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Genetics

One hundred base pairs of 5′ flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription

(homologous *in vivo* recombination/promoter translocation/gene regulation)

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ABSTRACT **A vaccinia virus late gene coding for a major structural polypeptide of 11 kDa was sequenced. Although the 5′ flanking gene region is very A+T rich, it shows little homology either to the corresponding region of vaccinia early genes or to consensus sequences characteristic of most eukaryotic genes. Three DNA fragments (100, 200, and 500 base pairs, respectively), derived from the flanking region and including the late gene mRNA start site, were inserted into the coding sequence of the vaccinia virus thymidine kinase (TK) early gene by homologous *in vivo* recombination. Recombinants were selected on the basis of their TK⁻ phenotype. Cells were infected with the recombinant viruses and RNA was isolated at 1-hr intervals. Transcripts initiating either from the *TK* early promoter, or from the late gene promoter at its authentic position, or from the translocated late gene promoters within the early gene were detected by nuclease S1 mapping. Early after infection, only transcripts from the *TK* early promoter were detected. Later in infection, however, transcripts were also initiated from the translocated late promoters. This RNA appeared at the same time and in similar quantities as the RNA from the late promoter at its authentic position. No quantitative differences in promoter efficiency between the 100-, 200-, and 500-base-pair insertions were observed. We conclude that all necessary signals for correct regulation of late-gene expression reside within only 100 base pairs of 5′ flanking sequence.**

Vaccinia virus, a member of the poxvirus family, contains a large double-stranded DNA genome of 180 kilobase pairs. Expression of this large amount of genetic information is temporally well-regulated. Early genes are transcribed shortly after penetration of the virus particles into the host cell. After DNA replication, late genes encoding predominantly structural polypeptides are expressed. The molecular basis for this temporal regulation is not understood.

In contrast to other animal DNA viruses, which replicate in the nucleus of the infected cells and which use the host cell RNA polymerase to transcribe their genes, vaccinia replicates in the cytoplasm and utilizes its own transcription system. A multisubunit RNA polymerase (1, 2) and enzymes involved in modification of RNA (3, 4) have been isolated from purified virus particles. The mRNAs made by these enzymes are not spliced (5, 6) but have cap structures (7) and poly(A) tails (8), which are characteristic features of eukaryotic mRNAs. As might be expected, recent evidence suggests that vaccinia virus has evolved its own regulatory signals for gene expression. First, vaccinia genes are transcribed in cell-free extracts prepared from infected cells but not in extracts from non-infected cells (9), which suggests that the host-cell transcriptional machinery does not recognize vaccinia promoter elements. Second, the 5′ flanking regions of four early genes that have been sequenced (10–13) lack the regulatory elements characteristic of the corresponding regions of most cellular and viral genes transcribed by RNA polymerase II. A prerequisite for identification of the regulatory elements involved in gene regulation in vaccinia virus is additional nucleotide sequence information, particularly for the late genes.

An exciting recent discovery is that foreign DNA can be introduced into the vaccinia virus genome by homologous *in vivo* recombination (14–19). Expression of the inserted DNA was shown to be dependent upon the use of putative vaccinia virus promoter sequences (15). Apart from providing further support for the view that vaccinia virus has evolved its own promoter elements, such recombinant viruses should also be powerful tools for the study *in vivo* of DNA sequences involved in gene regulation.

In this communication, we present the nucleotide sequence of a vaccinia virus late gene and its flanking regions. Furthermore, we show that the 5′ flanking sequence, when translocated into the thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) early gene by homologous *in vivo* recombination, correctly regulates late transcription.

MATERIALS AND METHODS

Cells and Viruses. Primary chicken embryo fibroblasts from 11-day-old embryos were grown in Eagle's basal medium supplemented with 10% calf serum and 3 g of bactotryptose phosphate broth per liter. Human TK⁻ 143B cells (20) were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ; repository no. GM5887). Cells were grown in Eagle's minimal essential medium supplemented with 8% fetal calf serum and containing twice the regular amount of essential and nonessential amino acids and vitamins. Mouse LTK⁻ cells and African green monkey kidney cells (CV-1) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The vaccinia virus strain WR (obtained from B. Moss, National Institutes of Health, Bethesda, MD) and the Copenhagen strain were used in this study.

Molecular Cloning. Various vaccinia virus DNA restriction fragments were cloned in pBR322 by using standard procedures. The recombinant plasmids used to generate recombinant vaccinia virus were constructed as follows. A *Cla* I fragment containing the *11kDa* (the gene encoding the 11-kDa structural polypeptide) was cloned in pBR322. The DNA was cleaved with *Xba* I, *Rsa* I, or *Cla* I, and *Eco*RI linkers were added after filling in the staggered ends with deoxynucleoside triphosphates and DNA polymerase. The DNA then was digested with *Eco*RI, and fragments of 500, 200, and 100 base pairs (bp) derived from the 5′ flanking

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Abbreviations: *11kDa*, a vaccinia virus late gene encoding a major structural polypeptide of 11 kDa; TK, thymidine kinase; bp, base pair(s).

region of the *11kDa* gene were isolated and ligated into the unique *Eco*RI site located in the middle of the coding sequence of the *TK* early gene (ref. 13 and Fig. 1). As acceptor DNA, we used a recombinant pBR322 plasmid containing the left-hand portion of the *Hin*dIII J fragment, extending from the *Hin*dIII site to the *Hpa* II site (Fig. 1) and in which the *Eco*RI site had been eliminated from the pBR322 DNA.

Construction of Recombinant Vaccinia Virus. Primary chick embryo fibroblasts in 35-mm tissue culture dishes were infected with the vaccinia virus temperature-sensitive mutant t_S7 (21). After 2 hr at the permissive temperature, the cells were transfected with a calcium-phosphate DNA precipitate essentially as described (21). For each dish, we used 60 ng of wild-type vaccinia DNA that had been coprecipitated with 20 ng of the appropriate recombinant plasmid containing a putative promoter fragment of the late gene (see *Results*) inserted into the body of the *TK* gene. After 2 days of incubation at 39.5°C, the cells were disrupted and the amount of TK^- virus in the progeny was determined by titration in LTK^- cells in the presence of 5-bromodeoxyuridine at 100 μg/ml. An ≈200-fold increase in TK^- virus was found in cells that had been transfected with wild-type DNA and the recombinant plasmids over that found in cells that had been transfected with wild-type DNA alone. Plaques were picked and the virus was plaque-purified a second time in human TK^- 143B cells in the presence of bromodeoxyuridine at 30 μg/ml. Virus stocks then were produced in CV-1 cells in the absence of bromodeoxyuridine.

Isolation of RNA. RNA was purified from infected cell monolayers by a procedure, communicated by H. Koblet (University of Berne, Berne, Switzerland), that has been described previously (22).

Nuclease S1 Analysis. RNA transcripts were mapped by nuclease S1 analysis (23), with 5'-end-labeled DNA fragments as hybridization probes (24), essentially as described (5) except that hybridization and S1 treatment were carried out at 37°C. The DNA fragment used as probe 2 in the experiment shown in Fig. 5 was derived from the chimeric plasmid containing the *Cla* I–*Eco* RI fragment of 500 bp inserted in the *TK* gene. The DNA was partially digested with *Taq* I and partially digested with *Eco*RI. The appropriate partial digestion product was isolated and subcloned. After end-labeling, a fragment, which also contained the pBR322 sequences from the *Eco*RI site to the *Pst* I site, was isolated and used as the probe.

RESULTS

Nucleotide Sequence of a Vaccinia Virus Late Gene. In this laboratory, the gene (*11kDa*) coding for a major late structural polypeptide of 11 kDa has recently been mapped in the vaccinia virus genome (22). Fig. 1 shows a physical map of the vaccinia virus genome and the location of the *11kDa* coding sequence. The mRNA is transcribed from left to right and is initiated about 130 bp to the left of the *Hin*dIII site at the junction between the *Hin*dIII F and E fragments (22). The relevant region of the DNA encoding the late polypeptide was sequenced by the chemical degradation method (25), using appropriate end-labeled DNA fragments. The nucleotide sequence and the derived amino acid sequence are shown in Fig. 2.

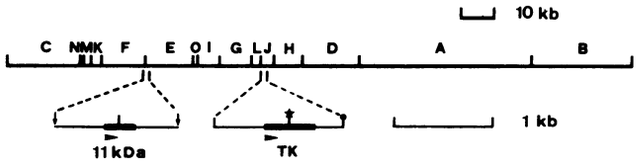

FIG. 1. Positions of the *11kDa* and *TK* genes in the vaccinia virus DNA. A *Hin*dIII restriction map of the vaccinia virus genome is shown. Letters designate *Hin*dIII restriction fragments of the vaccinia genome. The coding sequences are represented by the thick lines and the direction of transcription of the mRNAs is indicated by an arrowhead. Symbols for restriction sites: |, *Hin*dIII; ↓, *Cla* I; ↟, *Eco*RI; ↟, *Hpa* II. kb, Kilobase pairs.

Location of the 5' End of the mRNA for the 11-kDa Structural Protein. The 5' end of the mRNA encoding the 11-kDa protein was determined by nuclease S1 analysis (24). A *Cla* I–*Hin*dIII fragment of 650 bp and 5'-end-labeled at the *Hin*dIII site was hybridized to late RNA from vaccinia virus-infected cells. After nuclease S1 digestion, resistant material was electrophoresed alongside a "sequence ladder" prepared from the same DNA fragment (Fig. 3). The position of the two major S1-protected fragments permitted the localization of the 5' end of the mRNA to around nucleotides 189 and 190 in the sequence shown in Fig. 2. Multiple RNA transcription initiation ("start") sites have also been observed in vaccinia virus early genes (10, 11).

Late vaccinia virus transcripts are very heterogeneous in length (26), presumably as a consequence of incorrect termination of transcription late in infection. No attempt was made, therefore, to map the 3' end of the RNA.

Amino Acid Sequence of the 11-kDa Polypeptide. In the sequence shown in Fig. 2, the first translation initiation codon occurs at position 194, only 4 or 5 bases downstream of the mRNA start site. Similar short untranslated leader sequences have also been found in early mRNAs (11, 13). The open reading frame of 101 codons that follows ends with a TAA termination codon at position 497. The molecular weight of 11,400 calculated from the deduced amino acid composition agrees well with the size of the polypeptide determined by gel electrophoresis (27, 28). Furthermore, 17 of the 101 specified amino acids are basic whereas only 9 are acidic, which accounts for the basic nature (28) of the polypeptide. The 11-kDa polypeptide has been shown to be

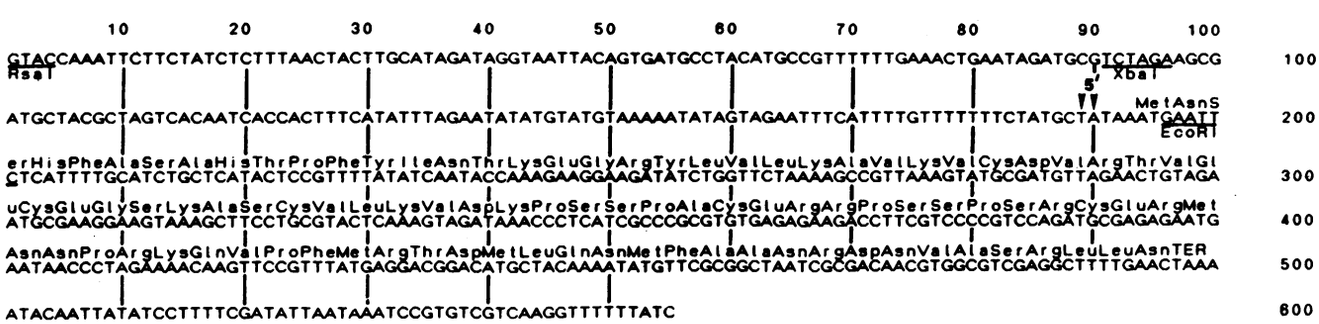

FIG. 2. Nucleotide sequence of the *11kDa* gene and flanking regions. The mRNA start sites are indicated by arrowheads. Three restriction sites (*Rsa* I, *Xba* I, and *Eco*RI) of particular interest (see text) are underlined. The derived amino acid sequence of the 11-kDa polypeptide is also shown.

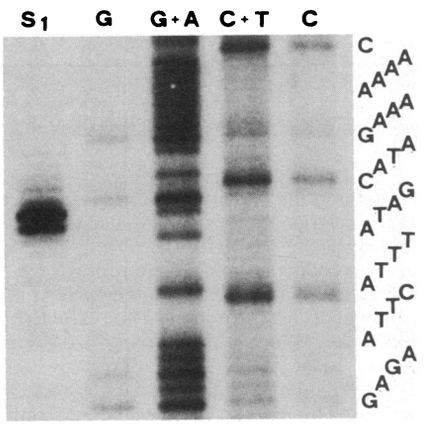

FIG. 3. Mapping of the 5′ end of the mRNA encoding the 11-kDa polypeptide. A DNA fragment of 650 bp, extending from a *Cla* I site to the *Hin*dIII site (see Fig. 1) was 5′-end-labeled at the *Hin*dIII site and hybridized to 25 μg of total late RNA from infected cells. Nuclease S1-resistant material (lane S1) and the products of the sequence reactions (lanes G, G+A, C+T, and C) of the same fragment were resolved in an 8% polyacrylamide sequencing gel.

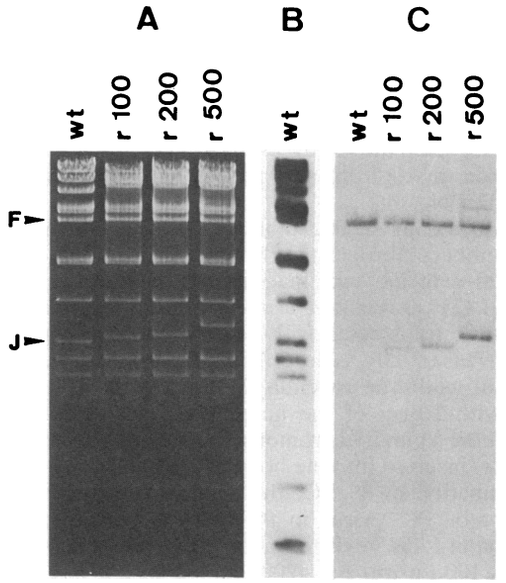

FIG. 4. Characterization of recombinant vaccinia virus. The DNA of wild-type virus (wt) and of TK⁻ virus isolated from cells that had been transfected with recombinant plasmid DNA containing insertions of 100, 200, and 500 bp in the *TK* gene (r100, r200, and r500, respectively) was cleaved with *Hin*dIII and analyzed by electrophoresis in an agarose gel followed by ethidium bromide staining (*A*). *Hin*dIII-digested DNA was transferred to nitrocellulose membranes and hybridized either with total vaccinia DNA (*B*) or with the 100-bp *Xba* I–*Eco*RI (see also Fig. 2) fragment (*C*). The positions of the *Hin*dIII J and F fragments are indicated.

phosphorylated (28). A very likely phosphorylation site is the serine encoded by the triplet at position 374. This serine lies in the amino acid sequence Arg-Arg-Pro-Ser, which is a natural substrate for the cAMP-dependent protein kinase that phosphorylates the serine in the sequence Arg-Arg-Xaa-Ser (29).

Construction of Recombinant Vaccinia Virus. To delineate the amount of DNA sequence information required for late gene regulation in vaccinia virus, we have constructed vaccinia virus recombinants. Three DNA fragments were inserted into the *TK* gene; these fragments were derived from the 5′ flanking region of the *11kDa* gene and included the initiation site of transcription. They extended from the *Eco*RI site (Fig. 2) located 6 or 7 bp downstream from the mRNA start site to the *Xba* I, *Rsa* I (Fig. 2), and *Cla* I sites (Fig. 1), respectively, that are about 100, 200, and 500 bp upstream of the mRNA start site. Plasmids that contained the putative late promoters inserted in the same orientation as the *TK* promoter were used to generate recombinant vaccinia virus by homologous *in vivo* recombination. Chick embryo fibroblasts were transfected with the DNA together with total vaccinia DNA as a calcium phosphate precipitate. Prior to transfection, the cells were infected with a temperature-sensitive (ts) mutant of vaccinia virus. The use of the ts mutant greatly reduces the background of nonrecombinant progeny virus, because virus multiplication at the nonpermissive temperature occurs only in cells that are successfully transfected by wild-type DNA and, therefore, most likely also be the recombinant plasmid DNA. After 48 hr at the nonpermissive temperature, the desired recombinants were isolated on the basis of their TK⁻ phenotype.

To verify that the altered phenotype was not due to spontaneous mutation but due to insertion of the *11kDa* promoter into the *TK* gene, DNA was purified from several TK⁻ virus isolates and cleaved with *Hin*dIII (Fig. 4*A*). The increments in size of the *Hin*dIII J fragments were consistent with the expected insertions of 100, 200, and 500 bp. Recombinants carrying the 100-, 200-, and 500-bp insertions in the *Hin*dIII J fragment were designated r100, r200, and r500, respectively (Fig. 4). To demonstrate that the increments in size were indeed due to DNA derived from the *Hin*dIII F fragment, the *Hin*dIII digests from the recombinants were transferred to nitrocellulose and probed with the radioactively labeled 100-bp *Xba* I–*Eco*RI fragment (Fig. 4*C*) derived from the 5′ flanking region of the *11kDa* gene. As expected, the probe hybridized to the *Hin*dIII F fragment of wild-type virus and, in addition for all three recombinants, also to the *Hin*dIII J fragment which contains the *TK* gene into which the 5′ flanking region of the *11kDa* gene had been inserted. As an alternative means of demonstrating DNA insertion, the *Hin*dIII J fragments of the recombinants were isolated and cleaved with *Eco*RI. The resulting fragments were electrophoresed alongside corresponding digests of the recombinant plasmids used for transfection. From the *Hin*dIII J fragments of all three recombinants, *Eco*RI fragments of the expected size were observed (results not shown). Thus, a *TK* gene containing DNA fragments from the 5′ flanking region of the *11kDa* late gene had successfully been incorporated into the vaccinia virus genome by homologous recombination.

Transcription from the Translocated Promoter Fragments. To determine whether the DNA fragments derived from the 5′ flanking region of the late gene and inserted into the *TK* gene are sufficient to temporally regulate late transcription, cells were infected with the three recombinants and with wild-type virus as a control. RNA was isolated at 1-hr intervals until 8 hr after infection. The time course of appearance of RNA was followed by nuclease S1 analysis, using 5′-end-labeled DNA fragments as probes (Fig. 5 *Upper*). Each probe was labeled at a restriction site within the coding sequences of the genes. These probes detected transcripts from the *TK* early promoter (probe 1), from the *11kDa* translocated promoter (probe 2), or from the late promoter at its authentic position (probe 3).

At 2 hr and 3 hr after infection, only transcripts from the *TK* early promoter are detected (250-bp protected fragment in Fig. 5 *Lower*). This early RNA appears at about the same time in cells infected with wild-type virus as in cells infected with the recombinant viruses. Moreover, similar levels of RNA are observed at any one point in infection. Thus, insertion of the late promoters into the *TK* gene does not influence the temporal regulation of early gene expression.

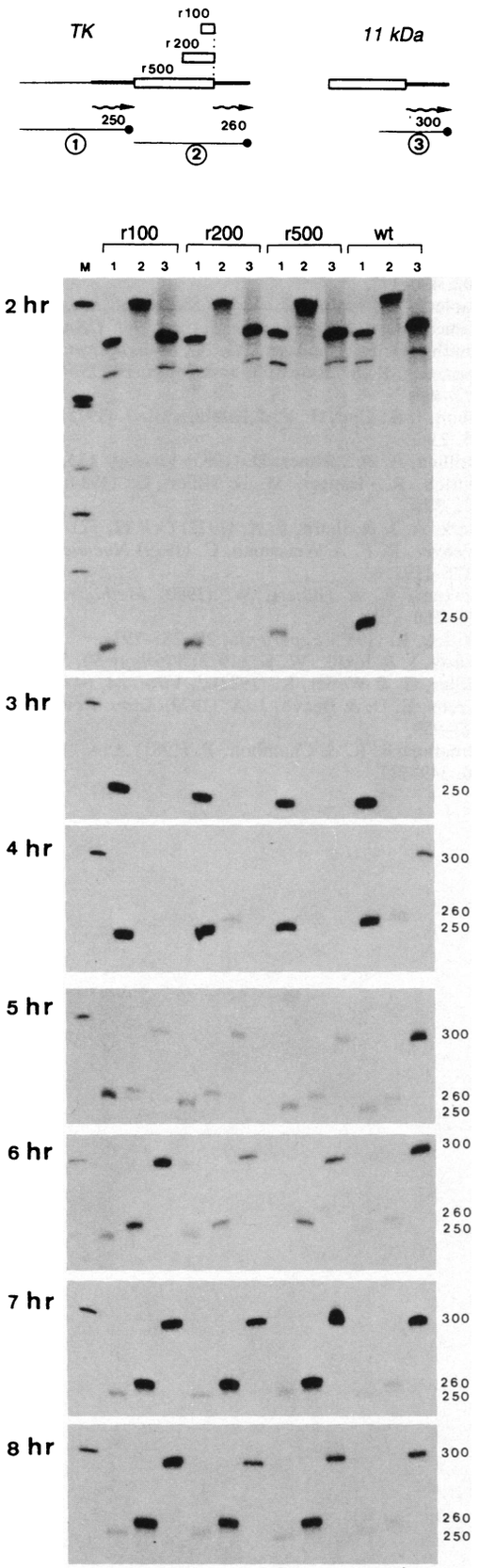

FIG. 5. Nuclease S1 analysis of RNA transcripts. (*Upper*) The 5'-end-labeled (solid circles) probes 1, 2, and 3 used to detect transcripts from the *TK* and *11kDa* promoter. The coding sequences are indicated by thick lines, and the 5' flanking sequences of the *11kDa* gene, by boxes. Probe 1 consisted of a DNA fragment from the *Hin*dIII site to a *Cla* I site located close to the *Eco*RI site within *TK* (Fig. 1 and ref. 13). For probe 2, a fragment from the *Cla* I site at the left of the *11kDa* gene (Fig. 1) to the *Taq* I site (13) close to the end of the *TK* coding sequence was used. Probe 3 consisted of a DNA fragment from a *Sau*3A site to the *Taq* I site (Fig. 2) near the end of the *11kDa* coding sequence. The sizes of the nuclease S1-protected fragments (wavy arrows) are also indicated. (*Lower*) Cells were infected with recombinant vaccinia virus r100, r200, or r500 or with wild-type virus (wt). Samples (25 μg) of total cytoplasmic RNA, which was isolated at 1-hr intervals after infection, were hybridized with each probe (lanes 1–3). After nuclease S1 digestion, resistant material was analyzed in a 6% polyacrylamide sequencing gel. The entire autoradiogram is shown for the first time point. For subsequent time points only the relevant portions are shown. Times after infection are given at left, and fragment size (in bases), at right. Lane M, end-labeled *Hin*fI fragments from pBR322, used as length markers.

At 4 hr after infection, transcripts originating from the late promoters are detectable as faint bands. These bands increase in intensity during the course of infection. RNA initiated from the *11kDa* late promoter at its authentic position accumulates again with similar kinetics, characteristic of late gene expression, in wild-type as well as recombinant virus. Most significant, however, the appearance of RNA initiated from the translocated promoters follows precisely the kinetics of that initiated from the *11kDa* promoter at its correct position. Furthermore, no major qualitative or quantitative differences between the insertions of 100, 200, or 500 bp can be detected. Thus, 100 bp of 5' flanking sequence are sufficient to regulate late transcription correctly.

DISCUSSION

In this communication, we present the nucleotide sequence of a vaccinia virus late gene and its flanking regions. The gene encodes a major structural polypeptide, which contributes about 10% of the total protein mass of the virus particle (27) and which is phosphorylated and basic (28). Inspection of the coding sequence reveals an open reading frame encoding a polypeptide of the predicted size and basic nature.

The sequence upstream of the initiation site of transcription of the *11kDa* late gene is extremely A+T rich (82% adenosine plus thymidine in the first 50 bp). In the 5' flanking region of most eukaryotic genes transcribed by RNA polymerase II, the consensus sequence T-A-T-A-(A/T)-A-(A/T) is located about 25–30 bp upstream from the mRNA start site (30). A second region of homology, the sequence G-G-(C/T)-C-A-A-T-C-T, has been found 70–80 bp from the RNA start sites of several genes (30). In the corresponding regions of the vaccinia virus gene, the sequences T-A-T-A and C-A-A-T-C, respectively, show some homology to these consensus sequences. Their functional significance, if any, remains to be determined. Apart from the very high A+T content, the 5' flanking sequence of the late gene shows little homology to the corresponding regions of early genes.

We show here that a relatively short stretch of DNA contains all the necessary regulatory signals for late gene expression. This was demonstrated by inserting fragments derived from the 5' flanking region of the late gene into the coding sequence of the *TK* early gene. This gene was chosen as the acceptor for these translocation experiments to rule out any positional effect on the proper functioning of the late promoter. In its entirely new sequence environment, even the smallest fragment tested, of only 100 bp, correctly regulated late transcription. Further, this fragment was as active in promoting transcription as the late promoter at its authentic location. A search for putative late-specific regulatory elements within these 100 bp is hampered by the lack of sequence information on other vaccinia virus late genes.

Clearly, more work is required in order to identify the regulatory elements involved in gene regulation in vaccinia virus. Since all information required for correct initiation and temporal regulation of transcription are contained within a maximum of 100 bp, this stretch of DNA should be readily accessible to further analysis.

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1. Baroudy, B. M. & Moss, B. (1980) *J. Biol. Chem.* **255,** 4372–4380.
2. Spencer, E., Shuman, S. & Hurwitz, J. (1980) *J. Biol. Chem.* **255,** 5388–5395.
3. Martin, S. A., Paoletti, E. & Moss, B. (1975) *J. Biol. Chem.* **250,** 9322–9329.
4. Moss, B., Rosenblum, E. N. & Gershowitz, A. (1975) *J. Biol. Chem.* **250,** 4722–4729.
5. Wittek, R., Cooper, J. A., Barbosa, E. & Moss, B. (1980) *Cell* **21,** 487–493.
6. Cooper, J. A., Wittek, R. & Moss, B. (1981) *J. Virol.* **39,** 733–745.
7. Wei, C. M. & Moss, B. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 318–322.
8. Kates, J. R. & Beeson, J. (1970) *J. Mol. Biol.* **50,** 19–33.
9. Puckett, C. & Moss, B. (1983) *Cell* **35,** 441–448.
10. Venkatesan, S., Baroudy, B. M. & Moss, B. (1981) *Cell* **125,** 805–813.
11. Venkatesan, S., Gershowitz, A. & Moss, B. (1982) *J. Virol.* **44,** 637–646.
12. Hruby, D. E., Maki, R. A., Miller, D. B. & Ball, A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 3411–3415.
13. Weir, J. P. & Moss, B. (1983) *J. Virol.* **46,** 530–537.
14. Panicali, D. & Paoletti, E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4927–4931.
15. Mackett, M., Smith, G. L. & Moss, B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7415–7419.
16. Panicali, D., Davis, S. W., Weinberg, R. L. & Paoletti, E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5364–5368.
17. Smith, G. L., Mackett, M. & Moss, B. (1983) *Nature (London)* **302,** 490–495.
18. Paoletti, E., Lipinskas, B. R., Samsonoff, C., Mercer, S. & Panicali, D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 193–197.
19. Smith, G. L., Godson, G. N., Nussenzweig, V., Nussenzweig, R. S., Barnwell, J. & Moss, B. (1984) *Science* **224,** 397–399.
20. Rhim, J. S., Cho, H. Y. & Huebner, R. J. (1975) *Int. J. Cancer* **15,** 23–29.
21. Drillien, R. & Spehner, D. (1983) *Virology* **131,** 385–393.
22. Wittek, R., Hänggi, M. & Hiller, G. (1984) *J. Virol.* **49,** 371–378.
23. Berk, A. J. & Sharp, P. A. (1977) *Cell* **12,** 721–723.
24. Weaver, R. F. & Weissman, C. (1979) *Nucleic Acids Res.* **7,** 1175–1193.
25. Maxam, A. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
26. Wittek, R. (1982) *Experientia* **38,** 285–297.
27. Sarov, I. & Joklik, W. K. (1972) *Virology* **50,** 579–592.
28. Hiller, G. & Weber, K. (1982) *J. Virol.* **44,** 647–657.
29. Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48,** 923–959.
30. Breathnach, R. & Chambon, P. (1981) *Annu. Rev. Biochem.* **50,** 349–383.