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Molecular Dissection of *cis*-Acting Regulatory Elements from 5′-Proximal Regions of a Vaccinia Virus Late Gene Cluster†

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Promoter elements responsible for directing the transcription of six tightly clustered vaccinia virus (VV) late genes (open reading frames [ORFs] D11, D12, D13, A1, A2, and A3) from the *Hin*dIII D/A region of the viral genome were identified within the upstream sequences proximal to each individual locus. These regions were identified as promoters by excising them from the VV genome, abutting them to the bacterial chloramphenicol acetyl transferase gene, and demonstrating their ability to drive expression of the reporter gene in transient-expression assays in an orientation-specific manner. To delineate the 5′ boundary of the upstream elements, two of the VV late gene (A1 and D13) promoter:CAT constructs were subjected to deletion mutagenesis procedures. A series of 5′ deletions of the ORF A1 promoter from −114 to −24 showed no reduction in promoter activity, whereas additional deletion of the sequences from −24 to +2 resulted in the complete loss of activity. Deletion of the ORF A1 fragment from −114 to −104 resulted in a 24% increase in activity, suggesting the presence of a negative regulatory region. In marked contrast to previous 5′ deletion analyses which have identified VV late promoters as 20- to 30-base-pair cap-proximal sequences, 5′ deletions to define the upstream boundary of the ORF D13 promoter identified two positive regulatory regions, the first between −235 and −170 and the second between −123 and −106. Background levels of chloramphenicol acetyltransferase expression were obtained with deletions past −88. Significantly, this places the ORF D13 regulatory regions within the upstream coding sequences of the ORF A1. A high-stringency computer search for homologies between VV late promoters that have been thus far characterized was carried out. Several potential consensus sequences were found just upstream from RNA start sites of temporally related promoter elements. Three major conclusions are drawn from these experiments. (i) The presence of promoters preceding each late ORF supports the hypothesis that each is expressed as an individual transcriptional unit. (ii) Promoter elements can be located within the coding portion of the upstream gene. (iii) Sequence homologies between temporally related promoter elements support the notion of kinetic subclasses of late genes.

Vaccinia virus (VV), the prototype of the poxvirus family, is a large double-stranded DNA-containing animal virus, which replicates in the cytoplasm of infected cells (27). The relative independence of VV replication from host cell nuclear functions (18, 25, 31, 36, 37), coupled with its complex assembly process (45), necessitates that the virus be capable of modulating the temporal expression of the approximately 200 genes encoded within its 185-kilobase genome. Penetration of VV into susceptible host cells is followed by the expression of early viral functions which are responsible for the shutoff of host-directed macromolecular syntheses and the initiation of viral DNA replication (25–28, 34). Concomitantly with DNA replication, viral late genes are transcribed and translated, producing both structural proteins and viral enzymes necessary for morphogenesis and the production of infectious-progeny virions (11, 25, 26). Early genes are not expressed at late times during infection. The mechanisms governing the switch between the early and late modes of viral gene expression are not yet known.

Although only a limited number of VV late genes have been analyzed in detail, previous studies suggest that transcription of late genes is regulated primarily by the 5′-flanking sequences (4, 5). Deletion analyses have indicated that promoter sequences are within approximately 30 base pairs (bp) of the transcriptional start site. These elements do not contain obvious homologs of the usual eucaryotic or procaryotic consensus signals (5, 15, 43). Since many of the VV late genes encode essential viral functions, it is not possible to study and manipulate these loci in their normal genomic context. Therefore, to circumvent this problem, VV late promoters have been studied either by using transient-expression assays (8) or by recombination of promoter elements into alternate locations within the viral genome (5, 15, 43). The results of both approaches have been similar. The VV transient-expression system uses plasmids containing chimeric viral promoter-reporter gene cassettes, which are transfected into susceptible host cells, followed by superinfection with VV to provide a source of VV RNA polymerase and potential *trans*-acting factors. Measurement of reporter gene activity gives relative values for VV promoter function. Transient-expression assays have been used extensively in other viral systems, such as simian virus 40 and herpesvirus, to define the sequences and *trans*-acting factors involved in the regulation of gene expression (1, 9, 19, 24). This approach affords the advantage of rapid assay of native and mutated promoter sequences. For example, Cochran et al. (8) have shown that the 7.5 kilodalton (kDa) VV promoter, when linked to the coding portion of the bacterial chloramphenicol acetyltransferase (CAT) gene in a recombinant plasmid, will catalyze *cat* expression only in

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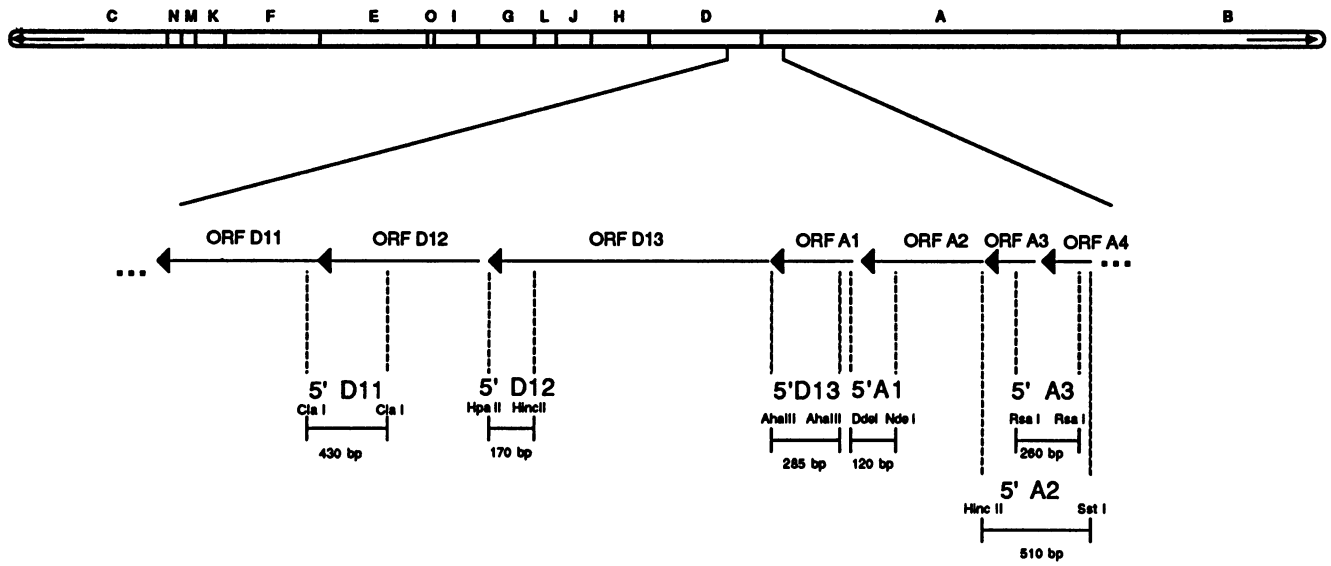

FIG. 1. Organization of the *Hin*dIII D/A region of the VV genome. At the top of the figure is the *Hin*dIII restriction map of the 185-kilobase VV genome with the closed ends and inverted terminal repeats represented. The 5.1-kilobase *Hin*dIII D/A region of interest has been expanded with the position and orientation of the ORFs indicated, as previously determined by Weinrich and Hruby (40). The sizes of the ORFs and the distances between them are drawn to scale. ORF designations are according to the conventions proposed by Rosel et al. (33). The lower portion of the figure indicates the genomic location, nomenclature, and restriction enzyme cleavage sites for each of the putative promoter fragments. The numbers below each potential promoter fragment indicate its size in base pairs.

cells transfected with this plasmid and superinfected with VV. Transcription was initiated correctly and had kinetics of expression consistent with the late gene promoter element found within the 7.5-kDa promoter (8).

In our efforts to understand VV late gene expression, we have focused on a region of the genome that is expressed primarily at late times. We previously mapped a 65,000-molecular-weight polypeptide (65K polypeptide)-encoding late gene near the *Hin*dIII D/A junction (42). Designated L65, this protein was shown to be a major viral product late in infection that was not associated with the mature virion. Tartaglia et al. (38, 39) mapped rifampin resistance to an A-T to G-C transition mutation within the coding sequences of L65. In the presence of rifampin, both VV morphogenesis and cleavage of precursor polypeptides to mature viral structural proteins are inhibited. Hence, the late protein L65 may play a role in virion assembly. Sequence analysis of the genomic region surrounding the L65 locus indicated the presence of six densely packed, tandemly oriented open reading frames [ORFs], of which L65 was the fourth ORF of the cluster (40). (Using the new VV genetic nomenclature proposed by Rosel et al. [33], L65 is now synonomous with ORF D13.) All of these ORFs are transcribed late in infection in a leftward direction towards the *Hin*dIII H fragment (31, 42). Kinetic data from an S1 nuclease analysis suggested that, rather than being transcribed as a single long polycistronic message, these ORFs were independently transcribed (41). In this communication, we further test this hypothesis by utilization of a transient-expression system to identify promoter elements preceding each ORF. The identification of six VV late promoters significantly increases the number of VV late promoters characterized to date. To further define sequences important in the regulation of viral gene transcription, a series of deletions were made in two of the promoters (A1 and D13) and the 5′ boundaries of the minimal promoter regions were determined. With the information gained from the deletion analysis, specific homology searches were conducted and several interesting homologies were observed.

MATERIALS AND METHODS

Plasmid construction. Recombinant plasmids were constructed, propagated, and analyzed essentially as described by Maniatis et al. (23). With the information previously published concerning the nucleotide sequence and RNA start sites used in the *Hin*dIII D/A portion of the genome (40, 41), the regions immediately upstream of, and including, the 5′ start sites were excised and cloned by using the restriction enzyme cleavage sites indicated in Fig. 1. The isolated promoter fragments were ligated into recombinant plasmids at sites immediately upstream of the coding sequences of the bacterial CAT gene, which was used as a reporter gene in these studies. Where possible, the promoter fragments were inserted in both the same and the opposite orientations relative to the *cat* gene. Final constructions were verified by both analytical restriction enzyme digestion and nucleic acid sequencing procedures. We created 5′ deletions of the D13 and A1 ORF promoters by unidirectional *Exo*III deletion mutagenesis as previously described (23; see Fig. 4). Deletion endpoints were determined by nucleotide sequencing.

Cells and virus. Growth, purification, and plaque titration of VV (strain WR) were carried out essentially as previously described (17). BSC-40 cells were grown in Earle minimum essential medium (GIBCO Laboratories), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine at 37°C, 5% CO_2, and 95% humidity. Ltk^- cells were maintained in the same medium plus 25 μg of 5-bromodeoxyuridine per ml. Before being used for transient expression, Ltk^- cells were passaged twice in medium lacking 5-bromodeoxyuridine.

Transient expression. Transient-expression assays were carried out essentially as previously described (8, 12, 13).

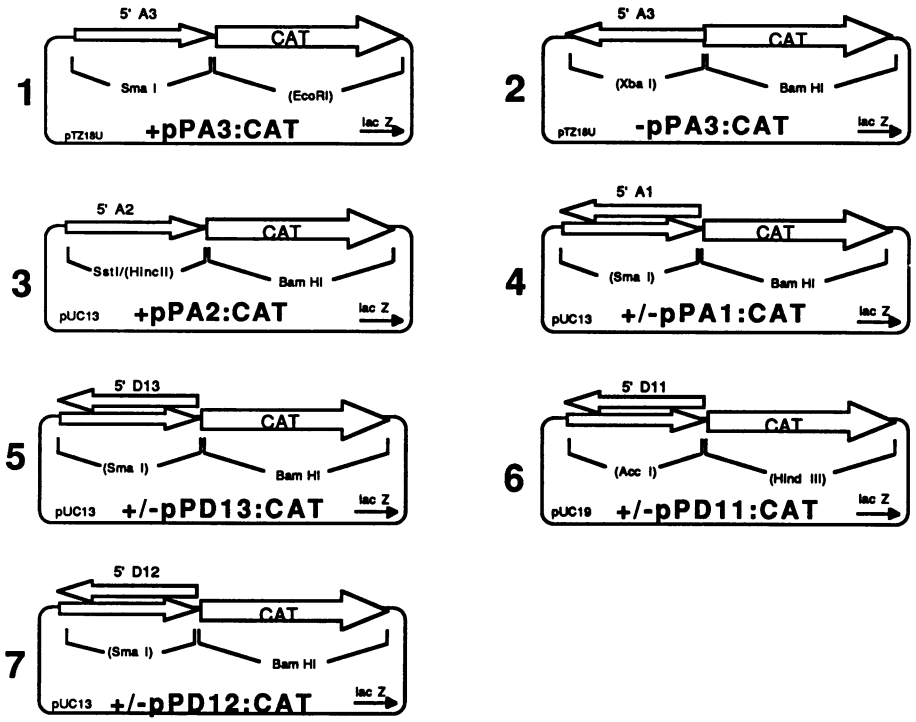

FIG. 2. Structures of recombinant plasmids containing chimeric VV late promoter-CAT gene cassettes. Potential VV late promoters from the *Hin*dIII D/A region were inserted into plasmids containing the coding sequences of the bacterial *cat* gene. Orientations of the promoter elements and *cat* genes are indicated by the directions of the arrows. The restriction enzyme cleavage sites used for insertion of each fragment are indicated (sites enclosed within parentheses were lost during cloning). The original parental plasmid, location of the *lac* promoter, and its direction of transcription are also indicated.

Briefly, recombinant plasmids (5 μg) were coprecipitated with carrier salmon sperm DNA by the calcium phosphate method (14). Precipitated DNA was layered onto monolayers of Ltk$^-$ cells. The monolayers were glycerol shocked 3 to 4 h later. After a 1-h recovery period, the cells were superinfected with VV at a multiplicity of 30 PFU per cell. The infected cells were incubated at 37°C for 24 h before being processed for CAT assays.

CAT assays. Cells were washed twice with ice-cold phosphate-buffered saline, pelleted, suspended in 1 ml of CAT assay buffer (40 mM Tris chloride [pH 7.8], 1 mM EDTA, 150 mM NaCl), and incubated at 25°C for 5 min (12, 19). The cells were pelleted and suspended in 0.1 ml of 0.25 M Tris chloride (pH 7.8). After the cells were subjected to three freeze-thaw cycles, the nuclei were pelleted at 12,000 rpm for 5 min in a TOMY 150A microcentrifuge and the supernatants were removed and stored at −20°C until subsequent CAT assays. CAT activity was measured by the ability of the extracts to transfer the acetyl group from acetyl coenzyme A onto [^{14}C]chloramphenicol (16). The acetylated chloramphenicol derivatives were resolved by thin-layer chromatography on silica plates, located by autoradiography, excised, and counted by liquid scintillation. Positive (CAT enzyme; Sigma Chemical Co.) and negative (water) controls were run with each set of reactions. Extracts were diluted to ensure that the assay was within the linear range.

Computer analyses. Nucleic acid sequences were analyzed for restriction enzyme cleavage sites and homologies using the Microgenie (Beckman Instruments, Inc.) program on an IBM PC computer.

Materials. Restriction endonucleases and other enzymes used for cloning manipulations were obtained from either Bethesda Research Laboratories, Inc., or New England BioLabs, Inc. Radioisotopes were from New England Nuclear Corp. All tissue culture supplies were purchased from GIBCO Laboratories.

RESULTS

Cloning of VV late promoter elements. Initial transcriptional mapping studies of the VV genome have not indicated any striking overall organizational motifs (3, 7, 29, 30, 32). However, at the subgenomic level, detailed analyses have demonstrated that VV genes belonging to the same kinetic class (i.e., early versus late) are often found grouped together in tandemly oriented clusters (10, 21, 22). The significance of such arrangements is not yet known. For example, it was previously shown that at least six VV late genes are found closely linked in the *Hin*dIII D/A region of the VV genome (Fig. 1). The ORFs predicted from the nucleotide sequence all read in the leftward direction and are so tightly packed as to suggest that the regulatory signals of each individual cistron may lie within the coding sequences of the neighboring upstream and downstream genes (40). This conclusion, however, presupposes that each gene represents an individual transcriptional unit, a fact which remains to be established. At late times postinfection, RNAs complementary to the VV *Hin*dIII D/A region were detected as a large heterodisperse population of transcripts. Although 5′ termini of RNAs were detected for each putative gene by S1 nuclease mapping (41), it was not obvious whether these ends represented transcriptional initiation from promoter elements upstream of each gene or whether they were generated by posttranscriptional processing of a larger polycistronic transcript.

OPEN READING FRAME	PROMOTER FRAGMENT			CAT ACTIVITY	
	5' LIMIT	CAP SITE [▼]	3' LIMIT	... promoter CAT ...	... promoter CAT ...
D11	-398		+32	18.8	2.2
D12	-141		+27	10.0	2.03
D13	-283		+1	280.3	2.5
A1	-114		+12	163.1	8.34
A2 & A3	-459		+33	120.1	NT
A3	-248		+12	42.2	2.3
7.5K	-241		+38	266.9	NT
pUC:CAT	NONE			2.7	NT

FIG. 3. Transient expression of VV late promoter-CAT genes in VV-infected cells. Promoter elements from the *Hin*dIII D/A region were assayed for their ability to express an abutted *cat* gene. The promoter fragments tested are indicated, along with the ORF from which they were excised and their 5′ and 3′ limits, relative to the major late start site of transcription (+1) previously determined (41). As positive and negative controls, plasmids containing the *cat* gene linked to the VV 7.5-kDa promoter or no promoter were included in these experiments. The recombinant plasmids were transfected into cells, the cells were superinfected with wild-type VV, and after 24 h, cytoplasmic extracts were prepared and assayed to determine the level of CAT activity present. The numbers represent nanomoles of chloramphenicol acetylated per 5×10^6 infected cells. The values given represent the average of at least three separate experiments in which the standard deviation of the measurements obtained was less than 18%. NT, Not tested.

To distinguish between these possibilities, it was of interest to excise the 5′-proximal region upstream of each gene and to determine whether it could direct the expression of an abutted reporter gene, i.e., function as a *cis*-acting promoter element. Therefore, the indicated VV subgenomic fragments (Fig. 1) were cloned, making use of available restriction endonuclease sites. Depending on the fragment, the 5′ boundaries of these putative promoters were between 114 and 283 bp upstream of the late RNA start sites previously identified (41). Based on earlier analyses of other VV late promoters, this should be more than enough DNA to encode a fully functional VV late promoter. On the 3′ side, the cloned fragments contained from 1 to 33 bases downstream of the RNA start site and all but one included sequences corresponding to 5′ codons of the downstream ORF. The isolated VV DNA fragments were inserted into recombinant plasmids which contained the coding sequences of the bacterial CAT gene (Fig. 2). The site of insertion was 5′ proximal to the *cat* gene. Except for the putative A2/A3 promoter, all fragments were inserted in both the correct and incorrect orientations. Recent experiments in our laboratory have indicated that the bacterial *lac* promoter is recognized, at least to some extent, by the VV transcriptional apparatus (unpublished data). Thus, it is of importance to note that in the chimeric constructions assembled here (Fig. 2), the *lacZ* gene contained in these plasmids is located downstream of the *cat* gene and reads in the antisense direction relative to the *cat* gene. This ensures that any CAT activity generated by these plasmids is not the result of *lac*-directed transcripts.

Transient-expression analysis of VV promoters. A transient-expression assay was used to assess the ability of the putative VV late promoter elements to be recognized by VV RNA polymerase and to direct the expression of functional CAT enzyme. Control experiments demonstrated that these plasmids were totally dependent on VV infection for activity. Cells were transfected with recombinant plasmids and infected with VV, and after 24 h, lysates were assayed for CAT activity (Fig. 3). As positive and negative controls, plasmids containing the *cat* gene linked to the VV 7.5-kDa promoter or no promoter element, respectively, were included in the experiments. The results indicate that each of the six fragments cloned from the *Hin*dIII D/A region behaved as a promoter element, directing the expression of the *cat* gene at levels ranging between 4- and 100-fold over the pUC:CAT control. In the inverted orientation, relative to the *cat* gene (except for the A1 fragment), the promoter elements were essentially inactive. The reason for the weak (threefold) activity of the negative orientation of the A1 fragment is unknown.

5′ deletion analysis of promoter sequences. To further delineate the nucleotide sequences that are important for the regulation of VV late gene transcription, the two most efficient promoter elements (A1 and D13) from the *Hin*dIII D/A cluster were chosen as substrates for 5′ deletion mutagenesis. Using the approach outlined (Fig. 4), a family of unidirectional deletions was constructed which extended from the 5′ boundary of the promoter fragment through the transcriptional start site. 5′-deleted promoter-CAT constructs were assayed by transient expression.

The results of applying this approach to the A1 promoter element are displayed in Fig. 5A. High-level promoter activity was maintained for Δ+pPA1:CAT plasmids containing sequences from −114 to −24 bp upstream of the RNA start site. A deletion to +2, which removed the normal start site, completely eliminated *cat* expression. Thus, characteristic of other VV late promoters analyzed thus far (11K [5, 15] and 28K [43]), the A1 promoter apparently requires only a short upstream region for full transcriptional activity in transient-expression assays. Deletion of sequences between −114 and −104 consistently resulted in an increase in CAT activity.

The results obtained from carrying out a similar analysis of the D13 promoter (Fig. 5B) were quite different. In this case, deletion of sequences between −235 and −170 caused a 40%

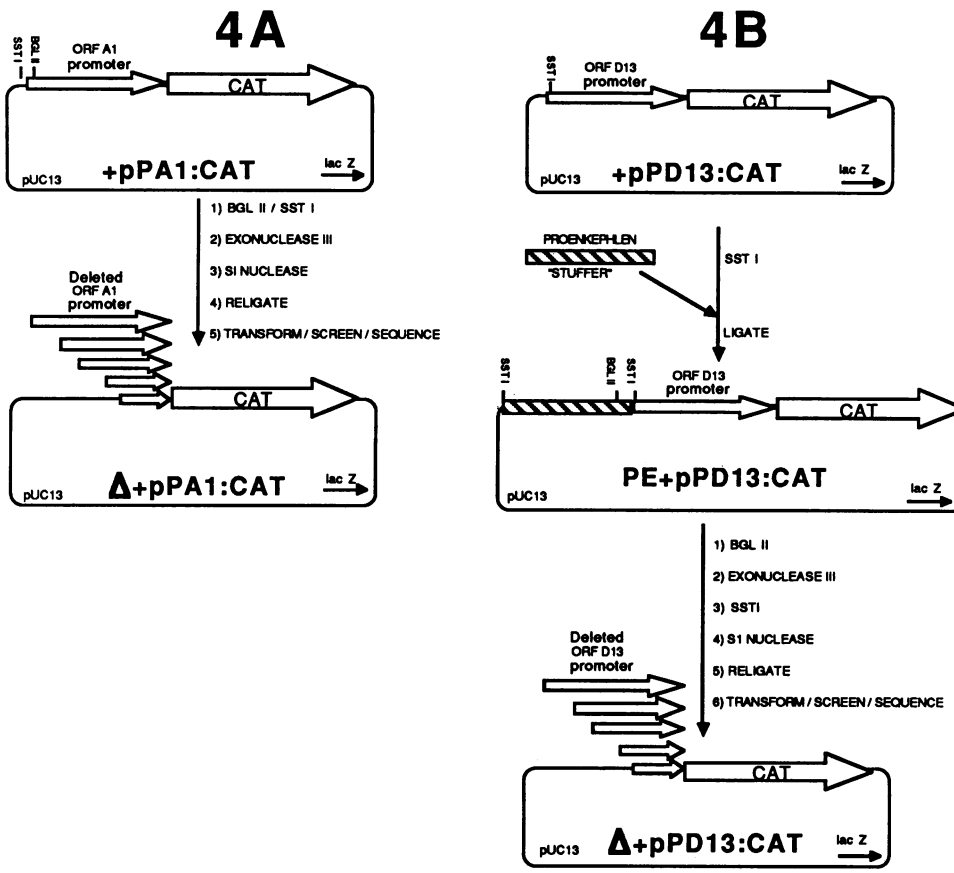

FIG. 4. *Exo*III deletion mutagenesis of the ORF A1 (4A) and D13 (4B) promoter elements. The +pPA1:CAT and +pPD13:CAT plasmids were progressively deleted from the 5′ direction, relative to the transcriptional start site, by the procedures outlined. The resulting contructs were screened by restriction enzyme analysis and nucleotide sequencing protocols to obtain an appropriate library of deletions extending into or through the regions suspected to contain the regulatory elements of the A1 and D13 late promoters.

loss in activity of the D13 promoter. Further deletions between −170 and −124 had little effect, whereas removal of the next 16 bp to −106 resulted in a substantial loss of promoter activity. None of these deletions removed the transcriptional start sites mapped by Weinrich and Hruby (41). The low level of D13 promoter activity was reduced to background levels by removal of the region between −88 and −41. Thus, the D13 promoter apparently contains at least two positive regulatory elements, one between −235 and −170 and the other between −124 and −106.

Occurrence of interpromoter nucleotide homologies. The deletion mutagenesis data provided more precise informa-

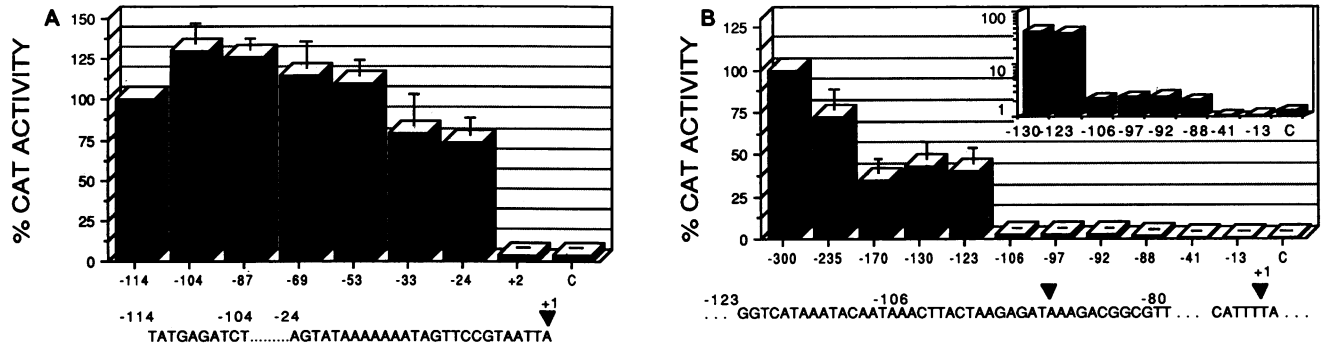

FIG. 5. Transient expression of the 5′-deleted ORF A1 (A) and D13 (B) promoter mutants. Recombinant plasmids containing the full VV promoter fragment or the indicated 5′ deletions abutted to the *cat* gene were assayed for transcriptional activity via the transient expression. Each experiment was repeated a minimum of three times. The average values are shown in bar graph form, with the standard deviation of the various trials indicated by the thin line. CAT activity was calculated relative to the intact promoter fragment. Below each graph is displayed the nucleotide sequence of the promoter fragment, along with the locations of various deletions and the major late transcriptional start site (41). The inset shows a log scale of activity levels of the indicated deletions. C, pUC:CAT control.

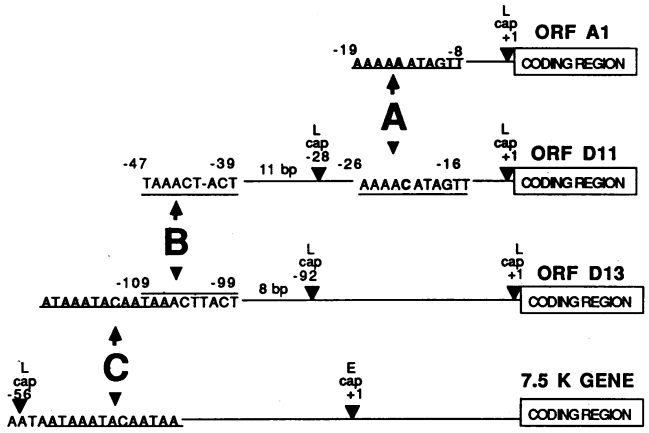

FIG. 6. Interpromoter sequence homologies present within kinetic subclasses of VV late promoter elements. VV late ORFs A1, D11, and D13 and the 7.5-kDa gene are diagrammed, showing nucleotide sequences and locations of the indicated regions upstream of the predicted coding regions. Both early and late RNA start sites (41) are shown. Three regions of high homology (A, B, and C) detected by computer analysis are indicated.

tion on the essential regions of the A1 and D13 promoters. Therefore, the nucleotide sequences between −24 and +1 of the A1 promoter and −124 and −92 of the D13 promoter were compared by computer at high stringency to one another, as well as with previously published VV promoter sequences (Fig. 6). We and others (15, 33) have noted the presence of the characteristic TAAAT(G) signal at or near the transcriptional start site of VV late genes. Although this signal is present at the 5′ end of most of the *Hin*dIII D/A gene cluster, three other regions of significant homology were noted. (i) The sequence TAAACT(T)ACT is found 8 and 11 bp 5′ to the upstream RNA start site of D13 and D11, respectively. (ii) The sequence AAAA(A/T)ATAGTT is found 8 and 16 bp, respectively, upstream from the first RNA start site for ORF A1 and the downstream RNA start site for ORF D11. (iii) The 13-nucleotide sequence ATAAATACAATAA is perfectly conserved between the late RNA start site of the 7.5-kDa gene promoter and the 5′ RNA start site of ORF D13.

DISCUSSION

Based on the ability of cloned VV DNA fragments to catalyze *cat* expression in a transient-expression assay and on differential appearance of 5′ ends by time course nuclease S1 analysis (41), we conclude that regions upstream of each ORF in the VV *Hin*dIII D/A late gene cluster contain a *cis*-acting promoter element. In two recent papers (6, 35), data have been reported which suggest discontinuous synthesis of VV late mRNA. These authors have detected a 35- to 4,000-nucleotide leader RNA at the 5′ end of late mRNA. The mechanism of addition is as yet unknown. The presumably extragenomic location of transiently expressed plasmids prevents any *cis* genomic regulatory functions from operating. This indicates that a nascent RNA strand is synthesized from the promoter fragment, in the absence of readthrough. The episomal assay condition argues strongly in favor of promoter activity and strongly against some other mechanism of transcript processing. The precise mechanisms of VV promoter function, i.e., leader-primed transcription and possible *trans*-splicing events occurring after RNA synthesis, are not addressed by these experiments. Differing levels of *cat* expression from individual promoter fragments would seem to support the hypothesis that the individual loci within this late gene cluster are differentially expressed and regulated. This latter conclusion must be qualified, however, since the six promoter fragments contain variable amounts of 3′- and 5′-flanking sequences which may or may not affect the measured level of expression of the abutted *cat* gene. Of the promoter fragments, five produced only background CAT activity when present in the opposite orientation relative to *cat*, whereas the ORF A1 promoter fragment demonstrated some measurable activity in both directions. It is not clear why this fragment is slightly active (threefold) in the negative orientation. Arguments could be advanced for either an enhancerlike activity or a cryptic VV promoter. The latter possibility seems more likely for two reasons. (i) The former requires postulation of a promoter, as well as an enhancer, within the inverted fragment. (ii) Weak VV RNA start sites at locations not thought to contain VV promoters have previously been recognized (41).

Nuclease S1 analyses of RNA generated from the chimeric genes during transient-expression assays showed a *cat* gene-specific RNA with a 5′ end corresponding to the in vivo 5′ end previously mapped by Weinrich and Hruby (41) for ORF A1 (data not shown). Likewise, the ORF D13 promoter fragment protected a 5′ end which corresponds to the upstream start site (−92) used in vivo. The cloning procedure used for the ORF D13 promoter fragment has eliminated the downstream site at +1. Therefore, regulatory signals described here are affecting expression of *cat* from the upstream D13 start site exclusively. Experiments are currently in progress to determine the limits of the upstream signals which regulate the expression of the downstream (+1) D13 start site.

The 7.5-kDa promoter represents one of the most active VV promoters thus far recognized. Because of this, it has been used to direct the expression of foreign genes in many of the recombinant VV strains which are currently being evaluated as candidate vaccines (20, 44). It is interesting to note that on the basis of the data shown in Fig. 3, the D13 promoter element is at least as efficient as the 7.5-kDa promoter element at expressing downstream *cat* gene sequences (280- versus 267-fold). Thus, the D13 promoter element may be of potential use in future recombinant virus constructions.

The use of 5′ deletion mutagenesis procedures revealed that, for the ORF A1 promoter fragment, only the 5′-proximal 24 bp is required for full *cat* gene expression. The small size of this promoter is in accord with other VV promoters studied to date (5, 15, 43). A run of eight A residues similar to the run of seven found in the ORF A1 promoter, has been shown to be important for function of the 28K gene promoter (43). It was also interesting that deletion of the bases between −114 and −104 consistently increased the activity of the A1 promoter to a level 124% of that of the intact fragment. This result could indicate that a negative regulatory element exists at this location. Although further refinement of the transient-expression system will be necessary before a detailed analysis of these sequences can be undertaken, there is evidence for negative regulatory (silencer) signals being involved in the attenuation of late genes at early times during viral replication in other viral systems (2). A similar analysis of the ORF D13 promoter showed that, in sharp contrast to other VV promoters analyzed, the ORF D13 promoter contains at least two positively acting regulatory regions. The significance of this higher level of

complexity is not known. Previous work indicated the presence of two transcriptional start sites for ORF D13 at +1 and −92 (41). The −92 site was used transiently from 2 to 3 h postinfection, and the +1 site was used from 3 to 12 h postinfection. The S1 analysis on *cat*-specific RNA mentioned earlier supports the notion that these two positive regulatory regions modulate levels of transcription from the upstream site at −92. The presence of essential regulatory signals over 100 bp from the transcriptional start sites represents a significant departure from previously described VV late promoters. Furthermore, these results suggest that nucleotide sequences in this region of the VV genome may contain information for both transcriptional initiation and protein sequences, since the coding sequences for ORF A1 end at −17, relative to the D13 RNA start site.

Sequence comparisons between these regulatory elements and other VV late promoters revealed the existence of short regions of homology between several of the promoters. Although the significance and possible function of these sequences remain to be established experimentally, it is interesting that in each case, these sequences are located in positions very close to established RNA start sites. More importantly, the kinetics of in vivo expression from the proximal RNA start are similar (41), suggesting a role in regulation of the timing of gene expression. Thus, a reasonable working hypothesis is that the general A/T richness and the TAAATG motif found within VV late promoter elements may play a general role in identifying late genes as such, and other *cis*-acting information, such as that identified in Fig. 6, may serve to activate the expression of particular kinetic subclasses of VV late genes.

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