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Identification of *rpo30*, a Vaccinia Virus RNA Polymerase Gene with Structural Similarity to a Eucaryotic Transcription Elongation Factor

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Eucaryotic transcription factors that stimulate RNA polymerase II by increasing the efficiency of elongation of specifically or randomly initiated RNA chains have been isolated and characterized. We have identified a 30-kilodalton (kDa) vaccinia virus-encoded protein with apparent homology to SII, a 34-kDa mammalian transcriptional elongation factor. In addition to amino acid sequence similarities, both proteins contain C-terminal putative zinc finger domains. Identification of the gene, *rpo30*, encoding the vaccinia virus protein was achieved by using antibody to the purified viral RNA polymerase for immunoprecipitation of the in vitro translation products of in vivo-synthesized early mRNA selected by hybridization to cloned DNA fragments of the viral genome. Western immunoblot analysis using antiserum made to the vaccinia *rpo30* protein expressed in bacteria indicated that the 30-kDa protein remains associated with highly purified viral RNA polymerase. Thus, the vaccinia virus protein, unlike its eucaryotic homolog, is an integral RNA polymerase subunit rather than a readily separable transcription factor. Further studies showed that the expression of *rpo30* is regulated by dual early and later promoters.

Poxviruses, of which vaccinia virus is the prototype, replicate and transcribe their DNA genomes in the cytoplasm of host cells (for reviews, see references 21 and 22). Use of the cytoplasm instead of the nucleus is correlated with the encoding by poxviruses of many, if not all, of the enzymes and factors needed for DNA and RNA synthesis. Thus, vaccinia virus encodes a multisubunit RNA polymerase, as well as transcription initiation and termination factors, and packages them in infectious virus particles. Poxvirus RNA polymerases resemble their eucaryotic counterparts in overall subunit structure, and the products of two large-subunit genes have significant similarity in predicted amino acid sequence to the two large subunits of several eucaryotic and procaryotic RNA polymerases (3, 6, 16, 23, 25). Of the several small RNA polymerase subunits, the sequences of two have been reported so far (1, 6), and no proteins homologous to these subunits were identified. It is not clear whether the small subunits have distinct transcriptional functions or serve as structural components in assembly of the enzyme. Identification and characterization of the additional small subunits might facilitate our understanding of their role in enzyme assembly and transcription of viral genes. In this report, we have identified a vaccinia virus gene encoding a 30-kilodalton (kDa) protein with significant sequence similarities to a eucaryotic transcription elongation factor. We found, however, that this protein is an integral subunit of viral RNA polymerase rather than an easily dissociable transcription factor.

MATERIALS AND METHODS

Preparation of viral RNA, in vitro translation, and immunoprecipitation. RNA from the virus-infected cell cytoplasm was isolated and purified by CsCl centrifugation as previously described (8). Early RNA was obtained from HeLa S3 spinner cells at 4 h after infection with 10 PFU of vaccinia virus (Western Reserve strain) per cell in the presence of 100 μg of cycloheximide per ml. Late RNA was isolated 6 h after infection in the absence of drug. A 100-μg sample of early RNA was hybridized to 5 μg of each subcloned DNA fragments of the vaccinia virus genome that had been immobilized to a 1-cm^2 nitrocellulose membrane as described previously (20). After washing, the RNA was eluted from the membrane and translated in a micrococcal nuclease-treated reticulocyte lysate (Promega Biotec) in the presence of [^{35}S]methionine. The labeled translation products were incubated successively with antibody to the viral RNA polymerase and staphylococcal protein A attached to Sepharose beads (Pharmacia). The bound polypeptides were eluted with sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. For in vitro synthesis of *rpo30* RNA, part of the *Hin*dIII-E DNA containing the *rpo30* gene was amplified by 20 cycles of polymerase chain reaction (PCR), cloned into pGEM3 vector (Promega Biotec) to construct plasmid pT7rpo30A, and transcribed with T7 RNA polymerase, using a cap analog as described previously (24).

Plasmid constructions and DNA sequencing. The 15.2-kilobase-pair (kbp) *Hin*dIII E segment of vaccinia virus genomic DNA cloned in pBR322 (plasmid named pHindIIIE) was used (4). *Bam*HI and *Cla*I restriction endonuclease fragments of pHindIIIE were inserted into the pUC19 vector. Plasmid DNA prepared by alkaline lysis was used for hybridization selection of viral RNA. For DNA sequencing, each insert was recloned into the phagemid vector Bluescript (Stratagene) in both orientations. Unidirectional deletion of insets was performed by exonuclease III digestion followed by nuclease S1, both of which were purchased from Pharmacia. Single-stranded DNA templates prepared from 2-ml overnight cultures of deletion clones in the presence of helper phage K-9 were used for dideoxynucleotide chain termination reactions, using Sequenase (United States Biochemical Corp.) and [α-^{35}S]dATP. Sequences at the junctions of subclones were confirmed by sequencing a

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double-stranded template of pHindIIIE with synthetic oligonucleotides as primers. Assembly, analysis, and translation of DNA sequences were performed by using the Staden program (37). Protein homology searches were performed with the FASTA program (26).

Purification and analysis of viral RNA polymerase. Vaccinia virus was purified twice by sucrose density gradient sedimentation, and the soluble proteins were extracted with a buffer containing 100 mM Tris hydrochloride (pH 8.4), 250 mM KCl, 10 mM dithiothreitol, 0.1 mM EDTA, and 0.2% (wt/vol) deoxycholate as described previously (7). The RNA polymerase was purified by successive chromatography on columns of DEAE-cellulose, heparin agarose, single-stranded DNA agarose, and phosphocellulose in a buffer containing 50 mM Tris (pH 8), 1 mM dithiothreitol, 0.1 mM EDTA, 0.01% (vol/vol) Nonidet P-40, 10% glycerol, and linear NaCl gradients. Silver-stained gels indicated that the purity of the enzyme was comparable to that previously described (3, 23). A 10-μg sample of pure enzyme was applied to a 12-ml 15 to 35% glycerol gradient in the latter buffer with 200 mM NaCl. Centrifugation was at 4°C for 16 h at 41,000 rpm in a Beckman SW41 rotor. Fractions of 0.5 ml were collected from the bottom of the tube. Three protein markers (100 μg each) were combined and sedimented in a parallel gradient, and their sedimentation peaks were detected with Bradford reagent (Bio-Rad Laboratories). RNA polymerase activity was measured in 50 μl of 60 mM Tris hydrochloride (pH 8)–50 mM NaCl–0.1 mM EDTA–3 mM $MnCl_2$–1 mM ATP, GTP, and CTP–0.1 mM UTP–2 μCi of [α-^{32}P]UTP–1 μg of M13mp18 DNA. After incubation for 20 min at 37°C, the reaction mixture was applied to Whatman DE81 paper, which was then successively washed three times with 0.5 M Na_2HPO_4 before scintillation counting. One unit of activity was defined as the amount of enzyme that incorporated 1 pmol of UMP into RNA. For Western immunoblot analysis, the proteins were separated by electrophoresis on a 10% polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore Corp.) in Tris-glycine buffer containing 20% methanol and 0.05% sodium dodecyl sulfate. The membranes were blocked with 0.1% Tween 20 and 5% nonfat dry milk in phosphate-buffered saline and incubated successively with antibody and ^{125}I-protein A. Anti-Rpo30 serum was prepared by immunizing rabbits with 100 μg of polyacrylamide gel-purified protein expressed in isopropyl-β-D-galactopyranoside (IPTG)-induced *Escherichia coli* BL21(DE3) containing the *rpo30* gene in the T7 vector pET3c as described previously (32).

RNA analysis. Nuclease S1 digestion and primer extension techniques were used to determine the 5′ and 3′ ends of the *rpo30* transcript as previously described (1). The single-stranded DNA probe used for S1 mapping of the 5′ end of the RNA was prepared by 30 cycles of asymmetric PCR of plasmid linearized at a *Bam*HI site (nucleotide [nt] 7859 according to the numbering in Fig. 2), using the *Taq* polymerase (Perkin Elmer Cetus) and a ^{32}P-5′-end-labeled primer complementary to the coding region (nt 5212 to 5237; hereafter referred to as the c primer). The 3′ polyd(T) probe used in the nuclease protection analysis of the 5′ poly(A) leader of the late transcript was prepared by two-step PCR. First, double-stranded DNA containing the 5′ terminus of the *rpo30* open reading frame (ORF) preceded by 50 d(A) residues was prepared by 20-cycle PCR amplification of pHindIIIE, using the c primer and another primer containing 50 d(A) residues, followed by 20 nucleotides of the coding sequence including the translation initiation codon. Amplified DNA was subjected to 30 cycles of a second asymmetric PCR by using the 5′-end-labeled c primer. The single-stranded, 5′-end-labeled DNA with a 3′ polyd(T) tail was purified in a 4% denaturing polyacrylamide gel. Typically, starting from 1 μg of plasmid DNA and 20 pmol of end-labeled primer, a probe of approximately 10^8 dpm/μg was obtained. The oligonucleotide used in primer extension analysis with avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals) was from nt 5297 to 5319. The probe for mapping the 3′ end of the *rpo30* RNA was prepared by filling in the 3′ recessive end generated by *Mlu*I digestion (at nt 4807) with Klenow enzyme and [α-^{32}P]dCTP, followed by *Bam*HI digestion at nt 789.

Nucleotide sequence accession number. The 15,212-bp sequence of the entire *Hin*dIII E fragment (see Fig. 2) has been entered into GenBank under accession number M36339.

RESULTS

Identification of the *rpo30* gene. To identify genes encoding viral RNA polymerase subunits, antiserum to the purified viral RNA polymerase was used to immunoprecipitate in vitro translation products of infected-cell mRNAs that had been selected by hybridization to cloned fragments of the vaccinia virus genome. We found previously that in micrococcal nuclease-treated rabbit reticulocyte lysates, the synthesis of several immunoprecipitable polypeptides of 32 to 37 kDa was programmed with mRNAs that hybridized to the 15.2-kbp *Hin*dIII E segment of the vaccinia virus genome (16). To aid in locating the gene(s) encoding these putative RNA polymerase subunits, we mapped the *Bam*HI restriction endonuclease sites of this unsequenced region of the vaccinia virus genome and cloned the subfragments numbered 2 to 4 (Fig. 1B). The early mRNAs that hybridized to the cloned DNA fragments were translated, and attempts were made to immunoprecipitate the products with antiserum to RNA polymerase. A positive result was obtained with *Hin*dIII-E subfragment 3; the pattern of immunoprecipitable polypeptides was identical to that obtained with mRNA that hybridized to the entire *Hin*dIII E fragment (Fig. 1A). To proceed further, we made a *Cla*I restriction endonuclease map of the 7.1-kbp *Bam*HI fragment and cloned the subfragments numbered 5 to 8 (Fig. 1B). This time, the mRNA encoding the putative RNA polymerase subunits exclusively hybridized to two adjacent fragments, indicating that the mRNA spanned the *Cla*I site (Fig. 1A).

Nucleotide sequence analysis of the 7.1-kbp *Bam*HI fragment revealed four complete putative ORFs. The predicted polypeptides were 86 kDa for E2L, 22 kDa for E3L, 30 kDa for E4L, and 40 kDa for E5R (Fig. 2). E4L was considered the most likely candidate on the basis of its location spanning the junction (at nt 4731) of the two *Cla*I restriction fragments (numbered 6 and 7 in Fig. 1) that hybridized to the mRNA encoding the putative RNA polymerase subunits. In addition, E4L had the capacity to encode proteins close to the observed size of 32 to 37 kDa.

To establish whether this single ORF encoded the multiple immunoprecipitable polypeptides observed, the gene was cloned next to a bacteriophage T7 RNA polymerase promoter so that it could be transcribed in vitro. Cell-free translation of the in vitro transcript yielded polypeptides identical in size to the translation products of in vivo mRNA (Fig. 1C), which reacted with antiserum to the viral RNA polymerase but not with preimmune serum, indicating that they were all derived from the single gene that we have named *rpo30*. Further evidence for the common origin of the polypeptides was obtained by a polyacrylamide gel electro-

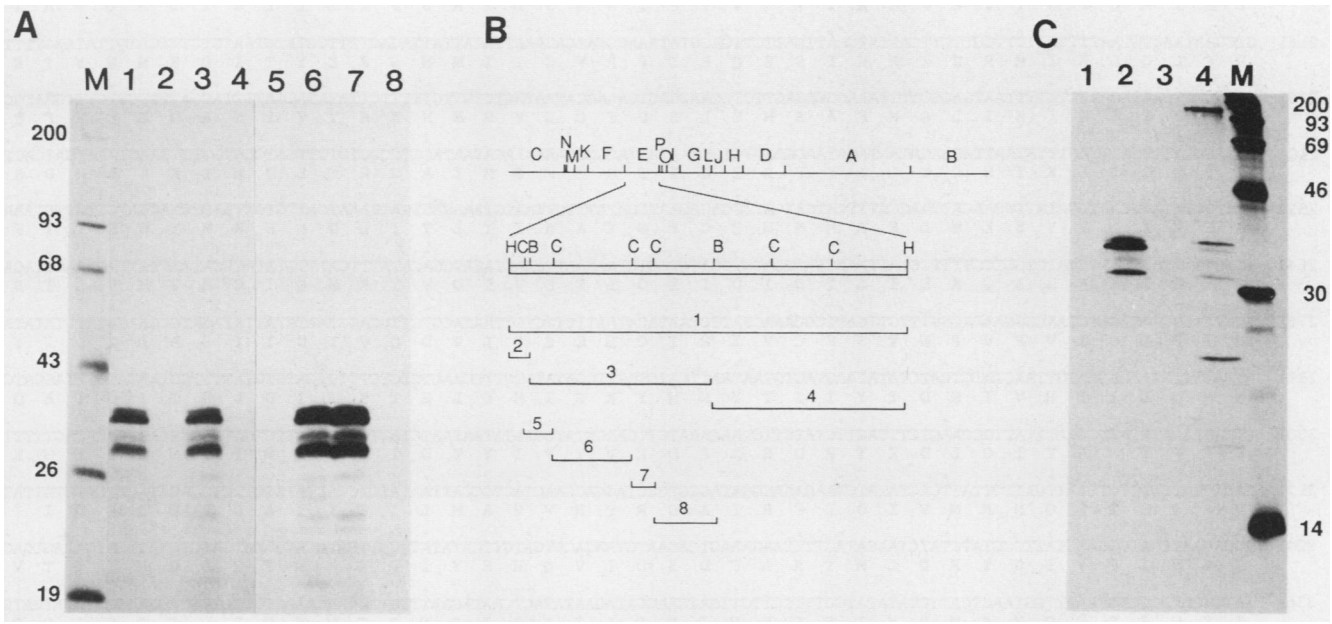

FIG. 1. Mapping of the *rpo30* gene. (A) Immunoprecipitation of the cell-free translation products of hybridization-selected viral mRNA. An autoradiograph of [^{35}S]methionine-labeled proteins separated by electrophoresis on a 10% polyacrylamide gel is shown. Lane numbers refer to the restriction fragments of viral genomic DNA shown in panel B and used for hybridization selection of mRNA. Lane M, ^{14}C-labeled protein markers. The numbers on the left refer to the molecular masses in kilodaltons of the markers. (B) Restriction endonuclease maps of the vaccinia virus genome. The upper line is the *Hin*dIII map of the entire 185-kbp genome. The 15.2-kbp *Hin*dIII fragment E is enlarged below; B refers to *Bam*HI sites located at nt 789 and 7859 (numbering according to the Fig. 2), and C refers to *Cla*I sites at nt 608, 2683, 4731, 5549, 9970, and 12259 (*dam*-modified *Cla*I site at nt 1871 is not shown), respectively. Numbered bars at the bottom represent the cloned DNA segments used for hybridization selection of mRNA. (C) Immunoprecipitation of the cell-free translation products of *rpo30* RNA synthesized in vitro by bacteriophage T7 RNA polymerase. An autoradiograph of a 15% polyacrylamide gel is shown. Lanes: 1 and 2, immunoprecipitation with preimmune and anti-RNA polymerase sera, respectively, of the translation products of in vitro-synthesized *rpo30* RNA; 3 and 4, immunoprecipitation with preimmune and anti-RNA polymerase sera, respectively, of the translation products of in vivo RNA from vaccinia virus-infected cells; M, ^{14}C-labeled protein markers, with molecular masses in kilodaltons shown on the right.

phoretic comparison of the endopeptidase V8 digestion products of the two major 32- and 36-kDa polypeptides that were eluted from a preparative polyacrylamide gel (E. V. Jones, unpublished data). A mutagenesis study (to be described below) suggested that the 32-kDa protein may have initiated from the second AUG codon located 48 bases downstream of and in frame with the first AUG.

Homology to SII. The nucleotide sequence indicated that ORF E4L potentially encodes a protein of 259 amino acids with a predicted molecular weight of 29,827. A search of the National Biomedical Research Foundation protein data base (release 23) and the translated GenBank sequences (release 63) using the FASTA program revealed an amino acid sequence similarity between the *rpo30* protein and the eucaryotic transcription elongation factor SII with an optimized score of 103 (13). Using program RDF2 (26), we found that this similarity score was more than 17 standard deviations over the mean score obtained by random shuffles of the sequences. Computer alignment of Rpo30 and SII showed that 23% of the residues were identical over a 180-amino-acid overlap (Fig. 3A), comparable to the relatedness of the largest RNA polymerase subunits of vaccinia virus and yeast cells (6). The dot matrix analysis demonstrated that the related sequences of the two proteins are linearly arranged (Fig. 3B). Furthermore, sequence motifs associated with zinc binding, Cys-X_2-Cys-X_{24}-Cys-X_2-Cys (12), are present near the C termini of SII and Rpo30, suggesting that these two proteins may interact with nucleic acids in a similar way. Although SII is about 4 kDa larger than Rpo30 and contains additional nonhomologous N-terminal sequences, there is evidence that the transcription-stimulatory activity of SII is located in the conserved C-terminal half of the protein (14).

Rpo30 is an integral subunit of viral RNA polymerase. Even though SII was shown to associate with RNA polymerase II in vivo and in vitro (15, 27, 29, 36), it is considered to be a eucaryotic transcription factor since it can be readily purified from RNA polymerase by conventional column chromatography at relatively low salt concentrations (28, 29, 34). The *rpo30* protein, in contrast, behaved as an integral subunit of RNA polymerase; it copurified with the viral RNA polymerase through several columns involving salt concentration of up to 0.5 M and under conditions in which transcription initiation and termination factors were separated (7, 35). As an aid in further substantiating the association of the *rpo30* protein with RNA polymerase, we expressed the *rpo30* ORF in *E. coli* and then obtained rabbit antiserum to the protein. This antiserum and antiserum to the purified whole viral RNA polymerase were then used to probe replicate Western blots of fractions from a glycerol density gradient to which some highly purified vaccinia virus RNA polymerase had been applied. Autoradiographs (Fig. 4) revealed that the *rpo30* 36- and 32-kDa products of cosedimented with RNA polymerase activity as well as with the other RNA polymerase subunits. A similar result was obtained upon sedimentation of the soluble virion extract before any enzyme purification, indicating that all of the packaged *rpo30* protein was associated with RNA polymerase (data not shown).

Efforts to separate the Rpo30 subunit from the viral RNA polymerase either by further column chromatography or with an Rpo30 antibody affinity column were unsuccessful

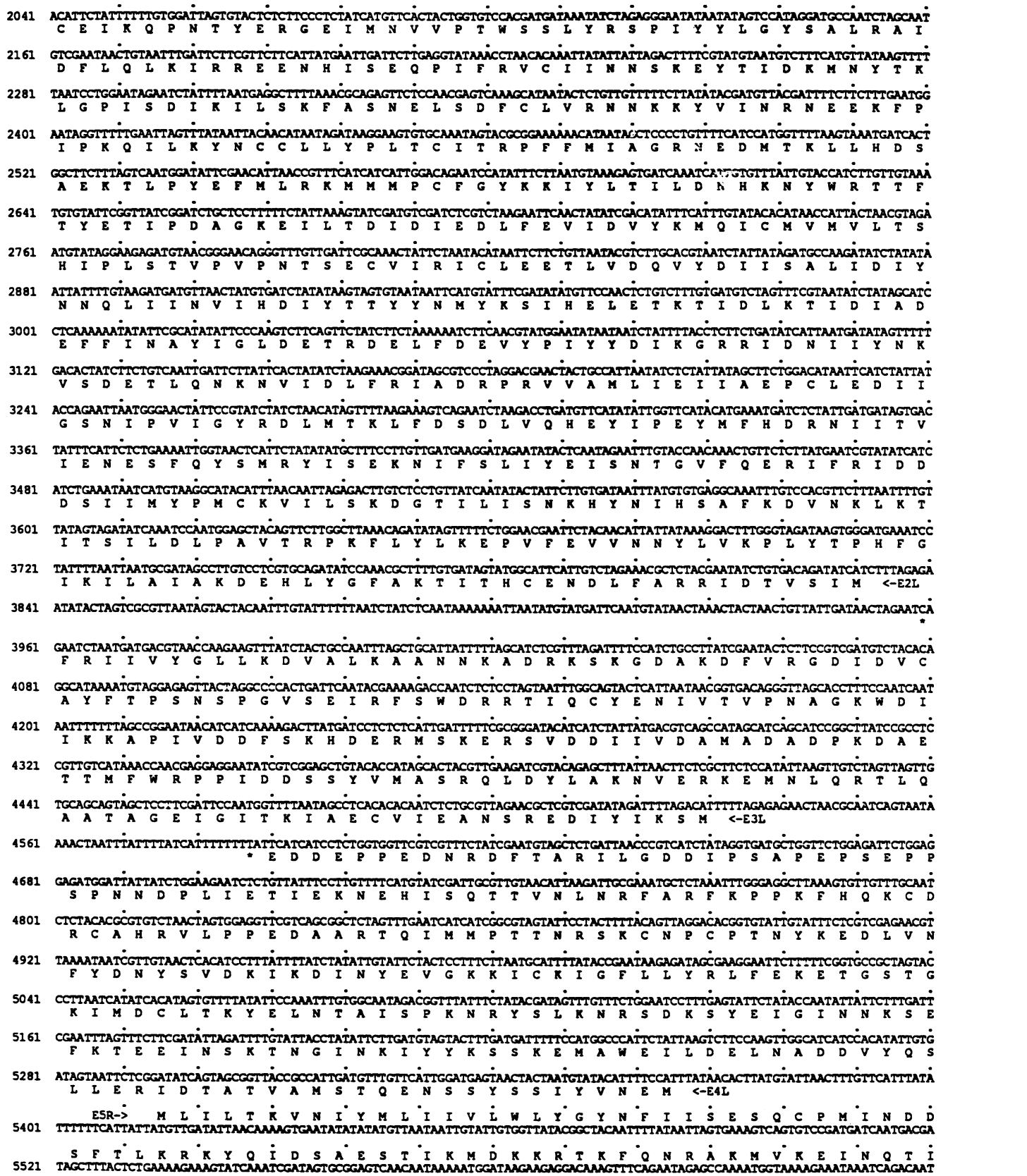

FIG. 2. Nucleotide sequences of vaccinia virus genomic *Hin*dIII E fragment. Numbering starts from the left *Hin*dIII site (at nt 1) in the same orientation as in the virus genome. Sequence of the top strand from nt 2041 to 8279 is shown. Naming of the major ORFs is according to Rosel et al. (31), with derived amino acid sequences displayed in single-letter code above the DNA sequence for rightward ORFs and below for leftward ORFs. Arrows indicate directions of the ORFs. The sequence from *Bam*HI (at nt 7859) to the end of ORF E6R (at nt 8279) was published previously (11).

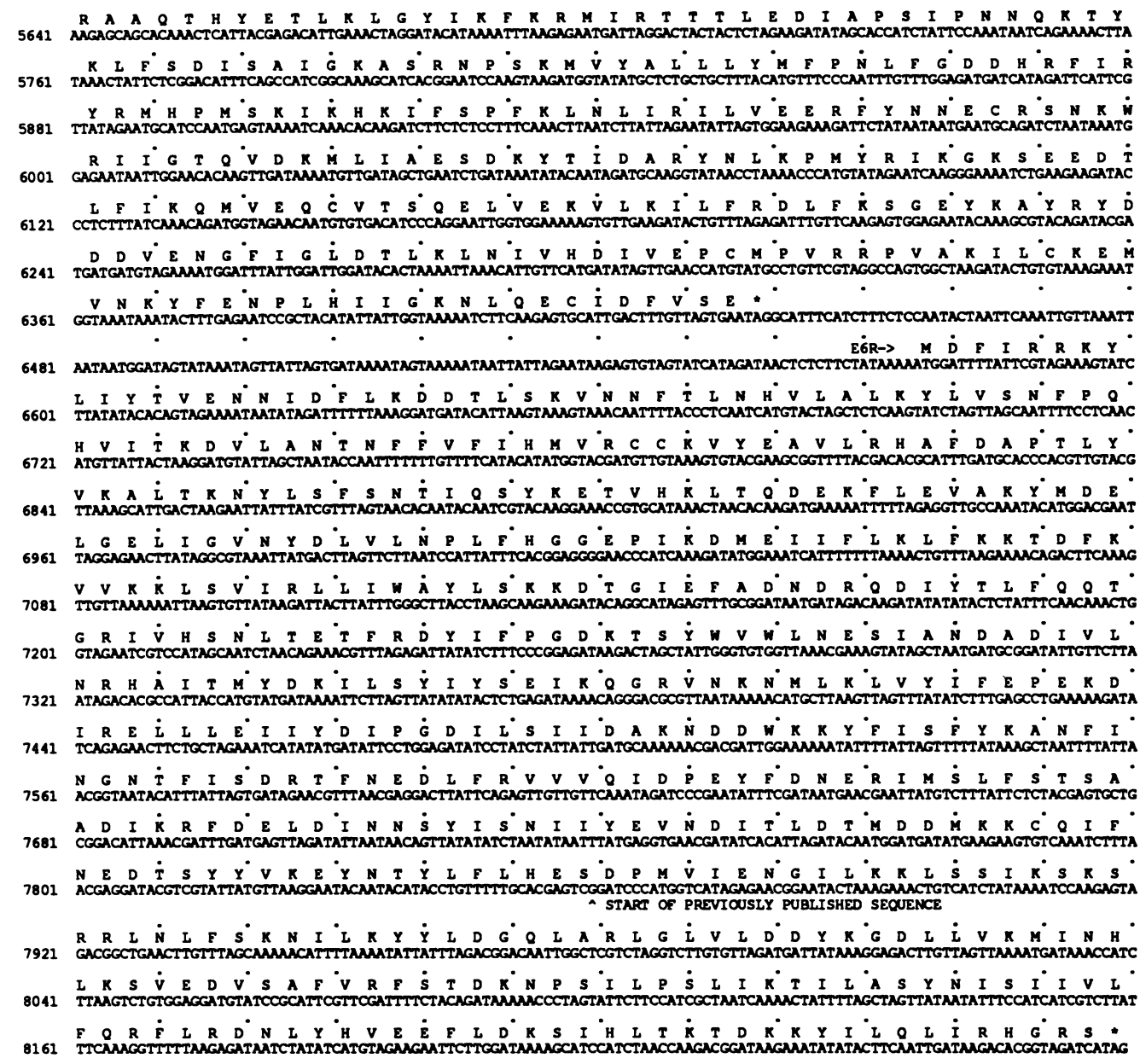

FIG. 2—*Continued.*

(B. Ahn, unpublished data). Therefore, we have not been able to determine directly the function of the Rpo30 subunit. Based on its sequence similarities with SII, however, a role in RNA chain elongation is suggested.

***rpo30* is expressed throughout viral infection.** To understand the expression and regulation of the *rpo30* gene during viral infection, we mapped the 5′ and 3′ ends of the *rpo30* transcript. A nuclease S1 protocol and primer extension procedures were used to analyze the 5′ end. Both methods mapped the 5′ end of a major early transcript within a stretch of three A residues (starting from nt 5389) located 24 bases upstream of the AUG codon (Fig. 5). The presence of an upstream sequence (from nt 5414 to 5399 as indicated in Fig. 6) resembling the consensus putative early promoter (9) is consistent with an early RNA start site. Although minor in abundance, one species of early RNA had its 5′ end mapped at nt 5340, located between the first and the second AUG codons (Fig. 5, lane 1). A protein 16 amino acid residues shorter than the full-size one would be synthesized from this minor RNA species if translation initiates at the latter AUG codon (Fig. 6). For further analysis, two 5′ deletions of the *rpo30* gene were constructed in which the first and the second ATG codons were successively deleted. When a 5′ truncated RNA starting from the second AUG codon was prepared in vitro and translated in the reticulocyte lysate, it yielded an immunoprecipitable polypeptide that migrated to the position of 32 kDa, whereas an RNA lacking the latter AUG codon produced a protein of about 28 kDa (Fig. 7). A protein of 32 kDa (as well as one of 36 kDa) was observed by Western blot analysis of purified RNA polymerase, suggesting that the 32-kDa protein is also made in vivo.

Nuclease S1 analysis indicated that the 5′ end of late RNA mapped immediately upstream of the first AUG located within a TAAATG motif, which is present in many vaccinia

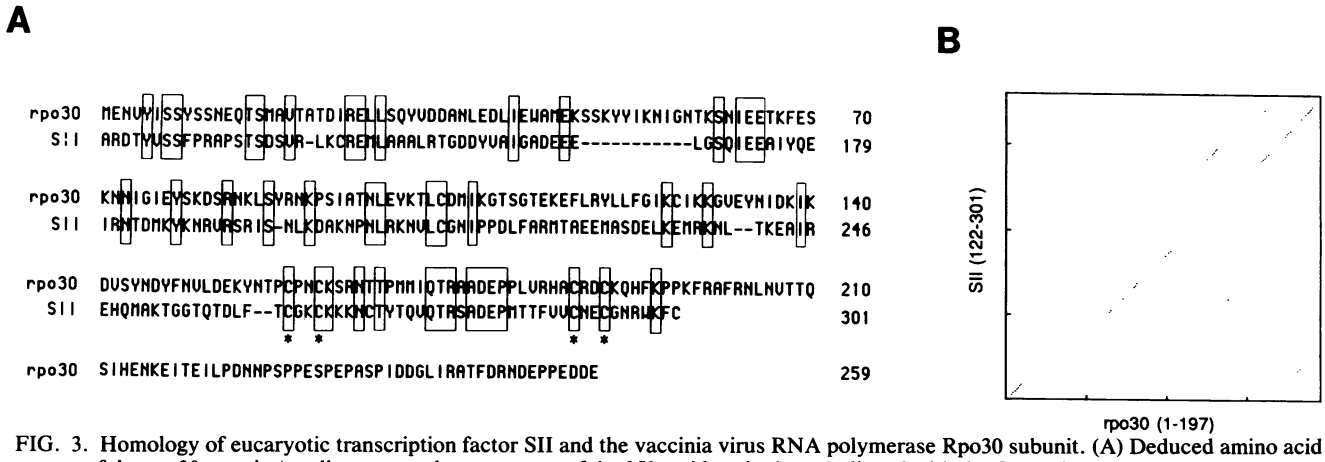

FIG. 3. Homology of eucaryotic transcription factor SII and the vaccinia virus RNA polymerase Rpo30 subunit. (A) Deduced amino acid sequence of the *rpo30* protein (top line; a complete sequence of the 259 residues is shown) aligned with the C-terminal half of SII (bottom line; sequence from residues 122 to 301 is shown). Amino acids are shown in single-letter code, with residue numbers on the right. The alignment, made by computer with the FASTA program, gave an optimized score of 103 with 23% identity in a 180-amino-acid overlap. Identical residues are boxed, and the four cysteine residues thought to be involved in zinc binding are indicated with asterisks. Indicated as dashed lines are the gaps made in the sequence of SII for the optimized alignment. (B) Linear dot matrix alignment of Rpo30 (x axis) and SII (y axis) made by using the Diagon program (37). Numbers in parentheses refer to the amino acid residues of each protein aligned.

virus genes of the late class (Fig. 5, lane 2). The transcripts of this gene class may contain a 5′ poly(A) leader that is not complementary to the DNA template (5, 33). Nuclease S1 analysis with a probe containing a 50-base poly(dT) tract attached to the sequence complementary to the coding region of the *rpo30* gene indicated the presence of a 5′ poly(A) leader in this case (Fig. 5, lane 3). It is noticeable that the protected probe is heterogeneous in size, suggesting that the leader varies in length, as do the leaders of other late RNAs characterized previously (2, 5, 33). These results indicated that the *rpo30* gene is expressed throughout the viral life cycle, consistent with observations made on the two large subunits of RNA polymerase.

The 3′ end of the *rpo30* RNA was regulated in a typical temporal pattern: the early transcript terminated 20 to 25 nt downstream of the conserved signal TTTTTAT located downstream of ORF E3L (at nt 3899); the majority of the late transcript extended heterogeneously far beyond the early termination signal up to more than a kilobase (Fig. 8). The band at the same position as the terminated early RNA might represent some residual early transcript present at 6 h after infection.

DISCUSSION

We have located the gene, *rpo30*, encoding one of the small subunits of vaccinia virus RNA polymerase. This brings the total number of mapped vaccinia virus RNA polymerase genes to five. The initial localization of *rpo30* was based on the reactivity of antibody to the purified vaccinia virus RNA polymerase with the cell-free translation products of mRNA that was selected by hybridization to immobilized viral genomic DNA fragments. Nucleotide sequence analysis of the positive region of viral genome identified an appropriately located ORF with a capacity to encode proteins of the observed size. To confirm this identification, the *rpo30* gene was expressed in *E. coli* and antibody to the protein was produced. This antibody reacted with the appropriate-size subunits of purified viral RNA polymerase. In fact, all of the *rpo30* product extracted from virus cosedimented with RNA polymerase in a density gradient centrifugation.

The sequence of *rpo30* predicted a polypeptide with a significant similarity to SII, a mammalian transcriptional elongation factor. The significance of the homology was underscored by the presence of a C-terminal zinc finger domain in each protein. Although the tight association of the Rpo30 subunit with RNA polymerase prevented us from determining its role directly, sequence homology to SII suggests that this protein may be required for efficient elongation of transcripts. It is intriguing, however, that the viral protein is an integral RNA polymerase subunit, whereas the eucaryotic homolog is an easily dissociable factor. The tight association of the *rpo30* polypeptide with the viral RNA polymerase may ensure its packaging during virion assembly, its introduction into infected cells in stoichiometric amounts with RNA polymerase, and a maximal and uniform rate of transcription. In contrast, the weaker association of the eucaryotic elongation factor with RNA polymerase may allow for more complex modulation of transcriptional efficiency. Such a role is consistent with evidence that SII stimulates RNA polymerase II to read through some transcription pause sites, suggested to play a role in regulation of elongation or termination of heterogeneous nuclear RNA synthesis (28–30).

The development of an in vitro transcription system either from vaccinia virus core or infected-cell extracts led to the identification of several specific transcription factors that complement purified RNA polymerase for accurate initiation and termination of transcription. These include a vaccinia virus early transcription factor (7), a late transcription factor (39), and a termination factor (35). In vitro study with mutagenized DNA templates also demonstrated that the sequence TTTTTNT, found approximately 20 to 50 bp upstream of the termination sites of many early genes, was necessary and sufficient for termination of early transcripts (40). In a few cases, the TTTTTNT sequences are present in the middle or even shortly downstream of the RNA start sites. It has not been determined, however, whether these sequences act as a signal for transcriptional attenuation of the latter genes. Interestingly, it was found that late transcripts of vaccinia virus do not have defined 3′ ends and often extend up to more than a kilobase past the ends of the

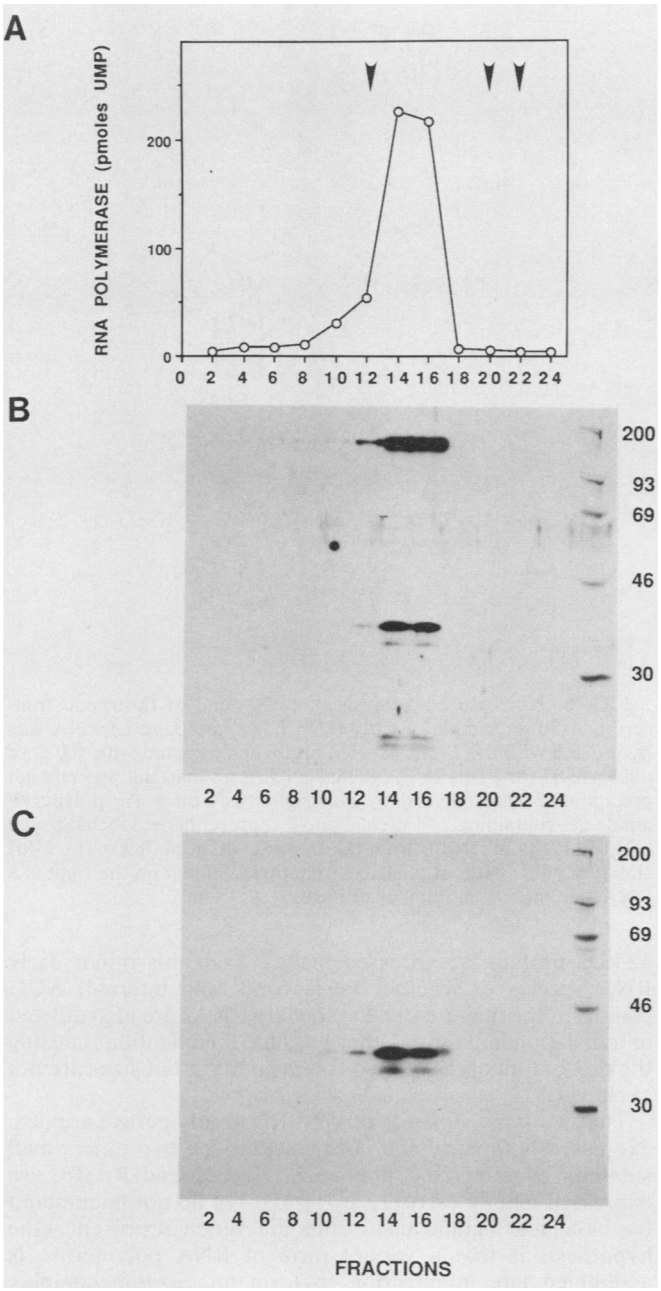

FIG. 4. Cosedimentation of the Rpo30 subunit with the RNA polymerase of vaccinia virus during glycerol gradient centrifugation. Purified RNA polymerase (10 μg) was applied to a 15 to 35% linear glycerol gradient and sedimented as described in Materials and Methods. (A) RNA polymerase activity; the number of RNA polymerase units in 5 μl of each fraction is indicated. The fraction numbers are in increasing order from the bottom to the top of the tube. Arrowheads indicate sedimentation positions of three protein markers, thyroglobulin (690 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). (B) Western blot analysis of glycerol gradient fractions with antiserum to the viral RNA polymerase. The proteins present in the individual fractions were resolved by polyacrylamide gel electrophoresis and transferred to a membrane, which was incubated successively with antibody to vaccinia virus RNA polymerase and ^{125}I-labeled staphylococcal protein A. Lane M, ^{14}C-labeled protein markers. (C) Western blot analysis of glycerol gradient fractions with antiserum to *rpo30* protein. Procedures were the same as for panel B except for the source of the antibody.

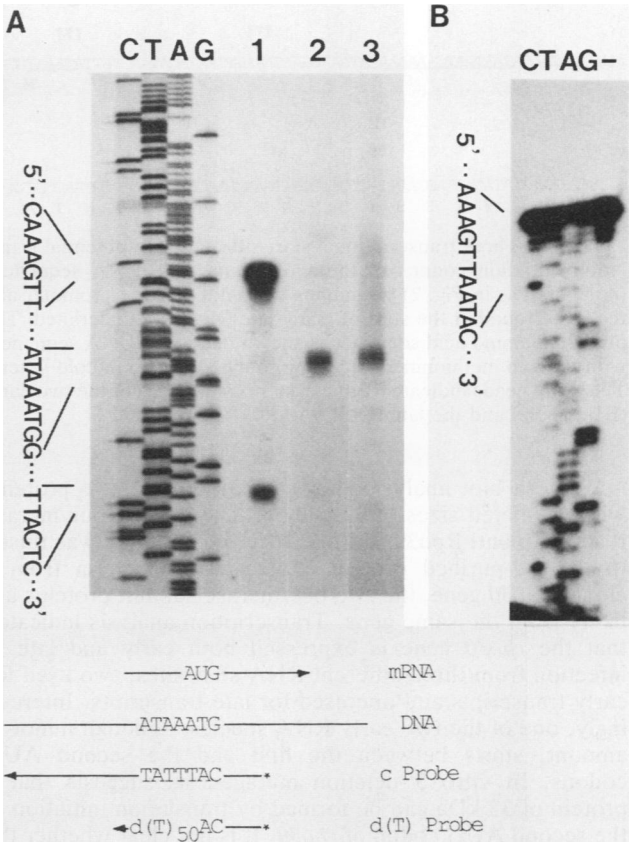

FIG. 5. Analyses of the 5′ end of the *rpo30* transcript. (A) S1 nuclease mapping. A 30-μg sample of total early RNA isolated from cells at 4 h after infection in the presence of cycloheximide or at 6 h in the absence of the inhibitor was hybridized with 1 pmol of ^{32}P-5′-end-labeled probe and digested with 400 U of S1 at 25°C for 1 h. The nuclease-resistant probe was ethanol precipitated and separated in a 5% polyacrylamide gel containing 7 M urea. The two probes used are indicated at the bottom. Also shown are the *rpo30* mRNA and 7.1-kbp *Bam*HI DNA fragment. The c probe contains a 153-nt segment of the *rpo30* gene and upstream sequence. The polyd(T) probe has the same 153 nt, followed by 50 d(T) residues immediately 3′ of the translation initiation codon. Asterisks and arrowheads indicate the labeled 5′ ends and polarity of the strands, respectively. Lanes contain the following: c probe with early (lane 1) or late (lane 2) RNA; d(T) probe with late RNA (lane 3). The sequence ladder produced by using the c primer is shown in the first four lanes, with three segments of sequence around each nuclease-resistant RNA end shown on the left (sequence of the RNA-like strand is shown). (B) Primer extension analysis of the *rpo30* early transcript. 5′-End-labeled primer (1 pmol) was hybridized to 30 μg of early RNA and elongated by avian myeloblastosis virus reverse transcriptase in the presence of ddGTP (lane C), ddATP (lane T), ddTTP (lane A), or ddCTP (lane G) or in the absence of dideoxynucleotide (lane −). Shown on the left is the sequence of the RNA-like strand starting from the stretch of three A residues where the 5′ end of RNA mapped.

coding regions. RNA-DNA hybridization studies revealed that nearly all of the genome is transcribed at late times. These data (reviewed in reference 22) suggest that transcriptional elongation and termination may play an important role not only in expression of individual genes but in the temporal regulation of the viral life cycle. A regulatory role of the *rpo30* gene product in the elongation of the in vivo mRNA remains to be determined.

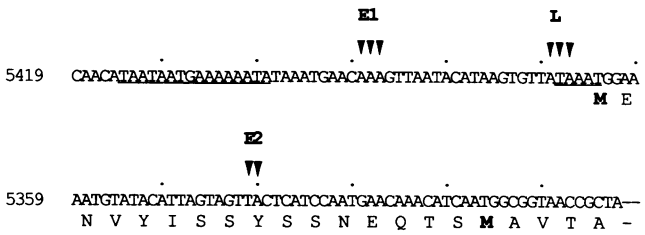

FIG. 6. Three transcriptional start sites and two potential translation initiation codons of the *rpo30* gene. The DNA sequences (numbered as in Fig. 2) resembling the putative early promoter and the motif found at the start of many late ORFs are underlined. The predicted amino acid sequence is shown below the DNA sequence, with the two methionine residues in frame indicated in bold letters. The arrowheads indicate S1 nuclease-resistant ends of the two early (E1 and E2) and the late (L) RNAs.

Western blot analysis showed that at least two proteins with estimated sizes of 32 and 36 kDa in RNA polymerase react with anti-Rpo30 serum. Since the antibody was raised to the gel-purified protein expressed in bacteria from a cloned *rpo30* gene, the two polymerase subunit proteins are likely from the same gene. Transcription analysis indicated that the *rpo30* gene is expressed both early and late in infection from three different RNA start sites, two used for early transcripts and one used for late transcripts. Interestingly, one of the two early RNA species, although minor in amount, starts between the first and the second AUG codons. In vitro 5′-deletion mutagenesis suggests that a protein of 32 kDa can be formed by translation initiation at the second AUG codon of *rpo30*. It is not clear whether the

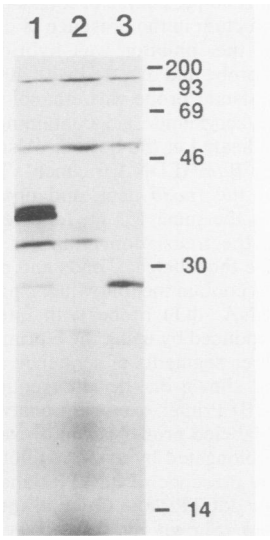

FIG. 7. Translation of 5′-truncated *rpo30* RNAs. RNA was synthesized in vitro by using T7 RNA polymerase and the $m^7G(5')ppp(5')G$ cap analog from three plasmid templates pT7rpo30A, -B, and -C. pT7rpo30A contains a full-length *rpo30* ORF cloned into the T7 vector pGEM3. pT7rpo30B and -C contain part of the ORF starting from the second (at nt 5317) and the third (at nt 5233) AUG codons, respectively. A 1-μg sample of RNA was translated in a reticulocyte lysate in the presence of [^{35}S]methionine. The translated products were immunoprecipitated with antiserum to viral RNA polymerase and separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. Lanes contain the translation products from pT7rpo30A (lane 1), pT7rpo30B (lane 2), and pT7rpo30C (lane 3). Shown on the right is a protein size marker in kilodaltons.

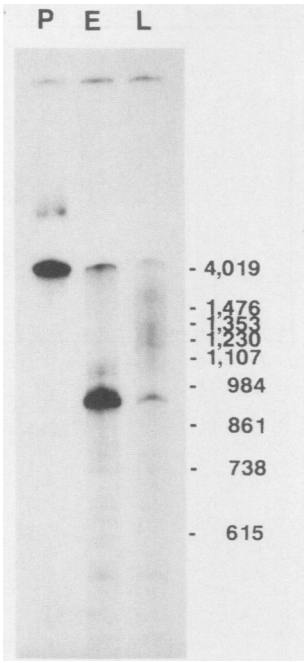

FIG. 8. Nuclease S1 analysis of the 3′ end of the *rpo30* transcript. A 30-μg sample of early (lane E) or late (lane L) RNA was hybridized with the 3′-end-labeled probe and digested with 400 U of nuclease S1 for 1 h at 25°C. Nuclease-resistant material was ethanol precipitated and analyzed by electrophoresis on a 4% polyacrylamide gel containing 7 M urea. Lane P represents the labeled probe of 4,019 bases in length (from the *Bam*HI site at nt 789 to the MluI site at nt 4807) without nuclease treatment. Shown on the right is a DNA size marker in number of bases.

32-kDa protein is expressed mainly from this minor early RNA species or whether the second (and internal) AUG codons of the major early and the late RNAs are also utilized in translation initiation. Other possible modifications causing the production of the *rpo30* proteins of different sizes are not excluded.

The two large subunits of viral RNA polymerase are also expressed both early and late, whereas the two other small subunits characterized previously, Rpo22 and Rpo18, are expressed mainly early (1, 6, 19, 25). We do not understand the biological significance of this difference at present. One hypothesis is that a variant form of RNA polymerase is assembled late in infection to form an enzyme complex capable of transcribing late-class genes. Whether distinctive forms of RNA polymerase more efficiently interact with specific transcription factors is not known. More information on the synthesis and assembly of each subunit is necessary to understand the role of RNA polymerase and its interaction with other regulatory factors.

The functions of the several small subunits of RNA polymerase are not well understood. Biochemical studies on the functions of individual subunits of RNA polymerase are hampered by the difficulty in dissociating single subunits from the native enzyme complex. Genetic alterations of some subunit genes were used as alternative approaches. Thus, analyses of temperature-sensitive mutants suggested that the alpha subunit of *E. coli* and the RPB3 subunit from yeast cells were involved in enzyme assembly, although they may have other roles as well (17, 18). The RPB4 subunit of yeast cells was shown to be required for the thermostability and efficiency of the enzyme (38). Identification of *rpo30* as

well as other genes of vaccinia virus RNA polymerase subunits provides an opportunity for such genetic approaches.

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