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In vitro synthesis of vaccinia virus late mRNA containing a 5′ poly(A) leader sequence

(poxvirus/*in vitro* transcription/late promoter)

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ABSTRACT **We have shown that an extract made from HeLa cells harvested 6 hr after infection with vaccinia virus can transcribe a duplex DNA template containing a late viral gene. S1 nuclease analyses using genomic and synthetic probes indicated that the 5′ ends of RNA synthesized *in vitro* are similar to those of RNA made *in vivo* and contain 5′ poly(A) sequences contiguous with the translation initiation codon. Kinetic analysis of RNA synthesized *in vitro* demonstrated that a correctly initiated and 5′ polyadenylylated product appeared within 5 min after transcription reactions were started. A cis-splicing mechanism of poly(A) addition can be ruled out because the DNA template used *in vitro* had no poly(dT) sequence and could contain as few as 37 base pairs upstream of the start of the RNA. In addition, we found that a point mutation in the first of two consecutively encoded adenylate residues preceding the ATG initiation codon abolished transcription *in vitro*. These data are consistent with at least three models: (*i*) RNA polymerase initiates RNA synthesis with a run of adenylate residues; (*ii*) a poly(A) primer is used for initiation; or (*iii*) the poly(A) leader is rapidly and efficiently attached to the RNA by ligation.**

The development of soluble *in vitro* transcription systems has greatly facilitated the analysis of protein factors required for eukaryotic RNA synthesis. Similarly, the demonstration that extracts of infectious vaccinia virus particles specifically and accurately transcribe DNA templates containing vaccinia early genes has accelerated progress in this field (1, 2). Three components essential to this early gene *in vitro* system have been described: a virus-encoded multisubunit RNA polymerase with amino acid sequence homology to the eukaryotic enzymes (3, 4), an early promoter-binding transcription factor (ref. 5; S. S. Broyles, personal communication), and a sequence-specific termination factor that copurifies with capping enzyme (6). The object of the present investigation was to develop an *in vitro* system that accurately transcribes vaccinia late genes.

Vaccinia virus transcription is temporally regulated; genes classified as early are expressed before DNA replication, and genes classified as late are expressed after virus uncoating and DNA replication have occurred. The mechanism of this early-to-late switch is not understood, but studies performed on both types of genes have shown that their regulatory sequences differ (7–9). Nuclease S1 analysis of late genes has revealed that the 5′ ends of the RNAs map within, or just upstream of, a run of three adenosines in a conserved sequence TAAAT that has been found 5′ to the coding region in all late genes examined to date (7, 10, 11). These start sites lie in very close proximity to the ATG translational initiation codons, such that the consensus sequence is often TAAATG, apparently leaving the mRNA with an extremely short leader sequence. However, recent results have shown that the 5′ ends of late RNAs mapped by nuclease S1 are actually a junction point between RNA encoded by the DNA template and a poly(A) sequence that is attached to the 5′ ends of late RNAs (12, 13).

In this communication we demonstrate that an extract from HeLa cells infected with vaccinia virus can accurately transcribe late gene templates. Using nuclease S1 analysis with genomic and synthetic DNA probes, we also show that the 5′ ends of these *in vitro* transcripts, like vaccinia late RNAs synthesized *in vivo*, contain a poly(A) sequence at their 5′ ends. Furthermore, the *in vitro* system is affected by a mutation in the conserved TAAAT sequence, as no transcription can be detected from a template containing a one-base change in this sequence.

MATERIALS AND METHODS

Plasmid Constructions. The plasmid pCFW2 was constructed by ligating a 670-base-pair (bp) *Hin*dIII/*Cla* I fragment from the *Hin*dIII F region of the vaccinia genome to a *Hin*dIII- and *Acc* I-cleaved pUC18 derivative that lacked an *Eco*RI site. This created a plasmid containing pUC sequences, 120 bp of the vaccinia late 11-kDa protein-coding sequences, and 550 bp of 5′ flanking sequences. pCFW8 was constructed by cleaving pCFW2 at the unique *Eco*RI site that exists just 3′ to the ATG initiation codon of the 11-kDa protein and inserting the oligonucleotide:

$$\begin{array}{l} 5'\ \mathrm{AATTC(A)_{50}TG}\ 3' \\ \quad 3'\ \mathrm{G(T)_{50}ACTTAA}\ 5', \end{array}$$

thus creating a poly(dA·dT) sequence just upstream of the ATG. The plasmid pCFW5 is also a derivative of pCFW2 constructed by cleaving pCFW2 with *Xba* I and ligating under dilute conditions such that an *Xba* I fragment of ≈450 bp, containing DNA upstream of the promoter of the 11-kDa gene and some pUC sequences, was deleted. The large *Xba* I/*Eco*RI fragment was isolated from pCFW5 and ligated to an oligonucleotide:

5′ CTAGAATTTCATTTTGTTTTTTTCTATGCTATAAATG 3′
3′ TTAAAGTAAAACAAAAAAAGATACGATACTTACTTAA 5′

that contains a mismatched base pair in the conserved TAAAT sequence, as indicated by the residues in boldface type. Replication of this plasmid in *Escherichia coli* yields plasmids that contain only the mutant (pCFW6) or only the wild-type (pCFW7) bp. The 5′ flanking sequences of these plasmids are ≈70 bp shorter than the *Xba* I/*Eco*RI fragment of pCFW5 because the oligonucleotides that were synthesized were only 37 nucleotides in length.

Oligonucleotides were purified from 12% denaturing acrylamide gels as previously described (14), suspended at 200–400 μg/ml and then hybridized by mixing equal amounts of each, heating to 65°C for 15 min, and cooling to room temperature in a beaker of water.

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Preparation of Whole-Cell Extracts and *in Vitro* Transcription Conditions. Whole-cell extracts were prepared from HeLa spinner cells 6 hr after infection with vaccinia virus strain WR as previously described (15). Protein concentrations were ≈10 mg/ml.

Standard transcription reactions were done essentially as described (15) but with the following modifications: 25 μl of dialyzed extract and final concentrations of 10 mM Hepes (pH 7.9)/6.2 mM $MgCl_2$/50 mM KCl/0.2 mM EDTA/1.0 mM dithiothreitol/8.5% (vol/vol) glycerol/800 μM (each) ATP, GTP, and CTP/100 μM UTP/5 μCi of [α-^{32}P]UTP (3000 Ci/mmol; 1 Ci = 37 GBq) were used in a final volume of 50 μl. The amount of plasmid template used varied from 10 to 40 μg/ml. Reactions were incubated without nucleotides for 10 min at 30°C, started by the addition of nucleotides, incubated at 30°C for 1 hr, and then terminated by the addition of 50 μl of a solution of 8 M urea/1% $NaDodSO_4$/10 mM EDTA containing 25 μg of tRNA. The reactions were then phenol/chloroform/isoamyl alcohol (25:24:1) extracted twice and ethanol precipitated. The ethanol precipitate was dissolved in 8 μl of 90% deionized formamide/10 mM EDTA/0.3% xylene cyanol/0.3% bromophenol blue, heated to 65°C for 5 min, and loaded on a 4% denaturing acrylamide gel.

Nuclease S1 Analysis. To prepare RNA for nuclease S1 analysis, standard transcription reactions were routinely scaled-up 2-fold, the labeled UTP was omitted, and all unlabeled nucleotides were at a final concentration of 800 μM. Reactions were terminated by the addition of 10 μg of tRNA and two phenol/chloroform/isoamyl alcohol extractions followed by ethanol precipitation. The precipitated material was then resuspended in 50 μl of 40 mM Tris·HCl, pH 7.5/6 mM $MgCl_2$ and incubated with 15 units of DNase I (Pharmacia) at 37°C for 10 min. Again the RNA was phenol extracted twice and ethanol precipitated. After centrifugation and washing with 95% ethanol, the RNA was hybridized to DNA probes.

To prepare the probes, pCFW2 and pCFW8 were cleaved with *Pvu* II or *Hin*dIII, treated with calf intestinal alkaline phosphatase (Pharmacia), and labeled with [γ-^{32}-P]ATP using polynucleotide kinase (Pharmacia) by the methods of Maniatis *et al.* (16). After labeling, the DNA was cleaved again with *Bam*HI, and the relevant fragments were purified from nondenaturing acrylamide gels by electroelution. RNA was hybridized to DNA (at 37°C for the *Hin*dIII-labeled probes; 42°C for the *Pvu* II-labeled probes) and digested with nuclease S1 (Pharmacia) as described (9) with the following modifications: temperature for the S1 digestion reaction was 13°C for 30 min, and concentration of S1 used was 110 units/ml.

Primer-Extension Analysis. For primer-extension analysis an oligonucleotide, 5′ CGGCTTTTAGAACCAGATATCTTCC 3′, 5′ end-labeled with polynucleotide kinase, was hybridized at 42°C to RNA isolated from infected cells under the same conditions used to hybridize DNA probes to RNA for the S1 analysis. The RNA·DNA hybrid was then ethanol precipitated and resuspended in 9 μl of reverse transcriptase buffer (50 mM Tris·HCl, pH 8.3/40 mM KCl/10 mM $MgCl_2$/8 mM dithiothreitol) to which 25 units of RNase-block (Stratagene Cloning Systems, San Diego, CA) and 48 units of reverse transcriptase (Seikagaku America, St. Petersburg, FL) were added for a final volume of 12 μl. This reaction was split into five 2-μl aliquots to which either 2 μl of deoxynucleotide mix (2.5 mM dATP/dCTP/dTTP/dGTP) was added or 2 μl of the appropriate dideoxynucleotide mix was added (see below). Reactions were incubated at 42°C for 45 min; then 2 μl of deoxynucleotide mix was added to all reactions, and incubation was continued at 42°C for another 45 min. Dideoxynucleotide mixes were as follows: cytidine mix contained 1 mM dTTP/dGTP/dATP, 0.5 mM dCTP, and 0.125 mM ddCTP; thymidine mix contained 1 mM dGTP/dATP/dCTP, 0.5 mM dTTP, and 0.25 mM ddTTP; adenosine mix contained 1 mM dGTP/dTTP/dCTP, 0.5 mM dATP, and 0.25 mM ddATP; guanosine mix contained 1 mM dTTP/dCTP/dATP, 0.5 mM dGTP, and 0.25 mM ddGTP.

Preparation of *in Vivo* RNA. *In vivo* early and late RNA were prepared as described by Rosel *et al.* (7).

RESULTS

Analysis of RNA Made *in Vivo*. As a model for late transcription, we chose a vaccinia virus gene encoding a major 11-kDa structural protein because it met the criteria of being a late gene, the product of which is abundant and the regulatory sequences of which have already been located (17, 18). The structure of the 5′ end of the mRNA made *in vivo* was determined by primer-extension and S1 nuclease analysis, so that later comparisons could be made between this RNA and RNA made by *in vitro* transcription. Fig. 1*A* shows the result of primer-extension and sequence analyses using a ^{32}P-labeled oligonucleotide. The primer-extension analysis suggested that the RNA molecules in this population terminate heterogeneously, with the majority having 5′ ends that extend ≈25–35 nucleotides 5′ to the ATG initiation codon. The sequence analysis demonstrated that a homopolymer tract of A residues begins immediately upstream of the ATG and extends to the ends mapped by primer extension. This result confirms recent reports from other laboratories of a nontemplate-encoded 5′ poly(A) sequence. In addition to the RNA molecules described above, a population of molecules with much longer 5′ ends also appears to exist.

To confirm the 5′ poly(A) sequence by an alternative approach, an S1 nuclease analysis was done on the RNA made *in vivo*. Two types of ^{32}P-labeled DNA probes were used: one that contained genomic sequences flanking the gene encoding the 11-kDa protein (wild-type probe) and one in which a 50-mer poly(T) sequence was engineered in the template strand of the gene immediately 5′ to the ATG [poly(T) probe]. Therefore, if a poly(A) sequence does exist in the mRNA, it should hybridize to the poly(T) sequence in the probe, creating a longer S1 nuclease-protected product than would be seen with a probe containing only the genomic upstream sequences. Both probes were labeled at a *Hin*dIII site that lies in the coding region of the gene.

Fig. 1*B* shows the result of the S1 nuclease analysis using both types of probe hybridized to *in vivo* RNA. Lanes 3 and 4 show the *Hin*dIII-labeled probes hybridized to vaccinia early RNA and, as expected, there is no signal with this RNA. Lanes 5 and 6 show these same probes hybridized to late RNA isolated from vaccinia-infected cells. The wild-type genomic probe maps the 5′ ends of the RNA to a set of bands centered over the TAAAT sequence, as has been observed (11). The poly(T) probe, however, maps the 5′ ends of the RNA to positions upstream of the conserved sequence, confirming the presence of a poly(A) tract on the *in vivo* mRNA. Much heterogeneity exists in the mRNA ends as mapped by the poly(T) probe, possibly due to "nibbling" by the S1 nuclease; however, the majority of the molecules appear to end 25–30 nucleotides 5′ to the ends mapped by the wild-type probe. There is also some protection of the full 50 thymidine residues indicating that some RNA molecules have longer poly(A) leader sequences.

***In Vitro* Transcription Runoff Assays.** To analyze transcription of the gene for the 11-kDa protein *in vitro*, a vector (pCFW5) was constructed that contained 110 bp of 5′ flanking sequences and 120 bp of protein-coding sequences in pUC 18. A 2-kb *Pvu* I fragment from this plasmid was routinely used as the template for the *in vitro* transcription system (Fig. 2). Initial experiments in which this template and others truncated with different restriction enzymes were used to pro-

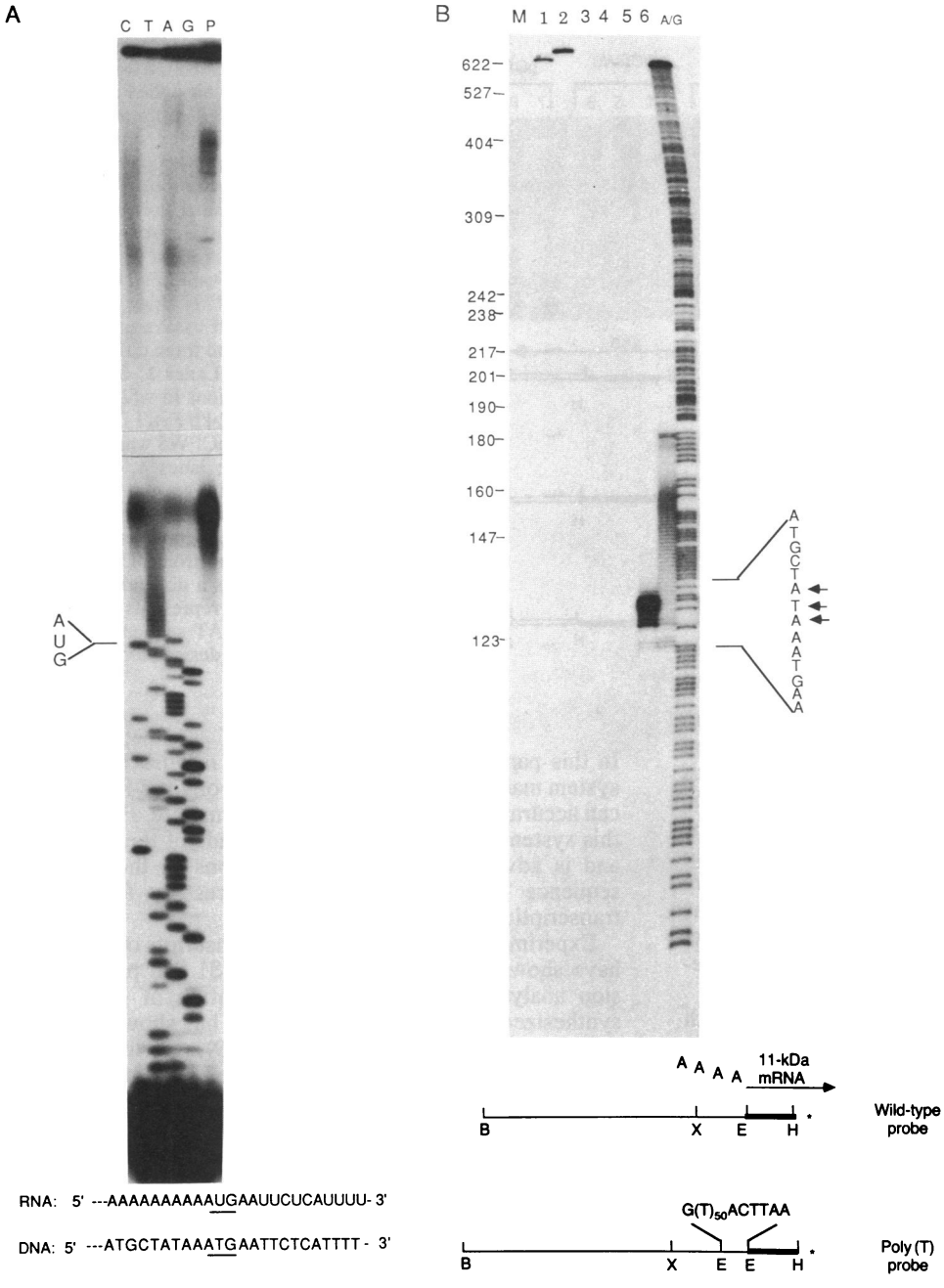

FIG. 1. (*A*) Primer-extension and sequence analysis of *in vivo* mRNA encoding the 11-kDa structural protein. A primer-extension analysis is shown in the lane designated P; other lanes are named for the dideoxynucleotide added to the reaction. The position of the AUG is indicated. Sequence of the RNA in the proximity of the AUG (which is the complement of the sequence as read from the gel) is shown at bottom of the figure. Also shown is the sequence of the corresponding region of the gene. (*B*) S1 nuclease analysis of *in vivo* mRNA. Lanes: M, ^{32}P-labeled pBR-322 *Msp* I markers; 1 and 2, untreated, labeled wild-type and poly(T) probes, respectively; 3 and 4, wild-type and poly(T) probes hybridized to early RNA; 5 and 6, wild-type and poly(T) probes hybridized to late RNA; A/G, A+G sequence ladder of the wild-type probe. The sequence to the right of the A+G ladder is the complement of the sequence within the brackets. Arrows indicate the 5′ ends of the mRNA as mapped by the wild-type probe. At bottom of the figure is a diagrammatic representation of the S1 nuclease mapping strategy. Closed boxes represent coding sequences for the 11-kDa protein, lines represent 5′ flanking sequences, and the asterisk represents the position of the ^{32}P label. Restriction sites are designated as follows: B, *Bam*HI; X, *Xba* I; E, *Eco*RI; H, *Hin*dIII.

gram the *in vitro* system showed the synthesis of runoff products of approximately the predicted size, demonstrating that the system could recognize a late promoter (Fig. 2*A*). In addition to these runoff products, we saw much larger products that also shifted in mobility, depending on the enzymes used to create the template, suggesting that these molecules initiated from the same point as did the smaller products. These molecules could be the result of either 3′ or 5′ modifications to the RNA. Synthesis of all of these RNAs was dependent upon the use of infected cell extracts; extracts from uninfected cells did not synthesize these products (data not shown).

Previous experiments with the gene for the 11-kDa protein have shown that deletion of the sequences more than 30 bp upstream of the initiation codon had very little effect on promoter activity but deletions that included the conserved TAAAT sequence completely abolished transcription (17, 18). Recent experiments using the promoter for the 28-kDa protein of vaccinia virus have shown that a single-base change converting the TAAAT sequence of this late gene to TGAAT drastically reduces gene expression (A. Davison, personal communication). As a stringent test of the system, we wondered whether the *in vitro* system was also sensitive to mutations in this sequence. Therefore, we constructed two new vectors by replacing the 110-bp *Xba* I/*Eco*RI fragment containing the promoter for the 11-kDa protein gene sequences with 37-bp oligonucleotides that either had the wild-type sequences (pCFW7) or contained a point mutation such that the conserved sequence was changed to TGAAT (pCFW6). These new constructions were also isolated on 2-kb *Pvu* I fragments (Fig. 2*B*), and varying amounts of each were used as the template in separate *in vitro* transcription assays. As can be seen in Fig. 2*B*, the template containing the shortened promoter region (pCFW7) synthesizes a runoff product of the same size as seen with the template containing the longer 110-bp promoter region (pCFW5), with no obvious decrease in amount of message. However, the template containing the mutated conserved sequence shows no synthesis of this runoff product, demonstrating that transcription in the *in vitro* system also depends upon this sequence.

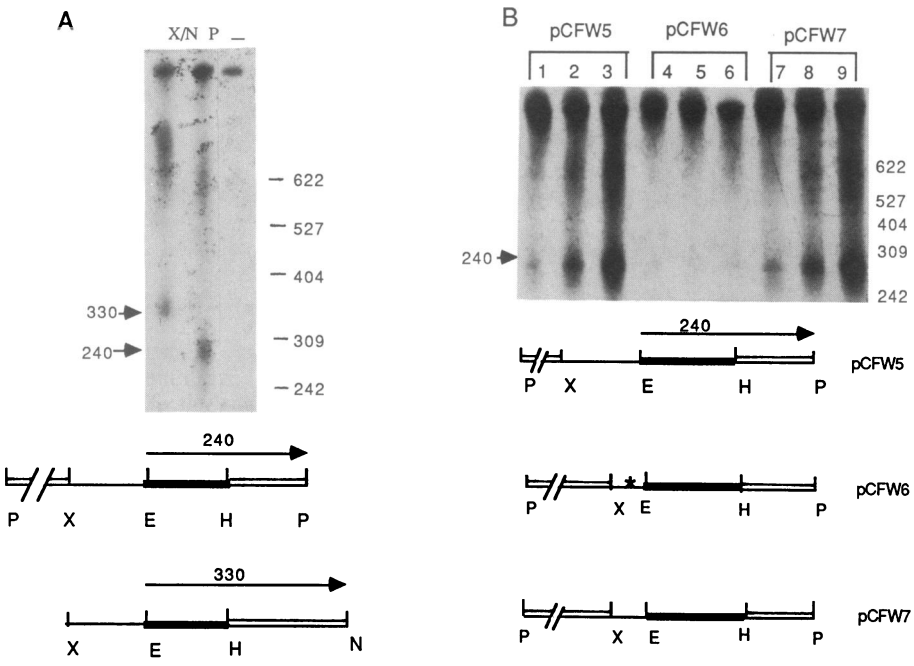

FIG. 2. (*A*) *In vitro* transcription runoff assays programmed with a 440-bp *Xba* I/*Nde* I fragment (X/N), a 2-kb *Pvu* I fragment (P), or with no added template (–). Position of pBR322 *Msp* I markers and runoff products are indicated. At bottom of the figure is a diagram of templates used; open boxes represent vector sequences, closed boxes represent the region coding for the 11-kDa protein, and lines represent 5′ flanking sequences. Restriction enzyme sites are designated as follows: P, *Pvu* I; X, *Xba* I; E, *Eco*RI; H, *Hin*dIII; N, *Nde* I. (*B*) *In vitro* transcription runoff assays using three different templates and three different concentrations of DNA. Lanes 1, 2, and 3 show transcription reactions to which 0.2 μg, 0.5 μg, or 1 μg of the 2-kb *Pvu* I DNA fragment from the plasmid pCFW5 was added to the whole-cell extract; lanes 4, 5, and 6 are analogous reactions, except the fragments were isolated from pCFW6; and lanes 7, 8, and 9 are again analogous, except the fragments were isolated from pCFW7. At bottom of the figure is a diagram of templates used. The asterisk represents the point mutation in the TAAAT sequence of plasmid pCFW6. All other designations are as in *A*.

S1 Nuclease Mapping of *in Vitro* RNA. Repeated attempts to analyze the structure of the *in vitro* RNA by primer-extension analysis were unsatisfactory, so S1 nuclease analysis was used to determine whether the 5′ end of this RNA was the same as that for RNA isolated directly from infected cells. The probes used to map the *in vitro* RNAs were similar to those used to map *in vivo* transcripts, except that they were labeled at a *Pvu* II site that lies in pUC sequences. This was done to prevent RNA endogenous to the whole-cell extract from protecting the probe and yielding an S1 nuclease-resistant fragment.

Fig. 3 shows the results of S1 nuclease analysis using both types of probes hybridized to RNA synthesized *in vitro* from the wild-type *Pvu* I DNA template. Lanes 2-5 and lanes 7–10 show the *Pvu* II-labeled probes hybridized either to RNA isolated from *in vitro* transcription reactions at 5, 15, 30, or 60 min, or, in the case of lanes 1 and 6, to RNA isolated from extracts in which the DNA template was not added to the system. These last two lanes prove that these probes, labeled in vector sequences, do not hybridize to any RNA endogenous to the extract.

Use of the wild-type probe in Fig. 3 demonstrates that within 5 min after initiation of the transcription reaction, the *in vitro* system can synthesize a transcript with the same 5′ endpoint as is made *in vivo*, with no evidence for a longer species of RNA complementary to the upstream DNA sequences. The poly(T) probe shows that these transcripts are also 5′ polyadenylylated within 5 min. However, the pattern of polyadenylylation appears slightly different with the *in vitro* transcripts than with RNA made *in vivo*; there is more protection of the full 50 thymidine residues of the probe with *in vitro* RNA than with *in vivo* RNA. This suggests that RNA made *in vitro* may contain more molecules with a 5′ poly(A) sequence longer than 30 nucleotides.

Fig. 3 also shows the result of S1 nuclease mapping with the templates from pCFW6 and pCFW7. These results are consistent with the results seen with the runoff assays in that no transcript is visible with the template containing the mutated TGAAT sequence, but a correctly initiated and polyadenylylated product is made with the template containing the authentic TAAAT.

DISCUSSION

In this paper we have shown that an *in vitro* transcription system made from a whole-cell extract of virus-infected cells can accurately transcribe a late-gene template. Furthermore, this system polyadenylylates the 5′ ends of these messages and is adversely affected by mutations in the conserved sequence TAAAT, shown to be necessary for late-gene transcription *in vivo*.

Experiments done to map *in vivo* transcripts of early genes have shown no discrepancy between S1 and primer-extension analyses. Furthermore, examination of early RNAs synthesized by purified virus particles has shown that these RNAs are initiated at their cap sites (19), proving that the 5′ ends of these messages are not modified in an unusual way. In agreement with previous results (15), we have found that the early promoter of the vaccinia virus gene encoding a 7.5-kDa protein is functional in the *in vitro* transcription system. Using an oligonucleotide containing a 20-mer poly(T) sequence, we mapped the 5′ end of the 7.5-kDa early transcript made *in vitro* using S1 nuclease analysis and found no evidence for a poly(A) sequence (unpublished results). Therefore, the *in vitro* system can also discern the difference between early and late promoters and only polyadenylylates transcripts of the latter.

Several mechanisms can account for the addition of the poly(A) sequence onto the 5′ end of late messages. One hypothesis is that the 5′ end of late messages is generated by a processing mechanism, and the TAAAT sequence represents a processing signal rather than a promoter element. Although our experiments do not bear directly on the mechanism of the 5′ polyadenylylation, several points can be made. First, a linear DNA segment containing only 37 bp of DNA upstream of the conserved TAAAT sequence can function as a template *in vitro*. Second, the time course of RNA synthesis *in vitro* shows that a correctly initiated and 5′ polyadenylylated product is synthesized within 5 min after transcription reactions are started. No evidence is seen for a longer transcript complementary to upstream sequences. Unless processing is rapid, this finding suggests that the 5′ end of the mRNA is not generated by cleavage of a longer transcript, with subsequent addition of the poly(A) sequence. The TAAAT mutation also addresses the latter point; if this

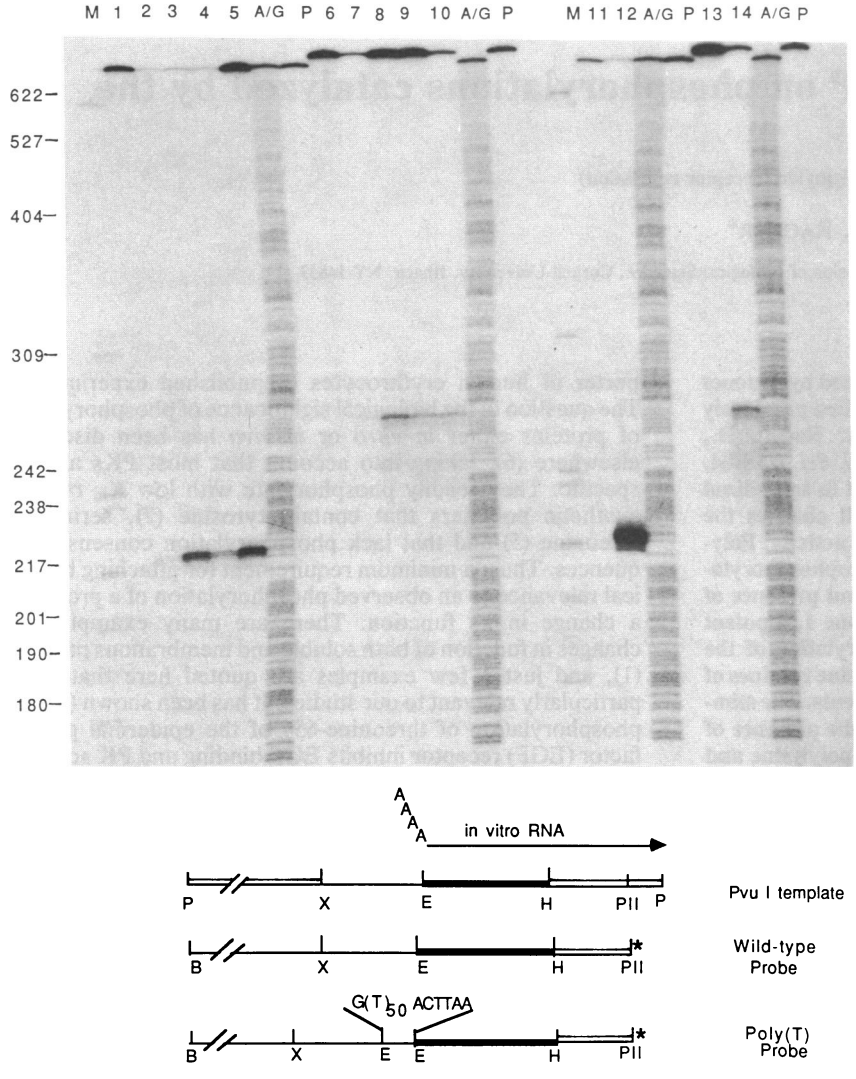

FIG. 3. S1 nuclease analysis of RNA synthesized *in vitro*. Lanes: M, pBR322 *Msp* I markers, A/G, A+G sequence ladder of *Pvu* II-labeled wild-type probe; P, untreated, labeled DNA probes, either wild-type or poly(T); 1 and 6, RNA isolated from transcription reactions in which no DNA template was added; 2–5 and 7–10, RNA isolated from transcription reactions in which the 2-kb *Pvu* I fragment of pCFW5 was used as the template, and reactions were terminated at 5 min (lanes 2 and 7), 15 min (lanes 3 and 8), 30 min (lanes 4 and 9), and 60 min (lanes 5 and 10). RNA in lanes 1–5 was hybridized to wild-type probe, and RNA in lanes 6–10 was hybridized to the poly(T) probe. Lanes 11 and 13 show RNA extracted from reactions (terminated at 60 min) in which the 2-kb *Pvu* I fragment of PCFW6 was used as the template and hybridized to the wild-type (11) or poly(T) (13) probes. Lanes 12 and 14 are similar to lanes 11 and 13, except the template was isolated from pCFW7. At figure bottom is a diagram of the mapping strategy. All restriction enzyme designations are as in Figs. 1 and 2 with the addition of P II representing *Pvu* II restriction site.

were a signal involved in cleavage a mutation here would be predicted to accumulate a larger transcript; instead, we see the complete abolishment of transcription.

At least three alternative mechanisms can be proposed for the 5′ polyadenylylation: (*i*) the primary transcript, beginning within the TAAAT signal, may be synthesized and released from the template with the subsequent rapid addition of the poly(A) sequence by ligation, (*ii*) a poly(A) sequence may serve as a primer for transcription initiating at the TAAAT sequence, or (*iii*) RNA polymerase may initiate RNA synthesis at the TAAAT signal with a run of adenylate residues. The *in vitro* transcription system should serve as a powerful tool to distinguish between these mechanisms.

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