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Discontinuous Transcription or RNA Processing of Vaccinia Virus Late Messengers Results in a 5' Poly(A) Leader

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Summary

We have demonstrated by primer elongation and cap analysis that mature vaccinia virus late transcripts are discontinuously synthesized. We have shown that RNA transcripts from a translocated 11K and from the authentic 11K and 4b late promoters are extended by approximately 35 nucleotides beyond the "start site" determined by S1 mapping using vaccinia genomic DNA as a probe. Sequencing of the RNA and of the first strand cDNA reveal that a homopolymeric poly(A) sequence is linked to the 5' terminus of the RNA transcripts. S1 mapping of RNA transcripts with a DNA probe containing an A-stretch, replacing promoter sequences upstream of position -1, confirms the existence of a poly(A) leader of approximately 35 A-residues.

Introduction

Transcription of the cytoplasmic vaccinia virus can be divided into two phases: an immediate early/early phase starting shortly after the infection of the cell, and a late phase starting with the onset of DNA replication (2-5 hr after infection). The mechanisms of the temporal regulation of gene expression, i.e., the switching from early to late transcription, are unknown.

The early genes are characterized by the presence of an untranslated leader sequence and their transcripts have discrete 3' ends (Venkatesan et al., 1961; Yuen and Moss, 1986). In contrast, late genes appear to lack an untranslated leader as well as termination signals at their 3' ends, which results in readthrough by RNA polymerase. Furthermore, early termination signals are not recognized in the late phase of infection (Smith et al., 1984). Basic promoter sequence elements such as a TATA box or CAAT sequence are not present in vaccinia promoters. The vaccinia early and late promoters are not recognized by prokaryotic or eukaryotic RNA polymerases (Smith et al., 1984). Vaccinia messengers appear to have a capped 5' end consisting of a 7-methyl-guanosine (m7G) residue (Wei and Moss. 1975; Urushibara et al., 1975), and they have an A-tail at their 3' ends (Nevins and Joklik, 1975). The regulatory signals controlling the transcription and the time of gene activation reside in very short stretches of approximately 20 to 30 bp. This has been shown by translocation of promoter fragments by means of homologous recombination (Cochran et al., 1985b; Rosel and Moss, 1985; Hänggi et al., 1986).

Vaccinia late promoters are characterized by the presence of a highly conserved TAAAT motif that overlaps the site of transcription initiation as determined by S1 mapping (Plucienniczak et al., 1985; Hänggi et al., 1986). The functional analysis of the vaccinia late promoter of the 11K basic polypeptide revealed that the sequences from position -29 to +8 (+1 is arbitrarily defined as the A-residue of the AUG) are sufficient for transcriptional activity (Hänggi et al., 1986). This was shown through the insertion of a chimeric gene consisting of the wild-type 11K promoter up to position +8, and the coding region of the mouse dihydrofolate reductase (dhfr) gene into the vaccinia thymidine kinase (tk) gene by homologous recombination (Panicali et al., 1982; Mackett et al., 1982). We have shown that mutations within the conserved TAAAT motif result in complete inactivation of promoter activity. We further show that mutations of sequences surrounding the TAAAT motif either have no effect or increase the overall promoter strength. The mutated regions of the translocated fragment include the start codon of translation; this sequence is partially conserved as reflected in the consensus sequence TAAAT (Hänggi et al., 1986). The AUG start codon of translation is either part of the TAAAT motif or immediately adjacent to this element.

This paper concerns the study of the structure of the 5' terminus of vaccinia virus late RNA transcripts. We demonstrate that the transcripts of both wild-type and translocated promoters are discontinuously synthesized and obtain a poly(A) leader sequence. We will discuss possible mechanisms involved in the synthesis of the discontinuous late transcripts.

Results

Preliminary observations made in our laboratory demonstrated that mutations downstream of the TAAAT motif result in a 4- to 5-fold increase in promoter strength as compared to the wild-type translocated promoter. In contrast, the efficiency of translation can be decreased more than 10-fold as compared to the wild-type translocated-dhfr construct. This phenomenon appears to be independent of the test gene cloned downstream of the mutated promoter (unpublished data). A detailed analysis of the different mutants will be presented elsewhere. The reduction of the translatability of these messengers indicates that the sequence at the 5' end of the mRNA might be directly or indirectly involved in a translational control mechanism. We have therefore analyzed the structure and sequence at the 5' end of late vaccinia messengers in more detail.

Primer Elongation

The site of transcription initiation of late vaccinia mRNA has been determined thus far by S1 mapping experiments using genomic DNA as a probe (Cochran et al., 1985b; Rosel and Moss, 1985; Bertholet et al., 1985; Hänggi et al., 1986). The reason for this is that primer elongation experiments are complicated by a high degree of complementarity in late vaccinia RNA transcripts, both DNA
strands are transcribed, genes can be overlapping, and there is readthrough of the RNA polymerase (Plucieniczak et al., 1985; Smith et al., 1984). If, however, the mature messenger is discontinuously synthesized, S1 mapping experiments can only reveal the putative junction site and not the 5' end of the mature messenger. We have been able to avoid a high nonspecific background in primer extension experiments by end-labeling the synthetic oligonucleotide primers. The results of such primer extension experiments using different gene internal primers and RNA derived from the translocated 11K promoter-dhfr gene construct are shown in Figure 1. If the 5' end of the RNA as determined by S1 mapping (Hänggi et al., 1986) represents the genuine 5' end of the mature transcript, we would obtain a primer elongated cDNA migrating at the position indicated by the arrows. However, the majority of the cDNA products are extended by approximately 35 bases beyond the S1 "start site" independent of the position of the synthetic oligonucleotide (Figure 1, lanes 2–4). As a negative control, we have performed primer extension with a dhfr primer and wild-type mRNA that does not contain dhfr sequences (Figure 1, lane 1). The fact that the primer extension does not coincide with S1 mapping indicates that the transcripts are discontinuous and that a leader RNA is linked to the transcripts. On a long exposure a faint band migrating at the position of the S1 "start site" is detectable (not shown). Furthermore, very long cDNAs are obtained, which might represent readthrough transcripts, and cDNAs shorter than the S1 "start site," which are probably premature stops of the reverse transcriptase.

Analysis of the Cap Structure
The 5' end of mature mRNA is characterized by the presence of a cap structure consisting of a 7-methylguanosine (m7G) residue linked with a triphosphate bridge to the RNA. Furthermore, the adjacent residue (in general G or A) is methylated at the 2'-O-ribosyl position (Banerjee, 1980). Vaccinia messenger RNAs also have a m7G cap.
A 5' Poly(A) Leader

Figure 3. Primer Extension of Late Transcripts

RNA from cells infected with the 11K-dhfr recombinant virus was primer extended using end-labeled oligonucleotides for the dhfr, the authentic 11K, and 4b genes. Lanes 1 and 2: dhfr transcripts with primers at position +24 to +46, respectively, +66 to +113; lanes 3 and 4: 11K transcripts with primers at position +14 to +33, respectively, +52 to +71; lane 5: 4b transcripts with a primer at position +10 to +29. The positions of the TAAAT motif representing the S1 "start sites" are indicated by the arrows. (M): [32P]-labeled HpaI-digested pBR-322 DNA size markers.

structure (Wei and Moss, 1975; Urushibara et al., 1975). In order to prove that the RNA transcripts containing the discontinuous leader sequence are mature messengers with a cap structure, we used a polyclonal antiserum against the m7G cap (Munns et al., 1982) to immune-precipitate the cDNA–RNA hybrids. The cDNA–RNA hybrids from the 11K translocated promoter, which are extended beyond the S1 start site (indicated by a bar), are selectively enriched by the immunoprecipitation (Figure 2, lane +). Some additional smaller cDNA fragments are also retained, as are some readthrough products. The cDNA band migrating at the position of the S1 "start site" is not precipitated by the antibodies, indicating that this RNA species does not have a cap structure.

The length of the "extension" appears to vary within 30–40 nucleotides. This heterogeneity is not due to premature stops of the reverse transcriptase: the cDNA–RNA hybrids can be retained by the antibody column after treatment with RNAase A prior to cap selection. Some smaller cDNA fragments are also retained, as some readthrough products. The cDNA band migrating at the position of the S1 "start site" is not precipitated by the antibodies, indicating that this RNA species does not have a cap structure.

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Primer Elongation of Wild-Type Late Vaccinia Messengers

We have demonstrated that transcripts from the 11K translocated promoter–dhfr gene construct are discontinuously synthesized with a leader RNA of approximately 35 bases 5' of the S1 "start site." The question now arises as to whether this is a general phenomenon of vaccinia late transcription. We have performed primer extension with synthetic oligonucleotides of two wild-type vaccinia mRNAs coding for the authentic 11K basic polypeptide (Wittek et al., 1984) and for the structural protein 4b (Rose1 and Moss, 1984). The results clearly demonstrate that both the authentic 11K (Figure 3, lanes 3 and 4) and the 4b (Figure 3, lane 5) messengers are extended by approximately 35 nucleotides beyond the S1 "start site" as we have shown for the translocated 11K messengers (Figure 3, lanes 1 and 2). After RNAase treatment–cap selection of the cDNA–RNA hybrids, we obtained the same results as for the wild-type translocated promoter–dhfr messengers (data not shown).

RNA Sequencing

The covalently linked leader RNA was sequenced by primer elongation in the presence of dideoxynucleotides using an end-labeled synthetic oligonucleotide. These experiments were again complicated by the presence of relatively high amounts of readthrough transcripts which initiate at upstream promoters. We therefore obtained two different RNA sequences: the promoter sequence which is present in the readthrough transcripts and the sequence of the discontinuous transcripts initiated at the respective promoter upstream of the putative junction. Downstream of the junction we obtained the uniform sequence of the coding body of the gene. The sequence of the RNA transcripts from the 11K translocated (Figure 4A) and both wild-type 11K (Figure 4B) and 4b (not shown) promoters show a stretch of minimally 10–15 T-residues in the complementary strand upstream of the AUG translation start codon. This sequence is present neither in the genomic sequence of the different promoters nor immediately upstream of them. We also observed on longer exposures promoter sequences present in the readthrough transcripts which are initiated at promoters further up...
RNA from cells infected with the wild-type virus (strain wr) or with the 11K-dhfr recombinant virus were primer extended in the presence of dideoxy-nucleotides using the 5'-labeled primers (+16 to +46) for dhfr transcripts (A) (respectively +14 to +33), and for 11K transcripts (B) as described in Experimental Procedures. The promoter-gene sequences surrounding the AUG are indicated.

Sequencing of the First Strand cDNA
The RNA sequencing revealed the presence of an A-stretch of at least 10–15 nucleotides 5' of AUG start codon. We wished to determine the complete sequence of the cDNA, but were unable to lower the dideoxy-nucleotide concentration without the introduction of unspecific ghost bands. Furthermore, standard cDNA cloning procedures (Oka- yama and Berg, 1982; Gubler and Hoffmann, 1983) using vaccinia late mRNA appeared to be, in our hands, highly susceptible to artifacts. We therefore decided to sequence the primer extended first strand cDNAs from the translocated 11K-dhfr gene construct and from the authentic 11K and 4b promoters by the method of Maxam and Gilbert (1980). For this purpose the extended cDNAs were extracted from denaturing gels and sequenced. In all three cases the sequence revealed a homopolymeric T stretch of more than 20 nucleotides in the complementary strand 5' of the S1 "start sites." Only the result of cDNA sequencing of the translocated 11K promoter transcripts is shown (Figure 5). We obtained bands in all four lanes, with the first ten nucleotides indicating the end of the cDNA. Alternatively, the cleavage in all four lanes might be due to misincorporations of the reverse transcriptase, which has been reported to occur following homopolymeric stretches (Murphy et al., 1986).

S1 Mapping of the Poly(A) Leader
We have analyzed thus far the 5' end of the mRNA in an indirect manner using reverse transcriptase. The obtained results now enable us to construct an artificial S1 probe complementary to the discontinuous in vivo mRNA in which a stretch of 80 A-residues replaces promoter sequences upstream of position −1 of the translocated 11K-dhfr gene construct and of the authentic 11K gene (Figures 6A and 6B, lane 2). As a control we have performed S1 mapping using genomic vaccinia DNA as a
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167 fragments corresponding to the discontinuous transcripts are indicated in the drawing. The Sl mapping of dhfr formed at low temperature to minimize nibbling of the transcripts. The digestion with nuclease Sl was performed by bars. (i) input DNA fragments; (M): [32P]-labeled HpaII- or probe from the Sl fragments protected by readthrough sequences. This allows the separation of the input DNA genomic Sl probes consist of nonhomologous plasmid artificial DNA probes complementary to the discontinuous mRNA (lane 2) of the dhfr(A) respectively authentic IIK gene (9). The Sl protected probe (Figures 6A and 6B, lane 1). The 3' ends of these genomic Sl probes consist of nonhomologous plasmid sequences. This allows the separation of the input DNA probe from the Sl fragments protected by readthrough transcripts. The digestion with nuclease Sl was performed at low temperature to minimize nibbling of the nuclease at the 5' end of the RNA. The results shown in Figures 6A and 6B (lane 2) confirm the presence of the poly(A) stretch of approximately 35 nucleotides at the 5' end of the mRNA. The Sl protected fragments of 350, respectively 290, nucleotides in length, which occur after mapping with the artificial DNA probe containing the 5' A-stretch (Figures 6A and 6B, lane 2), are mapping at the position of the Sl "start site" and correspond to readthrough transcripts that do not have an A-stretch at the 5' end. We observed the same percentage of transcripts initiated at the translocated 11K promoter as compared to readthrough transcripts after Sl mapping using either the genomic or the artificial Sl probes (Figure 6A, compare the band migrating at 485 in lane 1 with the band at 350 nucleotides in lane 2). This indicates that the majority of the transcripts initiated at the translocated promoter contain the A-stretch at their 5' ends. Readthrough of the RNA polymerase is again more prominent at the t(k locus in the middle of the genome than at the location of the authentic 11K gene in the left-hand side of the genome. We have observed the same phenomenon in the RNA sequencing experiments (Figures 4A and 4B). We conclude that vaccinia late mRNAs are discontinuously synthesized with a poly(A) stretch 5' of the Sl "start site."

Discussion

Unusual mechanisms of gene transcription have been reported for corona virus (Spaan et al., 1982) and trypanosomes (Murphy et al., 1986; Sutton and Boothroyd, 1986). Vaccinia virus, or the poxviruses in general, might also have developed unique mechanisms as a consequence of their cytoplasmic location. The virus does in fact have its own transcription and replication machinery (Moss, 1978). Furthermore, recent studies concerning the functional analysis of viral promoters confirm the earlier observation that they possess unique features that are not found in their eukaryotic counterparts (Cochran et al., 1985a; Rosel and Moss, 1985; Häggli et al., 1986).

The observation that mutations within the conserved sequence of the translocated 11K promoter can result in a strong reduction of translation without affecting transcription lead us to analyze the structure of the 5' end of the messengers. The possibility that the sequences following the conserved TAAAT motif might be involved in maturation of the RNA transcripts is further suggested by the fact that there is a sequence conservation downstream of the TAAAT motif (Häggli et al., 1986).

We have shown by primer elongation that the 5' end of the mature late messengers does not coincide with the site of transcription initiation as determined by Sl mapping (Figures 1 and 3). An RNA leader sequence of approximately 35 bases is linked to RNA transcripts originating from the 11K translocated promoter and from the authentic 11K and 4b promoters. We have shown by cap selection using a rabbit anti-m7G antiserum that the extended transcripts represent mature mRNAs with an m7G cap structure at their 5' terminus. Treatment of the cDNA-RNA hybrids obtained after primer extension with different RNAases prior to cap selection did not remove the cap structure (Figure 2). This demonstrates that we are obtaining full-length cDNA and not premature stops of the reverse transcriptase within the discontinuous leader RNA. Sequencing of the RNA transcripts and of the first strand cDNA revealed that the leader RNA consists of a homopolymeric stretch of at least 20 A-residues (Figures 4 and 5). The presence of other bases in front of the A-stretch cannot formally be excluded on the basis of the sequencing data. It has been described that reverse transcriptase can misincorporate nucleotides after homopolymeric stretches (Murphy et al., 1988). This phenomenon could explain the observed cleavage in all four lanes in the 5' end of the cDNA sequence (Figure 5). However, Sl mapping of RNA transcripts with an artificial DNA probe containing a stretch of 80 A-residues upstream of position −1 replacing promoter sequences confirms the presence of a poly(A) stretch of approximately 35 nucleotides (Figures...
of a poly(A) RNA and a subsequent capping of the RNA might also be possible since an RNA-specific phosphatase activity is detectable in vaccinia virions (Spencer et al., 1978). Furthermore, poly(A) polymerase and capping enzyme are reported to be present in purified vaccinia virions (Baroudy and Moss, 1980; Wei and Moss, 1974). We have recently established an in vitro transcription system specific for late vaccinia promoters. Preliminary results indicate that the poly(A) leader RNA is also present at the 5' end of late transcripts in a late specific in vitro extract.

Experimental Procedures

Purification of RNA and S1 Mapping

RNA of RK-13 or HeLa S3 cells infected with vaccinia virus recombinants or the wild-type strain was extracted with guanidinium hydrochloride followed by CsCl purification as described by Maniatis et al. (1982). S1 mapping of the mRNA and of the first strand cDNA was performed according to Maniatis et al. (1982). The hybridization was performed at 44°C and the nucleotide S1 digestion at 4°C.

Primer Elongation and RNA Sequencing

Primer elongation was performed using 32P-labeled synthetic oligonucleotides. The labeled oligonucleotide was incubated at 55°C with the RNA, slowly cooled down to 42°C and coprecipitated with 0.5 volumes of ethanol in the presence of 0.6 M NaCl. Ten units of reverse transcriptase and 20 U of RNAase inhibitor were used with 10 ng of total RNA. In the RNA sequencing experiments, the deoxy- to deoxyribonuclease ratios were b, 1.4, 1.4, respectively 2.7, for T, A, G, respectively C-reactions.

CAP Selection

The cDNA-RNA products from the primer elongation were phenol extracted and precipitated. The hybrids were incubated overnight at 4°C in binding buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% NP40 with the polyclonal rabbit anti-m7G in the presence of 1 mg/ml of Heparin (174,000 U/g)). The nucleic acid–antibody complex was subsequently incubated for 1 hr at room temperature with protein A-Sepharose in the same buffer containing 20 ng/ml of heparin. The beads were washed three times with binding buffer and twice with binding buffer containing 500 mM NaCl. The bound nucleic acids were removed by SDS-proteinase K treatment, phenolized, precipitated, and separated on a sequencing gel. RNAase treatment of the cDNA-RNA hybrid was performed with RNAases A at a final concentration of 10 μg/ml in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA for 10 min at room temperature, the nucleic acids were phenol extracted, and the cDNA-RNA hybrids immune-precipitated as described.

Sources of Materials

AMV reverse transcriptase and RNAase inhibitor were purchased from Genoht, Geneva, or Boehringer Mannheim. The RNAases A, T1, and restriction endonucleases were purchased from Boehringer Mannheim. Protein A-Sepharose CL-4B and nucleotide S1 were obtained from Pharmacia, Sweden, and heparin was obtained from Serva, Heidelberg. Ribonuclease nucleic acids were purchased from Amersham. RK-13 cells were obtained from Flow laboratories and the media for cell culture from Gibco.

Acknowledgments

We thank Claudio Schneider for his advice on cap selection experiments and for his generous gift of anti-cap antibodies, our colleagues for critical reading of the manuscript, Heide Seifert for secretarial help, and Jacky Schmidt for excellent technical assistance.

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