late messages were examined by nuclease S1 protection procedures (25). A 5′ end-labeled DNA segment of the 7.5-kD gene and upstream flanking region was hybridized to cytoplasmic RNA. The latter was obtained from cells at 2 h after virus infection in the absence of inhibitors or at 6 h after infection in the presence of cycloheximide to prevent protein synthesis. Under both conditions only early RNA is made. After nuclease S1 digestion of the unhybridized single-stranded DNA, the resistant material was compared electrophoretically to a sequence ladder generated by chemical degradation of the same 5′ end-labeled DNA segment used for hybridization (Fig. 1A). The protected fragment size obtained with early RNA made at 2 h after infection or at 6 h in the presence of cycloheximide corresponded precisely with the transcriptional start sites determined previously with in vivo and in vitro synthesized RNA (21, 23). However, when late RNA isolated at 6 h after infection in the absence of inhibitors was used, an additional start site 50 to 60 nucleotides upstream of the early one was detected.

Figure 1C shows a similar experiment in which the 5′ end-labeled DNA probe was prepared from a CAT gene fused to the 7.5-kD promoter region and the RNA was obtained from cells infected with a recombinant vaccinia virus containing the chimeric gene inserted into the thymi-

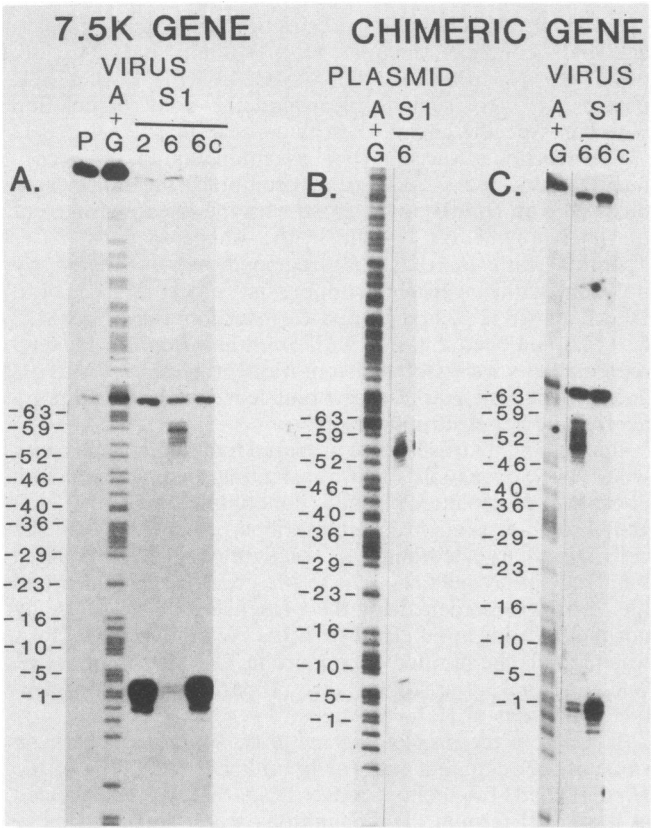

FIG. 1. Mapping of mRNA 5′ ends by nuclease protection. (A) The *Taq*I-*Hpa*II fragment of the 7.5-kD gene (21) was labeled at the 5′ end of the *Taq*I site and was hybridized to cytoplasmic RNA obtained from cells infected with vaccinia virus in the absence or presence of cycloheximide. After nuclease S1 digestion, the protected DNA was denatured and applied to a 6% polyacrylamide gel in 7 M urea next to the products derived from the A+G base-specific chemical cleavage reaction of Maxam and Gilbert (13). An autoradiograph is shown with the lanes corresponding to the probe (lane P), cleavage reaction (lane A+G), nuclease-resistant DNA after hybridization with RNA obtained at 2 h (lane 2) or 6 h (lane 6) after infection in the absence of cycloheximide or at 6 h after infection in the presence of cycloheximide (lane 6C). (B) The *Hin*dIII-*Pvu*II fragment of pCP1, which spans the junction between the 7.5-kD gene promoter and the CAT gene, was labeled at the 5′ end of the *Pvu*II site and hybridized to RNA purified from cells that had been infected with wild-type vaccinia virus and transfected with pCP1 6 h earlier. Hybrids were digested with nuclease S1 and analyzed as above. Lanes correspond to the A+G chemical cleavage reaction (lane A+G) and to the nuclease-resistant DNA (lane 6). (C) The same probe used in (B) was hybridized to early and late RNA purified from cells with recombinant virus vMM23, which contains the chimeric CAT gene inserted into the TK region of the vaccinia virus genome. Hybrids were digested with nuclease S1 and analyzed as above. The lanes correspond to the chemical cleavage reaction (lane A+G) and nuclease-resistant DNA after hybridization with RNA isolated at 6 h after infection in the absence (lane 6) or presence (lane 6C) of cycloheximide.

dine kinase locus. Despite the translocation, the same early and late RNA start sites were identified.

Figure 1B shows an analysis of the RNAs synthesized during a transient expression experiment. In this case, a recombinant plasmid containing the chimeric CAT gene described above was used to transfect cells which were also infected with wild-type vaccinia virus as helper to supply RNA polymerase and other trans-acting factors. Under these conditions significant expression first occurred at 6 h after infection (Cochran et al., in press), at which time the late RNA start site was predominant. The site of early gene expression within the virus core may account for the limited use of the early RNA start site of the transfected plasmid.

These experiments suggest that pre- and post-replicative expression of genes downstream of the 7.5-kD gene promoter results from the presence of separate early and late RNA start sites. However, since there is no ATG sequence between the sites, both classes of mRNA should give the same translation product.

Deletion of nucleotides within the 7.5-kD promoter region. To locate the regulatory signals within the promoter region of the 7.5-kD gene, two series of 5′ to 3′ deletions were made. The first mutants, prepared by Bal31 nuclease treatment of pMC4 (Fig. 2), contain deletions of 125 to 250 bp and are designated pMC4Δ−106 through pMC4Δ+17, according to the deletion endpoint relative to a major early transcriptional start site. The second series of deletions were derived from pMC24Δ−106 by treatment with exonuclease III and S1 nuclease (Fig. 2), and the mutants were designated pMC24Δ−86 through pMC24Δ−46. The locations of the deletion endpoints within the nucleotide sequence of the 7.5-kD gene promoter region are shown in Fig. 3.

DNA sequences required for transient expression. The vaccinia virus helper-dependent transient expression system (Cochran et al., in press) provided a facile way of analyzing the effects of promoter region deletions on CAT expression. Results of the CAT assay, depicted by unfilled bars in Fig. 4, indicate that nucleotides about 86 bp upstream of the early (or 31 nucleotides upstream of the late) RNA start site are sufficient for optimal expression, whereas expression is significantly diminished when the deletion extends to 71 bp upstream of the early (or 16 bp upstream of the late) RNA start site. Further deletions to 63 nucleotides above the early (or 8 nucleotides above the late) RNA start sites reduced CAT expression by 90%. Deletions from −63 to −30 had little further effect on expression; however, another small drop occurred with deletions beyond this site.

The kinetics of transient CAT synthesis and the nuclease S1 analysis of the RNA start site (Fig. 1B) suggest that this assay provides information primarily about late gene expression. Thus, AraC, an inhibitor of DNA replication, reduced transient CAT expression by more than 80% (Fig. 4, filled bars). Attempts to determine the RNA start site in the presence of AraC were unsuccessful, possibly because of the low levels of CAT RNA. For this reason, the effects of deletions on CAT expression in the presence of AraC are difficult to interpret. The major drop in expression occurred when nucleotides between −71 and −63 were deleted, and another drop occurred between −30 and −24.

DNA sequences required for expression of recombinant genomes. Since the transient expression system appears to preferentially use late promoter regions, recombinant viruses were constructed to carry out a more complete analysis. This was accomplished by inserting the sequenced −86, −71, −63, −46, −31, and −24 truncated 7.5-kD gene promoter regions into a plasmid, pMM22, specially made for such purposes (27). This plasmid contains the coding sequence for the CAT gene, restriction endonuclease sites for convenient insertion of vaccinia virus promoters, and flanking vaccinia virus DNA sequences needed to recombine the chimeric gene into the thymidine kinase locus and select infectious recombinant virus (Fig. 5). As described above,

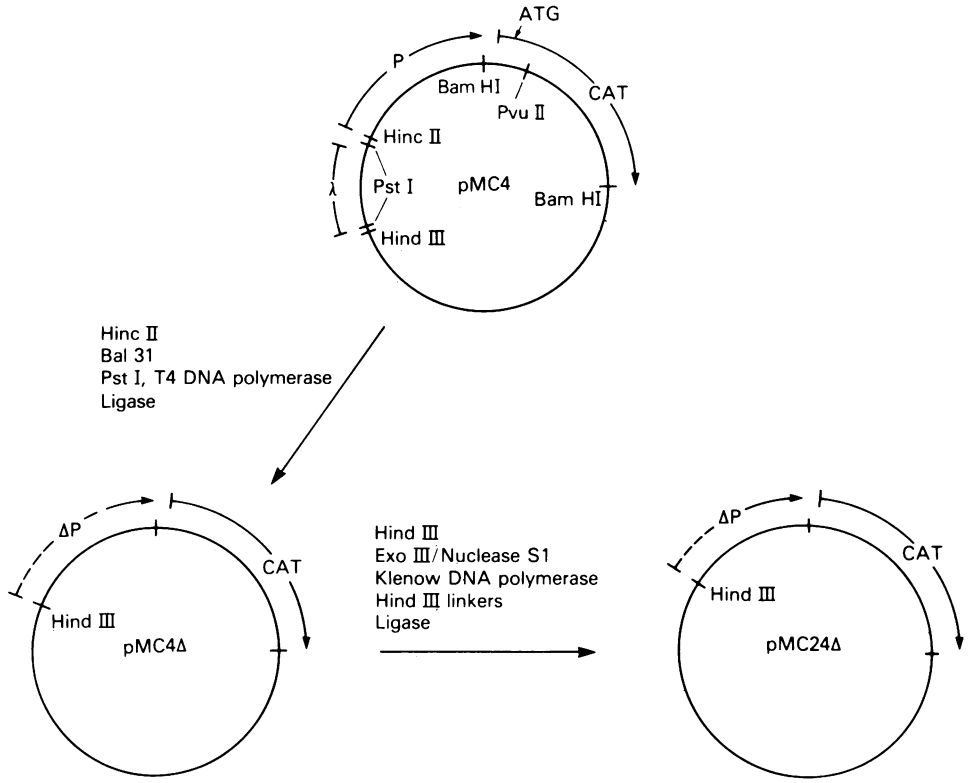

FIG. 2. Deletion of nucleotide sequences from the promoter region. Plasmid pMC4 contains a 265-bp fragment containing the promoter region and RNA start sites of the 7.5-kD gene flanked on one side by the entire coding sequence of the CAT gene and on the other by a 400-bp *Pst*I spacer fragment derived from lambda DNA. Sequences were deleted from the upstream region of the promoter segment by Bal31 digestion. The remainder of the lambda spacer segment was removed by digestion with *Pst*I, and the plasmid was recircularized. A second series of deletions was generated by digestion with exonuclease III and nuclease S1. Details of these constructions are described in the text.

the final step is carried out by homologous recombination in cells infected with wild-type vaccinia virus and transfected with the desired pMM22 derivative. Virus recombinants were then doubly plaque purified and designated vΔ−86, vΔ−71, vΔ−63, vΔ−46, vΔ−31, and vΔ−24 to correspond to the size of the deletion. In each case, the virus DNA was examined by restriction endonuclease analysis to verify that it was properly constructed. For vΔ−24, vΔ−31, and vΔ−46, the DNA was also hybridized to a ^{32}P-labeled *Hin*dIII-*Pvu*II segment of the 7.5-kD gene promoter region of pMC4 (Fig. 3), and then the nuclease S1-resistant segment was analyzed by gel electrophoresis.

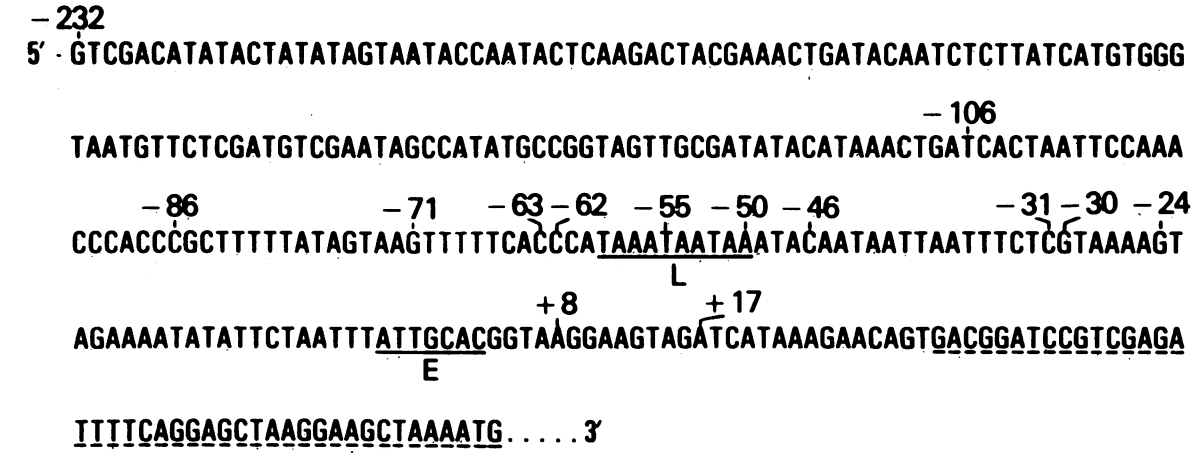

FIG. 3. Location of deletion endpoints within the nucleotide sequence of the promoter region. The early (E) and late (L) transcriptional start sites within the nucleotide sequence of the 7.5-kD gene promoter region are underscored with solid lines. The CAT gene sequence up to the ATG is underscored with a dashed line. The numbers refer to the base pairs of DNA upstream (negative) or downstream (positive) of a major early RNA start site present in the set of deletion mutants that were constructed.

The amount of CAT in cells infected with equivalent multiplicities of each recombinant virus was monitored at various times after infection in the presence and absence of AraC. The results are summarized in Fig. 6. The kinetics and amount of CAT synthesized was similar in cells infected with vΔ−232 and vΔ−86, indicating the absence of detectable control elements in that region. However, a further deletion of 15 bp from −86 to −71 resulted in a 50% decrease in expression of CAT in cells not treated with AraC. Since this deletion had no effect on CAT synthesis in the presence of AraC, it must affect only late transcriptional signals. No further effect on CAT synthesis was seen with deletions up to −31. However, removal of seven additional nucleotides caused almost complete loss of early promoter activity; the very low amount of CAT made in cells infected with vΔ−24 was similar to that occurring in cells infected with vMM22, which has no sequences derived from the 7.5-kD promoter region. In Fig. 7, the data from 24-h after infection are in the form of a bar graph to better compare the results obtained with recombinant virus with those obtained by transient expression. We interpret the results of both analyses as indicating that specific late expression is severely inhibited when sequences between 86 and 71 nucleotides upstream of the early RNA start site or more importantly 31 to 16 nucleotides upstream of the late RNA start site are deleted. Sequences important for early expression are located between nucleotides 31 and 24 above the early RNA start site.

Analysis of RNA synthesized by virus recombinants. To confirm the results obtained by analysis of CAT expression, the RNA synthesized in cells infected with recombinants containing deleted 7.5-kD promoter regions was analyzed by nuclease S1 procedures. For this purpose, the *Pvu*II site within the CAT gene in pCP1 was cleaved and 5′ labeled with polynucleotide kinase. The *Hin*dIII-*Pvu*II fragment was then isolated and hybridized to early RNA made in the presence of cycloheximide and late RNA made in its absence. After nuclease S1 digestion, the resistant hybrids were denatured and analyzed by polyacrylamide gel electrophoresis alongside a sequence ladder produced by chemical cleavage of the labeled *Hin*dIII-*Pvu*II fragment. The results showed that the early RNA start site was used when there were 31 or more nucleotides remaining upstream of the latter (Fig. 8A). The late RNA start site was used when there were 31 or more nucleotides remaining above the late start site corresponding to 86 nucleotides above the early site (Fig. 8B). Since the natural 7.5-kD gene is present in all of the recombinants, the early and late forms of the expressed RNA can be analyzed as a control. A *Sal*I-*Cla*I segment of the 7.5-kD gene, 5′ labeled at the *Cla*I site, was obtained from plasmid pAG4 (21). The probe was hybridized to the RNA samples, treated with nuclease S1, and analyzed by polyacrylamide gel electrophoresis. The data presented in Fig. 8C shows that the preparations of RNA used in Fig. 8B contained both early and late transcripts of the natural 7.5-kD gene. Thus, the absence of late transcripts of the chimeric CAT gene of vΔ−71 was a specific effect of the promoter deletion.

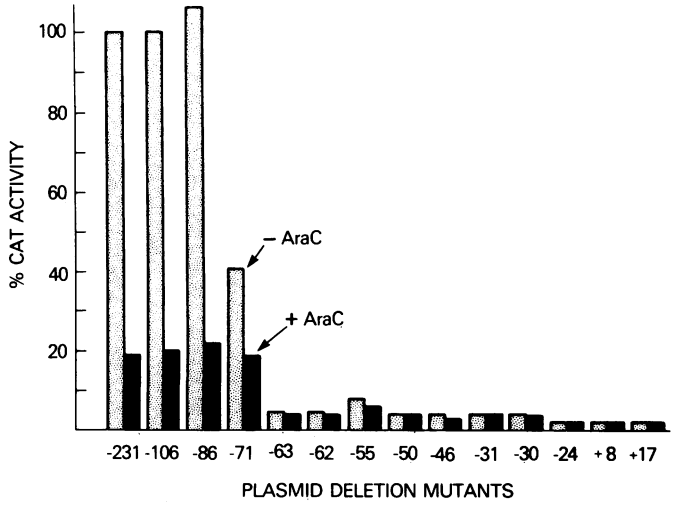

FIG. 4. Effect of 5′ deletions on transient expression of the CAT gene. Replicate cultures of CV-1 cells were infected with 30 PFU of wild-type vaccinia virus per cell and transfected with 10 μg of plasmid containing a chimeric CAT gene with the indicated length of DNA from the 7.5-kD gene promoter region as indicated in Fig. 3. Cultures were incubated in the absence (stippled bars) and presence (filled bars) of 40 μg of AraC per ml. At 12 h after infection, the cells were harvested and assayed for CAT. Activity is expressed as a percentage of that obtained with the longest promoter region in the absence of drug.

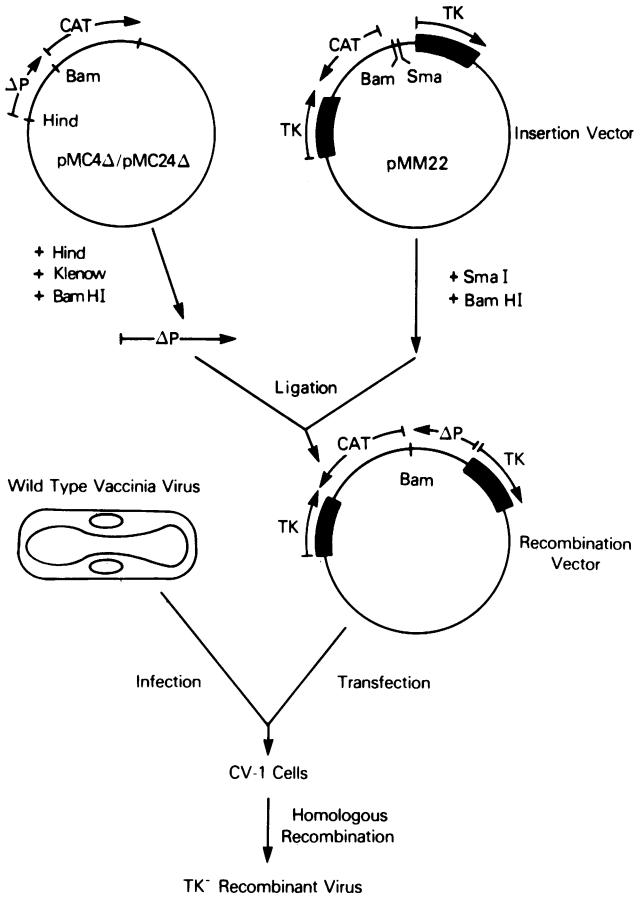

FIG. 5. Construction of vaccinia virus recombinants. DNA fragments containing the promoter regions (ΔP) were excised from plasmids used for transient expression assays and inserted into the insertion vector pMM22. The latter has the coding segment of the CAT gene interrupting the TK gene. The resulting recombination vector was transfected into cells that were infected with wild-type vaccinia virus. After homologous recombination was allowed to occur, TK⁻ recombinant virus was selected. Relevant restriction endonuclease sites are shown: Bam, Hind, and Sma are *Bam*HI, *Hin*dIII, and *Sma*I, respectively.

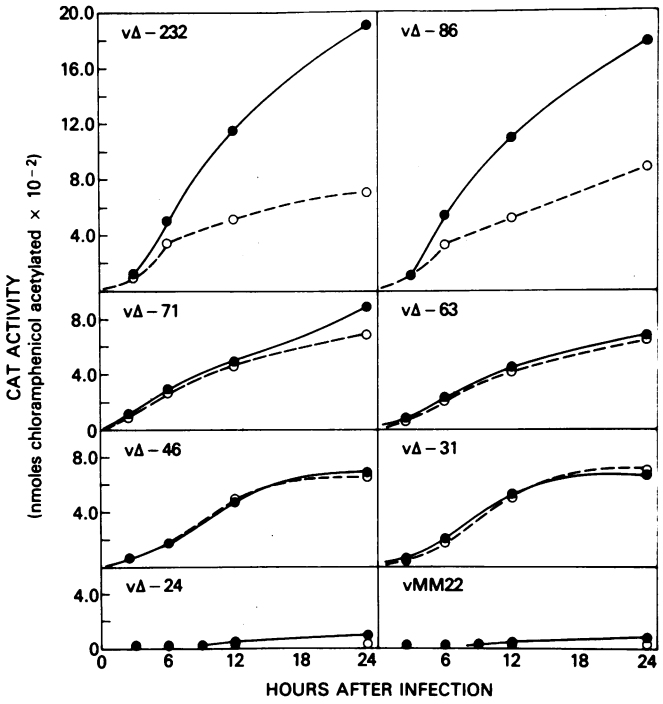

FIG. 6. Effect of 5′ deletions on CAT expression by recombinant virus. Replicate cultures of CV-1 cells were infected with 5 PFU of each recombinant virus per cell. Cultures were incubated in the absence (open symbols) or presence (filled symbols) of AraC. At the indicated times after infection, cells were harvested and assayed for CAT. Activity is expressed as nmol of chloramphenicol acetylated per 2.5×10^6 cells.

DISCUSSION

This study provides the first structure-function analysis of the promoter region of a vaccinia virus gene. Because of the nature of the promoter chosen, information concerning both early and late regulatory signals was obtained. Although the majority of vaccinia virus genes appear to be expressed either before or after viral DNA replication, some appear to be continuously active. Evidence that the promoter region of the 7.5-kD gene belongs to the latter class was obtained by using it to regulate the expression of the procaryotic CAT gene in vaccinia virus recombinants (12). The existence of two copies of the 7.5-kD gene, because of its location within the inverted terminal repetition, and a mechanism to enable continuous expression suggest an important although unknown role for this gene.

To initiate the current study, we inquired whether the 7.5-kD promoter region contained the same or separate RNA start sites for early and late expression. Evidence for the latter was obtained. The late RNA start site was located by nuclease S1 protection experiments at about 55 nucleotides upstream of the early site. For both sites, a cluster of nuclease-protected bands were resolved. Although some of the microheterogeneity could be due to nuclease S1 nibbling or incomplete digestion, there is independent evidence that the early 7.5-kD gene transcripts have multiple closely spaced start sites (21). Similar transcription sites were used for expression of the natural 7.5-kD gene and of chimeric genes translocated within the vaccinia virus genome.

To identify the nucleotide sequences important for the regulation of vaccinia virus transcription, 5′ to 3′ deletions were made in the promoter region of the 7.5-kD gene. These segments were ligated to the coding segment of the CAT gene to facilitate the analysis of expression. For simplicity, the helper vaccinia virus transient expression system was used for preliminary screening of the effects of the deletions. In this system, the helper vaccinia virus provides trans-acting factors for transcription of vaccinia virus genes in plasmids introduced into infected cells by transfection procedures (Cochran et al., in press). A major effect on expression occurred when the deletion was extended from 86 to 71 nucleotides upstream of the early RNA start site, which corresponds to 31 to 16 nucleotides upstream of the late site. Since expression occurs largely after DNA replication in the transient system, these data provided information regarding the location of late transcription signals within 31 nucleotides of the late RNA start site. Although it required additional steps, recombination of the chimeric genes into vaccinia virus provided more information. Again, a marked drop in expression occurred when the deletion was extended from 31 to 16 nucleotides before the late RNA start site. However there was no effect on early transcription, measured as CAT synthesis resistant to inhibition of DNA replication, until the deletion was extended to between 31 and 24 nucleotides before the early RNA start site. These results were confirmed by analysis of the transcripts. We concluded that the sequences regulating early transcription of the 7.5-kD gene are independent of those regulating late transcription. Whether, the opposite is true, however, still remains to be determined.

The presence of two promoter regions controlling a single coding sequence provides a mechanism for regulating the synthesis of a vaccinia viral gene product during the early and late phases of the replication cycle. The use of dual promoters for regulatory purposes is not unprecedented, since this has been found for the *E. coli* galactose operon (15), the lambda *cI* gene (17), the yeast invertase gene (5), the *Drosophila* alcohol dehydrogenase gene (2), and the mouse α-amylase gene (29). Moreover, the arrangement of early and late promoters preceding the simian virus 40 T antigen gene (1, 4, 8–10, 24) is similar to that described here for the vaccinia virus 7.5-kD gene.

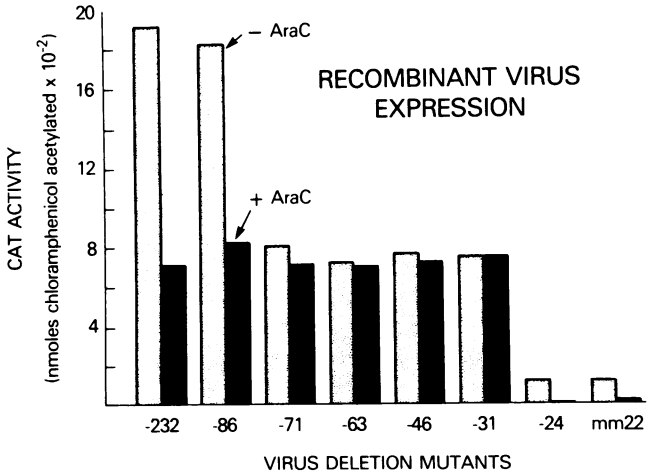

FIG. 7. Bar graph showing the effect of 5′ deletions on CAT expression. Values represent the 24-h samples in Fig. 6. Cells were incubated in the absence (stippled bars) or presence (filled bars) of 40 μg of AraC per ml. Activity is expressed as nmol of chloramphenicol acetylated per 2.5×10^6 cells.

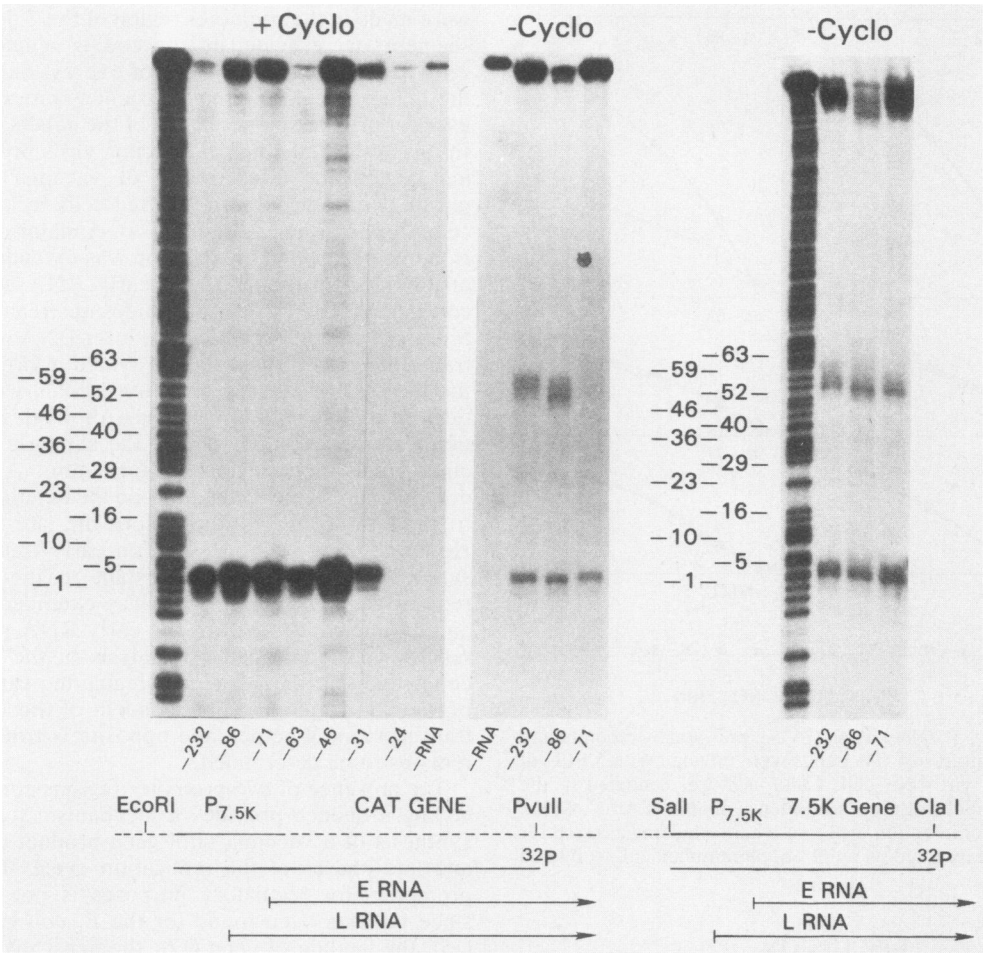

FIG. 8. Analysis of the 5′ ends of early and late RNAs. Cytoplasmic RNA was purified from cells at 6 h after infection with recombinant vaccinia virus in the presence (+Cyclo) or absence (−Cyclo) of cycloheximide. The RNA was hybridized to the indicated 5′ ^{32}P-labeled DNA fragment from the chimeric CAT gene (left) or authentic 7.5-kD gene (right). After nuclease S1 digestion, the resistant material was analyzed by polyacrylamide gel electrophoresis. An A+G Maxam and Gilbert sequence ladder is shown at the left of each set of autoradiographs. The numbers on the side indicate the nucleotides upstream of a major early RNA start site. The number below each lane defines the deletion of the recombinant virus used.

The signals within the promoter region of the 7.5-kD gene, for both early and late regulation of transcription, are contained in DNA segments of less than 31 nucleotides preceding their respective RNA start sites. Deletion of the sequences CGCTTTTTATAGTAA and CGTAAAA almost entirely eliminated late and early expression, respectively. To further delimit the regulatory regions, 3′ to 5′ deletions, internal deletions and point mutations of the 7.5-kD promoter region are planned. In addition, in vitro mutagenesis of stringent early and stringent late promoter regions is in progress.

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