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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., 1981; 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 (m^7G) 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 (Pluciennicczak et al., 1985; Hänggi et al., 1986). The functional analysis of the vaccinia late promoter of the 11 kd 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

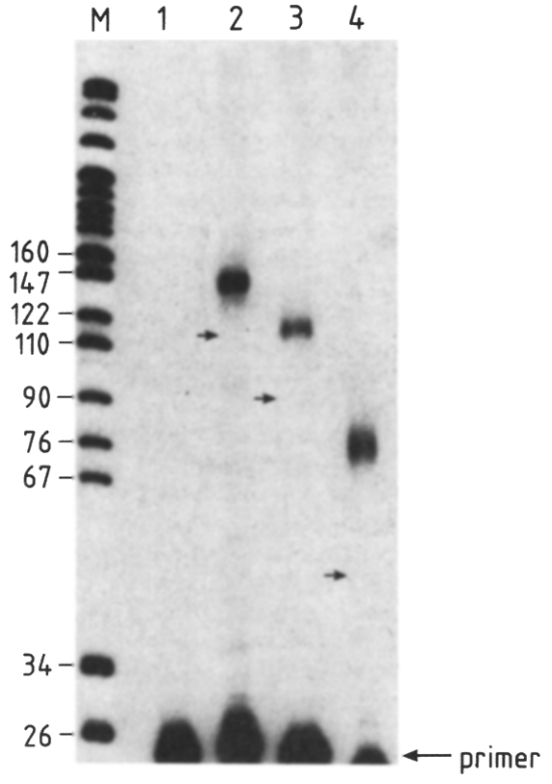

Figure 1. Primer Extension of *dhfr* Transcripts

RNA from cells infected with the wild-type virus (strain wr) or with the 11K-*dhfr* recombinant virus were primer extended using different 5′-labeled oligonucleotides as described in Experimental Procedures. Lane 1: wild-type RNA and the *dhfr* primer from position +63 to +87; lanes 2, 3, and 4: RNA from cells infected with the 11K-*dhfr* recombinant virus and the *dhfr* primers from position +88 to +112, +63 to +87, respectively, +24 to +46. The positions of the TAAAT motif representing the S1 "start site" are indicated by the arrows. (M): [^{32}P]-labeled HpaII-digested pBR-322 DNA size markers.

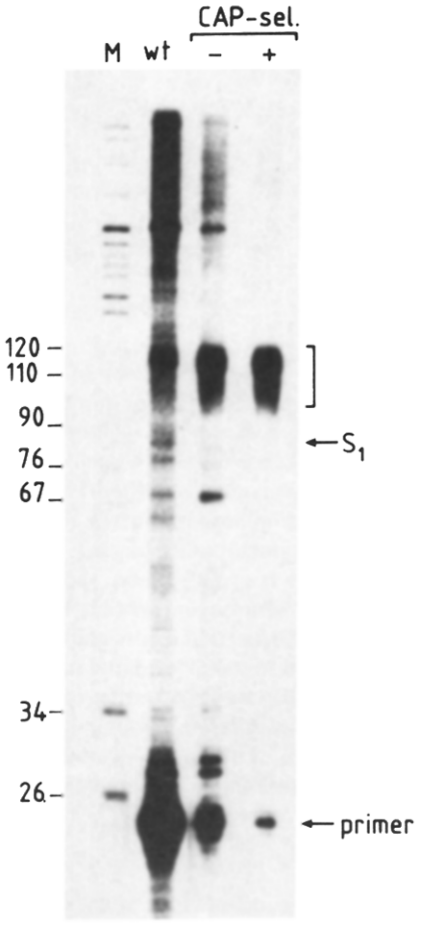

Figure 2. Primer Elongation and Cap Selection

RNA from cells infected with the 11K-*dhfr* recombinant virus were primer extended using a 5′-labeled oligonucleotide (+63 to +87) as described in Experimental Procedures. cDNA–RNA hybrids were incubated in the presence or absence of RNAase A at a final concentration of 10 μg/ml and the hybrids were cap-selected by immunoprecipitation using a rabbit anti-m^{7}G antiserum as described. (wt) primer extension without cap selection. (–) cap selection without RNase treatment. (+) treatment with RNAase A prior to cap selection. (M) [^{32}P]-labeled HpaII-digested pBR-322 DNA size markers. The position of the TAAAT motif representing the S1 "start site" is indicated by the arrow.

strands are transcribed, genes can be overlapping, and there is readthrough of the RNA polymerase (Pluciennic-zak 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-methyl-guanosine (m^{7}G) 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 m^{7}G cap

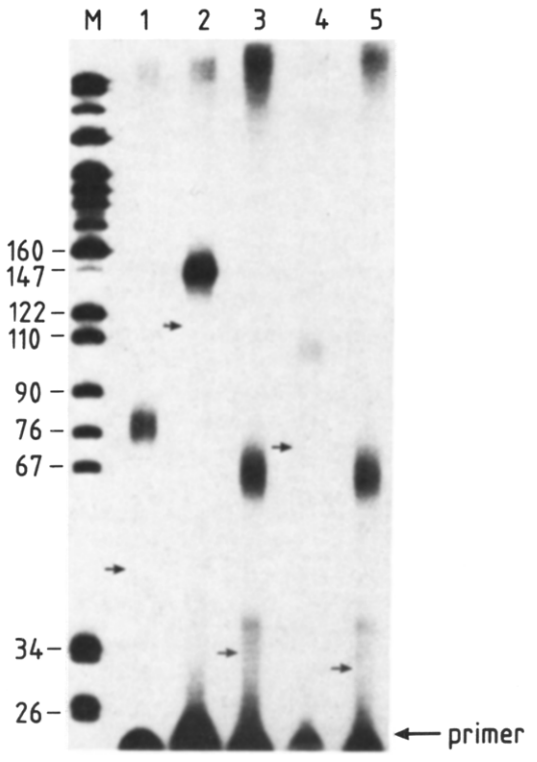

Figure 3. Primer Extension of Late Transcripts

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, +88 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): [^{32}P]-labeled HpaII-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 m^{7}G 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. In this experiment, the cDNA–RNA hybrids can only be retained by the antibody column if the RNA including the cap structure is protected from RNAase digestion by a full-length cDNA. The result shows that the RNA transcripts extended beyond the S1 "start site" are protected from the RNAase treatment by the cDNA (Figure 2, lane +). We conclude that these transcripts represent mature capped messengers and are not the result of premature stopping of the reverse transcriptase. The smaller cDNA–RNA hybrids as well as the majority of the very large cDNA–RNA hybrids are sensitive to RNAase treatment (Figure 2). This RNAase sensitivity shows that these cDNAs represent premature stops of the reverse transcriptase and not full-length cDNAs. The full-length cDNA–RNA hybrids are also insensitive to digestion with a combination of the RNAases A, T_1, and CL3 prior to cap selection (results not shown). The RNAase CL3 has a preference for Cp/N but cleaves also Ap/N bonds under the test conditions (Levy and Karpetsky, 1980).

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 (Rosel 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-

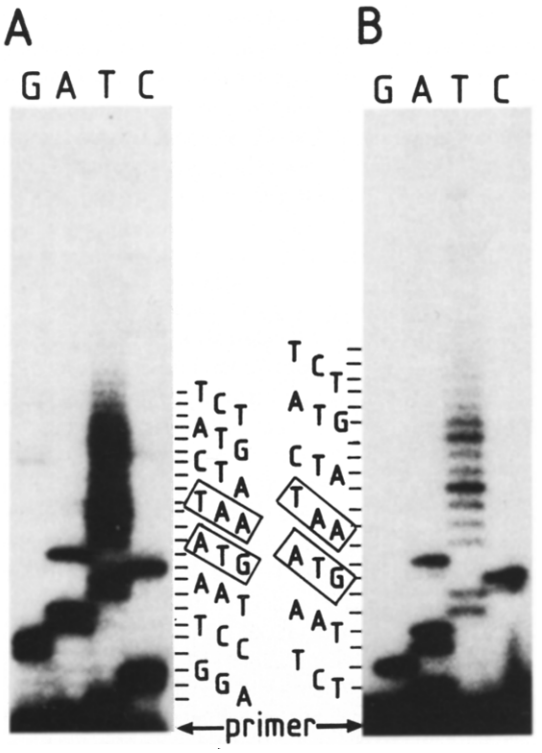

Figure 4. RNA Sequencing

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.

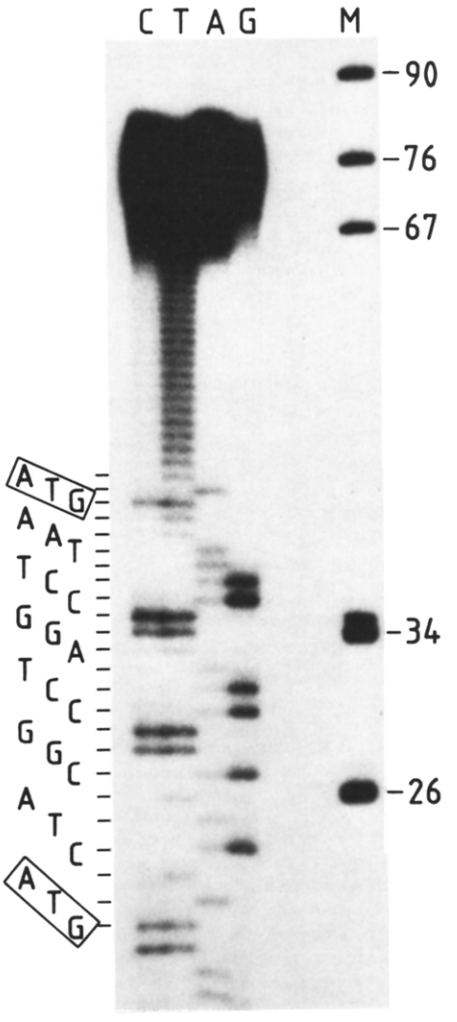

Figure 5. Sequencing of the First Strand cDNA

RNA from cells infected with the 11K–*dhfr* recombinant virus were primer extended using a 5′-labeled oligonucleotide (+24 to +46) as described in Experimental Procedures. The extended cDNA products of 70–80 nucleotides in length were isolated from a denaturing polyacrylamide gel. The cDNA was sequenced using the method of Maxam and Gilbert (1982). M: [^{32}P]-labeled HpaII-digested pBR-322 DNA size markers. The sequence of the coding region downstream of the AUG is indicated.

stream. The readthrough is, however, more prominent in transcripts from the translocated promoter construct within the *tk* locus than in the authentic 11K transcripts located in the left-hand side of the genome.

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 dideoxynucleotide concentration without the introduction of unspecific ghost bands. Furthermore, standard cDNA cloning procedures (Okayama 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

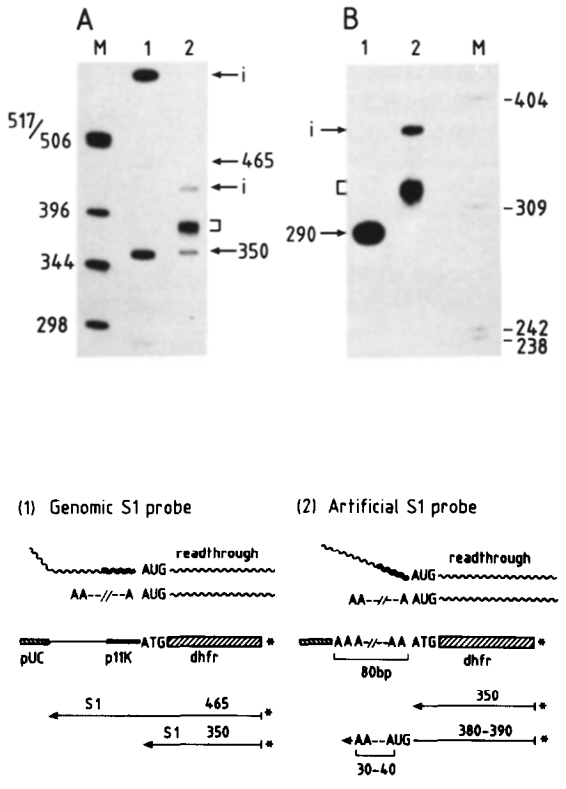

Figure 6. S1 Mapping of the Poly(A) Stretch

S1 mapping of RNA from cells infected with the wild-type virus (strain wr) or with the 11K–*dhfr* recombinant virus with genomic (lane 1) or artificial DNA probes complementary to the discontinuous mRNA (lane 2) of the *dhfr* (A) respectively authentic 11K gene (B). The S1 protected fragments corresponding to the discontinuous transcripts are indicated by bars. (i) input DNA fragments; (M): [^{32}P]-labeled HpaII- or HinfI-digested pBR-322 DNA size markers. The S1 mapping of *dhfr* transcripts is schematically indicated in the drawing.

probe (Figures 6A and 6B, lane 1). The 3′ ends of these genomic S1 probes consist of nonhomologous plasmid sequences. This allows the separation of the input DNA probe from the S1 fragments protected by readthrough transcripts. The digestion with nuclease S1 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 S1 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 S1 "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 S1 mapping using either the genomic or the artificial S1 probes (Figure 6A, compare the band migrating at 465 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 *tk* 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 S1 "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., 1985b; Rosel and Moss, 1985; Hänggi 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 (Hänggi 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 S1 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-m^{7}G antiserum that the extended transcripts represent mature mRNAs with an m^{7}G 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., 1986). This phenomenon could explain the observed cleavage in all four lanes in the 5′ end of the cDNA sequence (Figure 5). However, S1 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

6A and 6B). The ratio between the readthrough transcripts and the discontinuous mRNAs is constant in the S1 mapping experiments using either the genomic or artificial S1 probes. This indicates that the vast majority of the transcripts have a poly(A) stretch of approximately 35 bases upstream of the S1 "start site." We conclude that the addition of poly(A) stretch to vaccinia late transcripts is a general phenomenon. In contrast, early transcripts are not discontinuous; the 5′ end determined by S1 mapping coincides with the 5′ end of the cDNA clones (Venkatesan et al., 1981). The data that we have presented are to some extent ambiguous with respect to the length of the poly(A) stretch. Reverse transcriptase experiments point in the direction of a heterogeneity in the length of the A stretch of 5–10 nucleotides (Figures 1–5) which is not observed in the S1 experiment (Figure 6). This discrepancy might be due to an inability of the reverse transcriptase to read up to the methylated G-residue of the cap structure. Furthermore, homopolymeric stretches are relatively poorly transcribed even at high enzyme concentrations (Murphy et al., 1986). The RNAase–cap selection experiments cannot completely rule out this possibility because the poly(A) sequence might be less susceptible to the RNAases under the test conditions. The exact length of the poly(A) stretch in the leader RNA can probably only be determined after the elucidation of the mechanism of poly(A) addition.

The results that we have presented here do not elaborate on the mechanism for the addition of the A-stretch to late transcripts. *Cis* splicing seems to be unlikely because vaccinia promoter–gene constructs can be expressed and their transcripts translated in a transient assay in which the chimeric gene is not integrated into the viral genome (Cochran et al., 1985a; our unpublished observations). We are currently investigating whether these transcripts have a poly(A) leader sequence. Other mechanisms reported for synthesis of discontinuous transcripts, i.e., *trans* splicing in trypanosomes (Murphy et al., 1986; Sutton and Boothroyd, 1986) and primed transcription with a small RNA leader molecule in influenza virus (Beaton and Krug, 1981) or coronavirus (Spaan et al., 1982; Makino et al., 1986) might also apply to the synthesis of vaccinia late transcripts. The fact that we do not find a eukaryotic consensus splice acceptor site at or close to the putative junction of the discontinuous vaccinia transcripts does not exclude a *trans* splicing mechanism. Vaccinia as a cytoplasmic virus has developed its own transcription machinery; the conserved TAAAT motif might represent the vaccinia counterpart of the eukaryotic splice acceptor site.

The leader RNA addition might also be the result of a processing mechanism based on the addition of preexisting and possibly capped poly(A) RNA molecules. Initial attempts to detect such molecules in vivo have proved unsuccessful. It has been reported, however, that purified vaccinia virions produce high levels of polyriboadenylic acids in vitro (Bablanian and Banerjee, 1986). These authors postulated a role of the poly(A) RNA in translation control and shut-off of host translation. They did not determine, however, whether these poly(A) RNA molecules are present in vivo and whether they are capped. An addition of a poly(A) RNA and a subsequent capping of the RNA might also be possible since an RNA-specific phosphate kinase 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 nuclease S1 digestion at 14°C.

Primer Elongation and RNA Sequencing

Primer elongation was performed using 5′-labeled synthetic oligonucleotides. The labeled oligonucleotide was incubated at 55°C with the RNA, slowly cooled down to 42°C and coprecipitated with 0.6 volumes of iso-propanol in the presence of 0.6 M NaAc. Ten units of reverse transcriptase and 20 U of RNAase inhibitor were used with 10 μg of total RNA. In the RNA sequencing experiments, the deoxy- to dideoxynucleotide ratios were 5.5, 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-m^7G in the presence of 1 mg/ml of Heparin (174,000 IU/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 mg/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 Genofit, Geneva, or Boehringer Mannheim. The RNAases A, T_1, CI3 and restriction endonucleases were purchased from Boehringer Mannheim. Protein A-sepharose CL-4B and nuclease S1 were obtained from Pharmacia, Sweden, and heparin was obtained from Serva, Heidelberg. Radioactive nucleotides were purchased from Amersham. RK-13 cells were obtained from Flow laboratories and the media for cell culture from Gibco.

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