

Regulation of Expression and Nucleotide Sequence of a Late Vaccinia Virus Gene

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A subset of vaccinia virus genes are expressed only after DNA replication. To investigate the regulation of such transcriptional units, a representative gene encoding a major late polypeptide (M_r , 28,000) was mapped and sequenced. Translatable mRNAs were heterogeneous in length and overlapped several early genes downstream. The 5' end of the message was located, and the DNA segment upstream was excised and ligated to the coding sequence of the easily assayable procaryotic chloramphenicol acetyltransferase gene. The resulting chimeric gene was recombined into the thymidine kinase locus of the vaccinia virus genome, and infectious recombinant virus was isolated. Both the time of chloramphenicol acetyltransferase synthesis in infected cells and the requirement for DNA replication indicate that the sequence upstream of the late gene contains *cis*-acting transcriptional regulatory signals.

Vaccinia virus is a large, double-stranded DNA virus that replicates in the cytoplasm of infected cells (12, 27). The transcriptional program of this virus is unusual since the enzymes involved in the synthesis and processing of mRNA resemble those of eucaryotic cells yet are packaged within the mature virus particle and are probably virus encoded. The 187-kilobase-pair (kb) vaccinia virus genome contains more than 100 genes that are regulated in a temporal fashion. Early genes are transcribed within minutes after infection, and their expression is not prevented by inhibitors of either protein or DNA synthesis. The onset of DNA replication results in a dramatic shift in gene expression. From this time on, most early proteins are no longer synthesized, and a new group of late polypeptides, including the major structural components of the virus particle, are made.

Many early genes have been precisely mapped and some have been sequenced (16, 39, 41, 45). No evidence of splicing has yet been found, and the mature 5' ends of early mRNAs correspond to the sites of transcriptional initiation (39). In each case examined, a region of at least 40 to 60 nucleotides preceding the RNA start site was found to be greatly enriched in deoxyadenylate (A) and thymidylate (T) residues, and the sequences differed appreciably from eucaryotic or procaryotic regulatory signals. Evidence that vaccinia virus has evolved unique signal sequences is supported by recent transcriptional studies with extracts prepared from uninfected and virus-infected cells (34).

To learn more about early regulation of vaccinia virus transcription, chimeric genes composed of putative early vaccinia virus promoters linked to foreign structural genes were constructed and recombined into vaccinia virus. A 275-base-pair (bp) DNA fragment containing sequences preceding and including the RNA start site of a gene encoding a 7,500-dalton (7.5K) polypeptide has been shown to promote expression of a variety of genes, including herpesvirus thymidine kinase (TK), chloramphenicol acetyltransferase (CAT), hepatitis B virus surface antigen, and influenza virus hemagglutinin (19, 20, 36, 37). Similar results were obtained by

using a DNA segment preceding the vaccinia virus TK structural gene as a promoter.

For a long time, late mRNAs were known to have certain peculiarities. Although many early proteins are no longer synthesized after DNA replication, DNA-RNA hybridization studies have revealed that nearly the entire genome is transcribed (6, 31, 32). As a class, late mRNAs were found to be longer than early mRNAs and to include early sequences (31). In addition, late mRNAs were able to form intermolecular duplexes with themselves or with early RNAs (7, 8, 38). Further analysis of late transcripts revealed that they are extremely heterogeneous in size, such that an mRNA coding for a single protein varied several-fold in length (9, 11, 21). Partly for this reason, precise mapping and sequencing of late genes has been delayed, and little is known about the regulation of late transcription.

To determine the nature of a late transcriptional unit and to investigate the relationship of replication and expression, we mapped and sequenced a gene that encodes a major late protein of 28K. The RNA start site was located, and the putative regulatory sequence was isolated and used to construct a chimeric CAT gene. Expression of the latter was measured after recombination into vaccinia virus, and evidence for *cis*-acting regulatory sequences preceding the late structural gene was obtained.

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from Bethesda Research Laboratories, New England Biolabs, or Boehringer Mannheim Corp. T4 polynucleotide kinase and S1 nuclease were from P-L Biochemicals, T4 DNA ligase and *Escherichia coli* DNA polymerase 1 large fragment were from Bethesda Research Laboratories, and calf intestinal alkaline phosphatase and calf thymus tRNA were from Boehringer Mannheim Corp.

Preparation of DNA. Recombinant plasmids were purified as described by Birnboim and Doly (5), and fragments were isolated from agarose gels by electrophoresis onto DEAE paper (46). Routine manipulations were carried out as described by Maniatis et al. (23).

DNA sequencing. DNA sequencing was carried out on end-

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labeled fragments as described by Maxam and Gilbert (24) or by the dideoxynucleotide chain termination method (35), using single-stranded recombinant M13mp8 and M13mp9 phage (25, 42).

Purification of RNA. HeLa S-3 cells in suspension culture were infected with a multiplicity of 30 PFU of wild-type or recombinant vaccinia virus strain WR. Cells were collected by centrifugation, washed with phosphate-buffered saline, and Dounce homogenized. Cytoplasmic RNA was purified by CsCl gradient centrifugation as described previously (9). Early RNA was isolated 4 h after infection in the presence of 100 μ g of cycloheximide per ml; late RNA was isolated 6 h after infection in the absence of drugs.

Hybridization selection and cell-free translation. Selection of mRNA by hybridization to plasmid DNA, which was immobilized on nitrocellulose filters, was done essentially as described previously (1, 44). Cell-free translation was carried out for 1 h at 30°C in a micrococcal nuclease-treated reticulocyte lysate as described previously (9).

Mapping the 5' ends of mRNA by single-stranded nuclease digestion. DNA that was end labeled with polynucleotide kinase was mixed with approximately 20 μ g of cytoplasmic RNA in 30 μ l of 80% formamide–40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [pH 6.4]–1 mM EDTA–0.4 M NaCl. The solution was heated to 90°C for 5 min and then incubated at 42°C for 3 to 4 h. After hybridization, the mixture was placed in an ice bath, and 0.3 ml of cold 0.28 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM ZnSO₄–400 U of nuclease S1 per ml was added. After 5 min on ice, the mixture was incubated at 25°C for 1 h and extracted with phenol-chloroform. Carrier tRNA (10 μ g) was added, and the RNA was precipitated with isopropanol.

Marker rescue. Transfection and marker rescue were performed as described previously (44) to introduce foreign DNA into vaccinia virus at the TK locus (20).

CAT assay. Samples (5 ml) of infected HeLa cells were centrifuged, and the pellets were suspended in 0.2 ml of 0.25 M Tris-hydrochloride (pH 7.8). After freezing and thawing three times, the disrupted cells were dispersed by sonication, and the suspension was assayed for enzyme activity (20).

RESULTS

Mapping of a major late protein. The 187-kb vaccinia virus genome is conveniently divided by the restriction endonuclease *Hind*III into 15 fragments, ranging in size from 1.5 to 45 kb (13, 49). These cloned or purified fragments were used to make a preliminary cell-free translational map of hybrid-selected early and late mRNAs (3, 9). Although early and late mRNAs are distributed throughout the genome, there appears to be some local clustering. For example, the major proteins encoded within the 4-kb *Hind*III L fragment, located approximately 48 kb from the left end of the genome, are predominantly late. Of these, the most prominent polypeptide has a molecular mass of about 28K (Fig. 1B) and corresponds to one of a group of polypeptides estimated to be about 30K (3). A restriction map of the *Hind*III L and J fragments and the location of subcloned segments that were used for finer mapping are shown in Fig. 1A. Using DNA from these subclones immobilized on nitrocellulose filters for hybridization selection of mRNA, we found that the major 28K protein and additional proteins of about 38K, 26K, 23K, and 21K mapped to the right side of fragment L and into fragment J. Other polypeptides of about 30K and 27K mapped to the left side of fragment L and were previously resolved from the major 28K polypeptide by two-

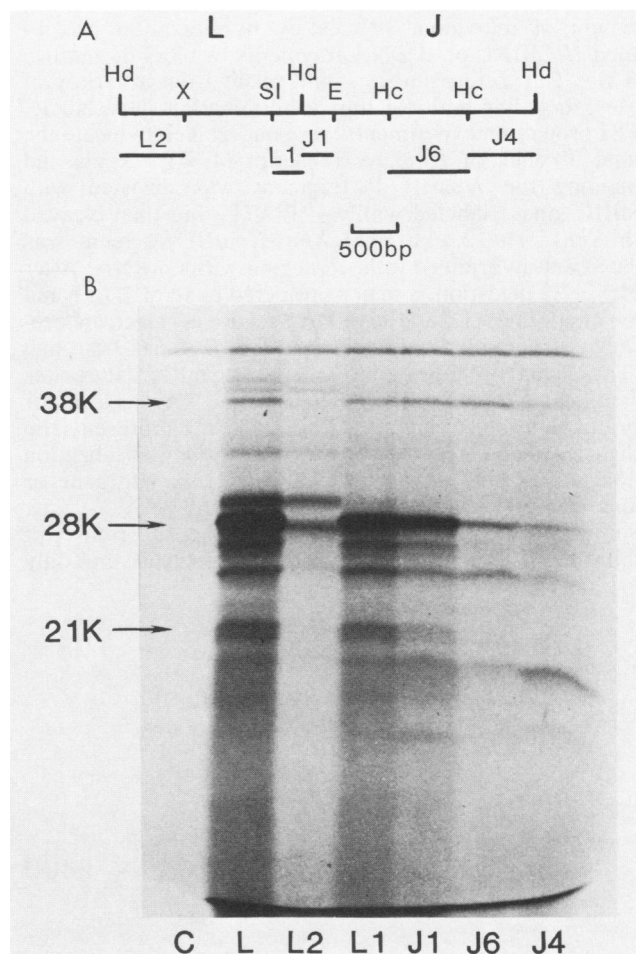


FIG. 1. Cell-free translation products of late vaccinia virus mRNA selected by hybridization to cloned subfragments of *Hind*III-L and -J. (A) A portion of the vaccinia virus genome containing the *Hind*III L and J fragments. Restriction endonuclease cleavage sites are abbreviated as follows: Hd, *Hind*III; X, *Xba*I; Sl, *Sall*; E, *Eco*RI; Hc, *Hinc*II. The designated segments were cloned in plasmid (L, L1, L2, J6, and J4) or single-stranded phage (J1) vectors. (B) Purified cytoplasmic RNA, isolated 6 h after vaccinia virus infection, was hybridized to recombinant DNA containing the *Hind*III L fragment or the indicated *Hind*III L or J subfragments. Selected mRNAs were eluted and translated in a reticulocyte cell-free system containing [³⁵S]methionine. The labeled products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18) and autoradiographed. Molecular masses (indicated to the left of the gels) were determined by coelectrophoresis of labeled protein markers. Lane C is a control translation performed without added RNA.

dimensional gel electrophoresis (3). One of the segments, J1, was cloned in the phage M13mp8 vector (25). Translation of mRNA that hybridized to the single-stranded DNA of the recombinant phage demonstrated that the direction of transcription of several genes, including the one coding for the 28K polypeptide, was from left to right. Since cloned subfragments of the right end of *Hind*III-J selected mRNA for the 28K and other proteins, some of the mRNA species must be several thousand nucleotides long and, consequently, overlap several early genes located within the *Hind*III J fragment (1).

Transcriptional mapping of the late gene. The length of heterogeneity of late mRNAs prevented the resolution and

detection of individual species by hybridization of ^{32}P -labeled *Hind*III-L or -J DNA fragments to blots of agarose gels (1). This heterogeneity could result from a variety of causes, including multiple start or termination sites. Nuclease S1 protection experiments were undertaken to locate the 5' and 3' ends of the late transcript (4, 43). A plasmid containing the *Hind*III L fragment was digested with *Hind*III, kinase labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, and then cleaved with *Xba*I. The 2.4-kb right *Xba*I-*Hind*III fragment was isolated and hybridized to late vaccinia virus mRNA. After nuclease S1 digestion, a major protected band of 1.1 kb and some minor ones were resolved by agarose gel electrophoresis and detected by autoradiography (Fig. 2, lanes 3 through 5). These bands correspond to specific late mRNA start sites since they were not observed when early RNA was used for hybridization (Fig. 2, lane 7). The 2.4-kb band represents the undigested probe. This could result from either hybridization to long transcripts arising from an upstream late gene or incomplete nuclease digestion of reannealed DNA.

Similar efforts to map the 3' end of the late transcript within the *Hind*III J fragment were unsuccessful, and only

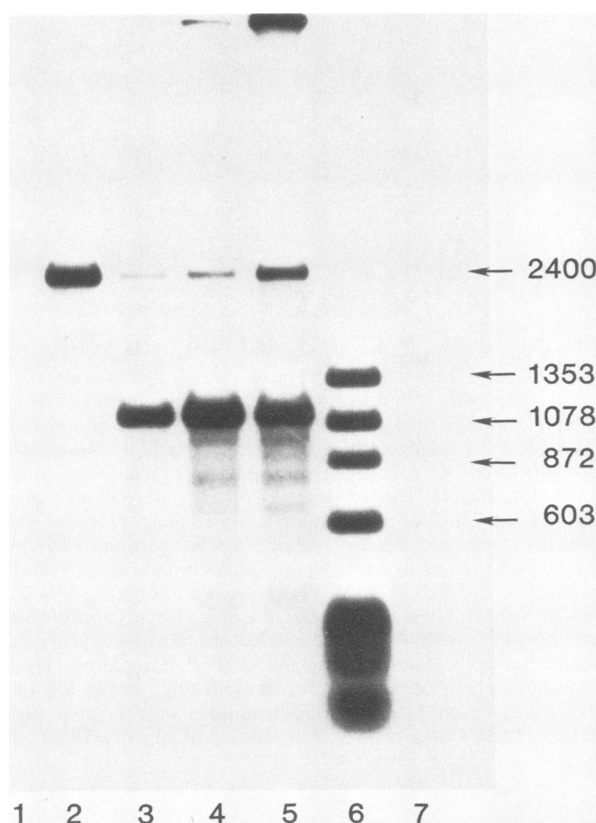


FIG. 2. Location of the 5' end of a late mRNA by nuclease S1 protection. A plasmid containing the vaccinia virus *Hind*III L fragment was cleaved with *Hind*III and end labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase. After secondary cleavage, the large *Xba*I-*Hind*III fragment was purified and then hybridized to RNA. Single-stranded DNA was digested with nuclease S1, and the resistant hybrids were analyzed by agarose gel electrophoresis and autoradiography. Hybridization was carried out with 20 μg of calf thymus tRNA (lane 1), 20 μg of late RNA (lanes 3 through 5), 20 μg of early RNA (lane 7), and one times (lane 3), four times (lanes 1, 4, 7), or nine times (lane 5) the amount of labeled DNA. Additional lanes contain the labeled DNA probe (lane 2) and defined-length DNA markers (lane 6). Marker lengths in bp are indicated to the right.

smears in a size range of 2 to 6 kb were revealed by autoradiography (data not shown). This result, along with the ability of cloned subfragments far to the right of *Hind*III-J to hybrid select the 28K message, indicates that the mRNA for this late protein is extremely long and heterogeneous. The heterogeneity can be accounted for by the absence of discrete 3' ends.

Nucleotide sequence of the late gene. The strategy used to determine the DNA sequence of the right 1.8 kb of the *Hind*III L fragment, which contains the start site of the late gene encoding the 28K polypeptide, is shown in Fig. 3A. Overlapping DNA fragments were cloned in mp8 or mp9 derivatives of M13 phage and sequenced by the dideoxynucleotide chain termination method (35). The nucleotide sequence derived in this manner is shown in Fig. 3B.

Further nuclease S1 protection experiments were performed to accurately locate the 5' end of the message. For this purpose, the *Bgl*II site located at position 884 (Fig. 3B) was cleaved and 5' labeled with polynucleotide kinase. An *Xba*I-*Bgl*II fragment was isolated and then hybridized to late RNA. After nuclease S1 digestion, the resistant hybrids were denatured and analyzed by polyacrylamide gel electrophoresis alongside a sequence ladder produced by chemical cleavage (24) of the *Bgl*II end-labeled DNA fragment (Fig. 4). A pair of protected major bands migrated alongside two T residues that were complementary to the A residues at positions 769 and 770 (Fig. 3B).

Inspection of the nucleotide sequence revealed that the first possible initiation codon occurs at or one nucleotide after the apparent start site of the message and is followed by an open reading frame of 250 codons. The second and third ATG sequences occur 27 and 40 codons downstream and in phase with the first one. Polypeptides of 28.5K, 25K, and 24K would result from initiations at the first, second, and third ATG codons, respectively. These predicted molecular weights correspond to the major and some comapping minor polypeptides detected by polyacrylamide gel electrophoresis (Fig. 1B).

Formation of vaccinia virus recombinants containing a chimeric CAT gene. To determine the sequences that are required for transcriptional regulation of the late gene, we wished to isolate the promoter region and ligate it to the coding sequence of the easily assayable bacterial enzyme CAT which is not present in eucaryotic cells (15). After recombination of the chimeric gene into a nonessential region of vaccinia virus, the enzyme activity could be used as a convenient measure of transcriptional activity (20).

The desired piece of vaccinia DNA was obtained from the replicative form of a recombinant M13 clone (mp9-L5) that was generated while sequencing the late gene. It contained an inserted piece of vaccinia DNA starting at the *Sau*3AI site at position 411 and extending past the *Acc*I site at position 774 (Fig. 3). Cleavage at the *Sma*I site, located within the M13 cloning region adjacent to the *Sau*3AI site, and at the *Acc*I site liberated a 369-bp segment. This contained 357 bp of vaccinia DNA preceding and 8 bp beyond the start site of the message. This fragment was used as the putative late promoter.

Plasmid vectors used for the insertion and expression of foreign genes in vaccinia virus have been described previously (20, 29). A new vector, pMM22, was designed to test vaccinia virus promoters (Fig. 5). This plasmid contains the coding sequences for the CAT gene, two restriction endonuclease sites for convenient insertion of vaccinia virus promoters, and flanking vaccinia virus DNA sequences needed to recombine the chimeric gene into vaccinia virus and select

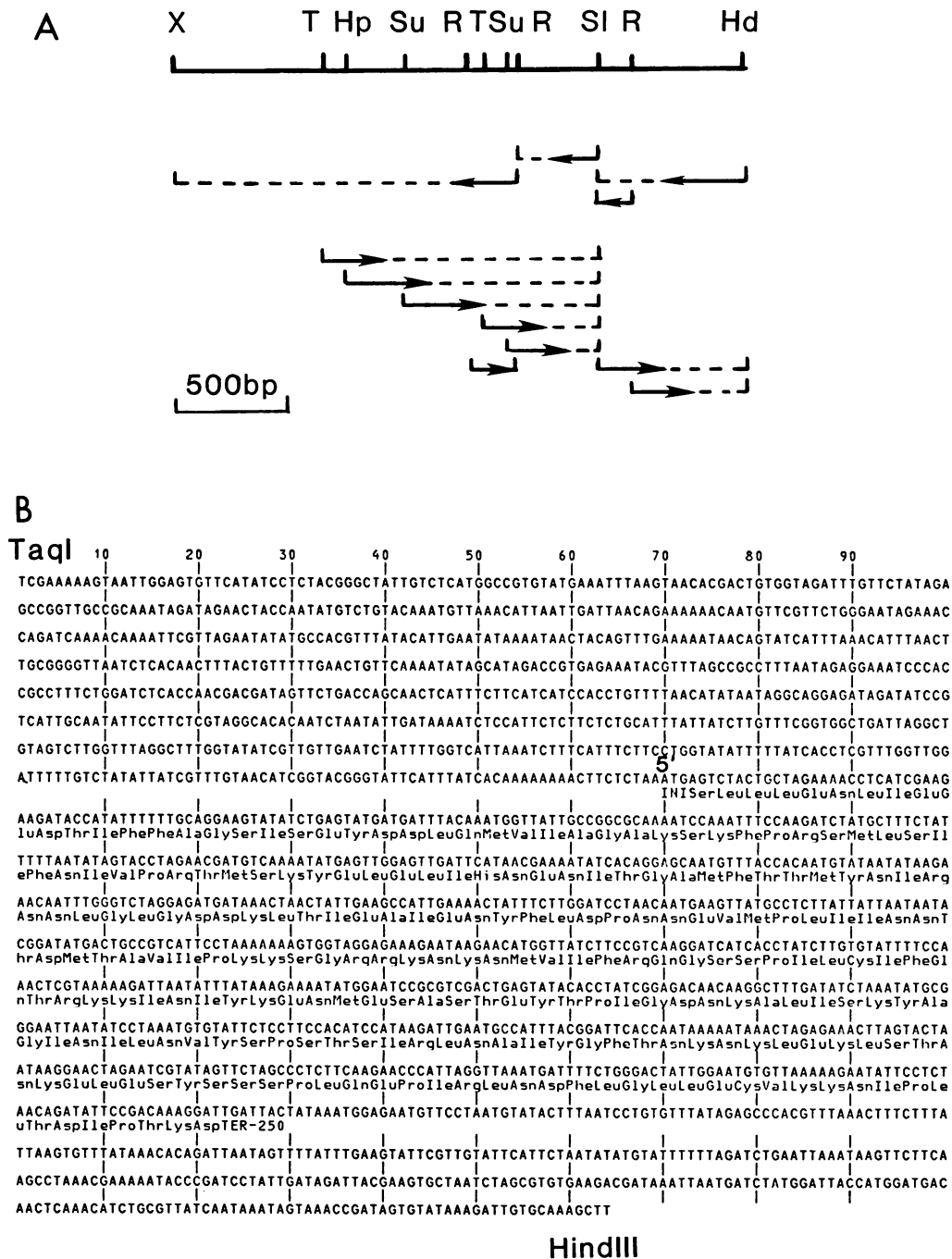


FIG. 3. Nucleotide sequence of the late gene and surrounding DNA. (A) A diagram of the right portion of the vaccinia virus *HindIII* L fragment, with restriction endonuclease sites abbreviated as follows: X, *XbaI*; T, *TaqI*; Su, *Sau3A1*; R, *RsaI*; SI, *SmaI*; Hd, *HindIII*. The indicated fragments were subcloned in M13mp8 or M13mp9 phage and sequenced by the dideoxynucleotide chain termination method. The solid arrows show the direction and extent of sequence determination. (B) The nucleotide sequence, the location of the 5' end of the late mRNA, and the derived amino acid sequence are shown. INI, initial methionine codon; TER, termination codon.

recombinants. The CAT coding sequence was inserted into the region from which the amino terminal half of the vaccinia virus TK gene was deleted so that the TK promoter was downstream and opposite to it in orientation. Upstream of the CAT gene are engineered *Bam*HI and *Sma*I restriction endonuclease sites followed by the carboxy-terminal half of the TK gene. The 366-bp putative late promoter fragment was inserted at the *Sma*I site of pMM22 in both orientations relative to the CAT gene (Fig. 5). In pLCAT1, the late

promoter was oriented correctly with respect to the coding sequence of the CAT gene, whereas in pLCAT2 the orientation was incorrect. The plasmid pMM23, which has a chimeric CAT gene containing a previously described early promoter for a 7.5K polypeptide (19), was constructed for comparative purposes (Fig. 5).

Vaccinia recombinants vLCAT1, vLCAT2, and vMM23 containing chimeric CAT genes inserted into the TK locus were formed by transfecting virus-infected cells with the



FIG. 4. Determination of the nucleotide sequence of the 5' end of the late mRNA. An *Xba*I-*Bgl*II fragment 5' labeled at the latter site was hybridized to late RNA and then digested with nuclease S1. The resistant DNA was denatured and applied to an 8% polyacrylamide gel in 7 M urea next to the products of the four chemical cleavage reactions described by Maxam and Gilbert (24). An autoradiograph is shown with the lanes corresponding to the cleavage reactions labeled G, G/A, T, T/C, and S1, containing nuclease-digested DNA. A portion of the nucleotide sequence is shown at the right, and a representation of the mRNA and DNA fragments is shown at the bottom.

corresponding plasmids pLCAT1, pLCAT2, and pMM23. TK⁻ virus plaques were selected in medium containing 5-bromodeoxyuridine and then checked for CAT DNA by a dot blot hybridization procedure (36). After further plaque purification, large virus stocks were prepared, and the predicted structure of each recombinant virus DNA was verified by hybridization of electrophoretically separated *Hind*III digests to ³²P-labeled vaccinia virus and CAT DNA (data not shown).

Expression of chimeric CAT gene in recombinant virus. The vaccinia virus recombinants described above were used to study the expression of CAT under control of the putative late promoter. HeLa cells were infected with recombinant or wild-type vaccinia virus, and at appropriate times, the cells were lysed and the cytoplasmic extracts were assayed for the presence of CAT. In previous studies, it has been established that CAT activity is not detectable in extracts of uninfected or wild-type vaccinia virus-infected cells but is present within 2 h after infection with virus recombinants containing the CAT gene fused to either of two early vaccinia promoters (20). One promoter, obtained from the TK gene, was expressed only at early times, whereas the other, obtained from a gene encoding a 7.5K polypeptide,

was expressed throughout infection. Similarly, we found that CAT was detected soon after infection with vMM23, which contains the 7.5K promoter (Fig. 6). In contrast, CAT activity started to increase between 6 and 9 h after infection with vLCAT1 (Fig. 6). No CAT activity was detected in cells infected with vLCAT2 in which the late promoter was improperly oriented (data not shown). Addition of cytosine arabinoside, an inhibitor of DNA replication, before infection completely prevented CAT synthesis directed by the late promoter (Fig. 6), whereas synthesis directed by TK promoters was unaffected, and synthesis directed by the 7.5K gene promoter was only partially affected (20). Both the time of onset of CAT synthesis and the dependence on DNA replication indicate that the 366-bp vaccinia DNA segment contained the signals for late transcription.

DISCUSSION

The present investigations were undertaken to determine the nature of a representative late transcriptional unit and the factors involved in regulation of late gene expression. The strategy we used to map a late gene involved the following steps. First, the approximate location of a gene encoding a major 28K protein was determined by cell-free translation of late mRNA selected by hybridization to cloned DNA fragments immobilized on nitrocellulose filters. Next, the direction of transcription was established by translation of late mRNAs that hybridized to single-stranded recombinant phage DNA. A discrete 5' end of the message was then mapped by single-stranded DNA-specific nuclease digestion and polyacrylamide gel electrophoresis after hybridization of late RNA to 5'-³²P-labeled DNA probes. Finally, the open reading frame of the structural gene was identified by nucleotide sequencing.

Nuclease S1 mapping studies indicated that the size diversity of late mRNAs is due to heterogeneity at their 3' ends. If this were caused simply by the absence of appropriate termination or processing sites, then the late RNA would be expected to end after a downstream early gene. Instead, translatable mRNAs for the 28K polypeptide hybridized to cloned DNA fragments that extended for at least several thousand nucleotides past several early genes (1). The overlapping of early genes by the late mRNA suggests that either the RNA polymerase or other factors necessary for termination or processing were modified. These results provide an explanation for the anomalous characteristics of late mRNA, including its size, sequence complexity, and ability to form RNA-RNA hybrids. Results of other recent studies also support these conclusions (14, 21, 48, 50). Although early vaccinia virus transcripts are of discrete sizes, examples of overlapping transcripts have been noted (1, 14, 22, 47). Length heterogeneity is also exhibited by certain adenovirus late transcripts (26).

The structural gene for the 28K polypeptide contained a continuous protein-coding sequence appropriate for the determined polypeptide size, indicating the absence of introns. Similarly, no evidence of splicing was found during previous analyses of early genes. This apparent distinction from the majority of other DNA viruses presumably reflects the use of viral enzymes and the predominantly cytoplasmic site of transcription. From the DNA sequence, it appears that three in-phase AUG codons occur after the start of the message. The first AUG is at or close to the cap site, whereas the second has the favored CXXAUGG sequence, in which X may be any nucleotide (17). However, since several polypeptides of similar sizes were resolved by polyacrylamide gel electrophoresis, it is possible that more than one site is used

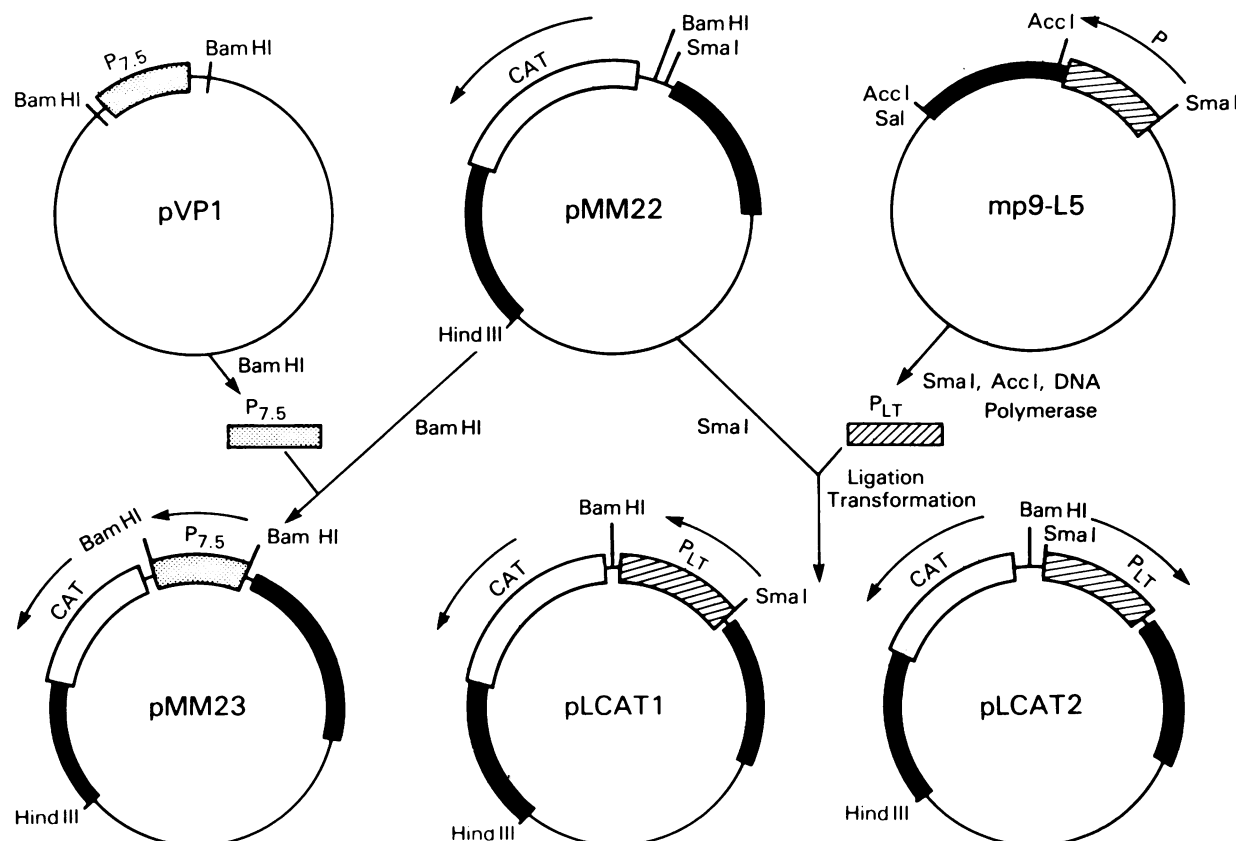


FIG. 5. Construction of chimeric CAT genes containing vaccinia virus promoters (P). Steps in the construction of pMM23, pLCAT1, and pLCAT2 are presented. The cross-hatched segment designated P_{LT} and the stippled segment designated P_{7.5} contain DNA preceding and including the RNA start site of the late 28K polypeptide gene and an early 7.5K polypeptide gene, respectively. Filled and unfilled segments represent vaccinia virus and CAT sequences, respectively. The functional orientation of promoter and coding segments are indicated with arrows.

for initiation. Preliminary studies involving pulse-labeling of infected cells suggest that the 28K polypeptide is a precursor of a virion protein.

The nucleotide sequence immediately preceding the late gene is of particular interest with regard to regulation.

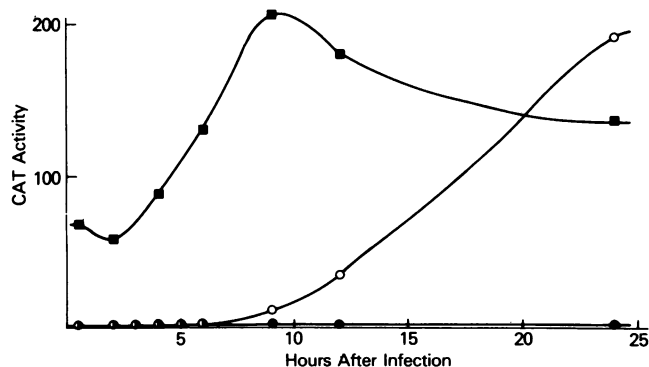


FIG. 6. Time course of CAT synthesis in cells infected with recombinant vaccinia viruses. HeLa cells in suspension culture were infected with 30 PFU of recombinant virus vLCAT1 per cell in the presence of 40 μ g of cytosine arabinoside per ml (●) or with vM23 (■) and vLCAT1 (○) in the absence of drug. At the indicated times, samples were removed and assayed for CAT activity. Activity is expressed as nanomoles $\times 10^{-2}$ of chloramphenicol acetylated per microliter of extract.

Perhaps the most notable feature is the presence of eight consecutive A residues centered about 13 nucleotides upstream of the RNA start site. This octamer corresponds precisely in position with a conserved sequence of A and T residues preceding several early genes (Fig. 7).

In spite of this A octamer, the late upstream sequence is less AT rich than the corresponding region of the early genes. The average AT content of the first 50 bases upstream of the four early genes is 83%, as opposed to 70% for the late promoter sequence. If the first 100 bases upstream are compared, the average AT content for the early promoters is 76% compared with 69% for the late promoter.

Evidence was obtained that the region preceding the late gene contains sequences required for the regulation of transcription. Those studies were carried out by isolating the putative promoter and ligating it to the coding sequence of CAT, an easily assayable procaryotic enzyme. The chimeric gene was then inserted into the vaccinia virus genome by homologous recombination. To avoid the influence of flanking sequences as much as possible, the gene was inserted so that several adjacent early genes (1, 45; unpublished data) were oppositely oriented and immediately upstream. Additionally, similar recombinant viruses were made that either contained no promoter or the promoter from an early gene. Since the recombinant viruses were stable and infectious, CAT expression could be assayed during a normal growth cycle. In the absence of a promoter, CAT activity was barely detectable. When the early promoter was present, CAT

	-100	-90	-80	-70	-60	-50	-40	-30	-20	-10	0
(I)	AACTGATCACTAATTC CAAAACCCACCGCTTTT TATAGTAAGTTTTT CACCCATAAATAAATAACAATAATTAATTTCTCGTAAAGTAGAAAAATATATTCTAATTTA										
(II)	TTTTTAACAGCAAACACATTCAATATTGTATTGTTATTTTATGTATTATTACACAATTAACAATATATTATTAGTTTATATTACTGAATTAATAATATAAAATCCCA										
(III)	TATAATATATACCTAATAATGTGTCTTAATAGTTCTCGTGATTCTGTC CAACAATCATTCTTATAAAATATAATAAGCAACGTAAACACATAAAAAAAGCGTAACATAA										
(IV)	CACCGCAATAGATCCTGTTAGATACATAGATCCTCGTCGCAATATCGCATTTTCTAACGTGATGGATATATTAAGTCGAATAAAGTGAACAATAATTAATCTTTATTG										
(V)	CATTTCTTCCTGGTATATTTTATCACCTCGTTGGTGGATTTTGTCTATATTATCGTTTGTAAACATCGGTACGGGTATTCATTTATCACAAAAAACTTCTCTAA										

FIG. 7. Comparison of the nucleotide sequences preceding four early and one late vaccinia virus genes. Nucleotide sequences preceding the RNA start sites of the 7.5K polypeptide gene (I) (39), 19K polypeptide gene (II) (41), 42K polypeptide gene (III) (41), TK gene (IV) (45), and 28K polypeptide gene (V). In each case, the major RNA start site is numbered 0. Conserved sequences preceding the four early genes (I through IV) (45) are underlined.

synthesis began almost immediately after infection, whereas with the late promoter, synthesis was delayed until the onset of DNA replication and was prevented by an inhibitor of the latter. Thus, *cis*-acting regulatory sequences that are translocatable immediately precede the late 28K gene.

The evident translatability of the chimeric CAT mRNA was of interest since cell protein synthesis is completely inhibited late in infection. This inhibition has been attributed to a variety of mechanisms, including specific viral effects on synthesis, stability, and translation of mRNA. Because of the way in which the chimeric gene was constructed, only the first eight nucleotides of the message can be of viral origin. The first in-phase AUG occurs at the start of the CAT gene, an additional 36 nucleotides downstream. Thus, if a viral mRNA leader sequence is required for late expression, it can be extremely short.

The manner in which regulation of gene expression is linked to replication is not understood. Prevention of virus DNA replication severely inhibited CAT expression when the CAT gene was under the control of a late promoter. *trans*-acting factors might be included in a small group of postreplicative proteins that are made before that of the majority of late species (10, 28, 33). Modification of the viral RNA polymerase is suggested by differences in the polypeptide composition of the enzyme isolated from virus particles (2) and from cells late in infection (30). Further elucidation of the regulatory mechanisms used by vaccinia virus should be of general interest since DNA replication acts as a selective switch mechanism for expression of procaryotic, eucaryotic, and other viral genes.

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