

Identification, Sequence, and Expression of the Gene Encoding a M_r 35,000 Subunit of the Vaccinia Virus DNA-dependent RNA Polymerase*

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The gene *rpo35*, encoding a subunit of the vaccinia virus DNA-dependent RNA polymerase, was identified, and its RNA and protein products were characterized. An M_r 35,000 polypeptide, which bound antibody to the purified RNA polymerase, was synthesized in reticulocyte lysates programmed with viral mRNA that hybridized to a 2,300-base pair segment of the viral genome. Determination of the sequence of the DNA segment revealed four potential protein coding regions, none of which had evident similarity to any described RNA polymerase subunit of prokaryotes or eukaryotes. One open reading frame that could encode a 35,400-Da protein was identified as *rpo35* on the basis of mRNA hybridization, cell-free translation, and immunoprecipitation. The identification was confirmed by sequencing tryptic peptides of the authentic M_r 35,000 RNA polymerase subunit. Antiserum to the purified recombinant protein, expressed in bacteria, reacted specifically with a M_r 35,000 polypeptide that was detected starting 2 h after virus infection and that co-sedimented with RNA polymerase purified from virions. RNA analyses indicated that the 5'-end of an early transcript started 25 nucleotides upstream of *rpo35*, which is consistent with the location of an early promoter consensus sequence.

Most DNA viruses replicate in the cell nucleus, where they make extensive use of the host transcription apparatus. The poxviruses, of which vaccinia virus is the prototype, are exceptions in that they replicate in the cytoplasm and encode many, if not all, of the enzymes needed for DNA and RNA synthesis (reviewed in Ref. 1). Indeed, the possession of a virus-encoded DNA-dependent RNA polymerase is a distinctive feature of members of this family. The RNA polymerase, along with a transcription factor, RNA guanylyl- and methyltransferases, a poly(A) polymerase, and other enzymes, is packaged within the virion and activated upon entry into the cell cytoplasm leading to the production of the early class of mRNAs and proteins. Following DNA replication, intermediate and late classes of mRNAs and proteins are synthesized. The RNA polymerase, isolated from either vaccinia virus-infected cells (2) or from purified vaccinia virions (3) is a multisubunit enzyme composed of two large polypeptides of

about 147 and 132 kDa and numerous smaller ones. Antibody, prepared to highly purified vaccinia virus RNA polymerase, was used to demonstrate that the subunits are virus-encoded and to determine their approximate genome locations by immunoprecipitation of the cell-free translation products of viral early mRNAs that hybridized to specific DNA fragments (4). The two large subunit genes, *rpo147* and *rpo132*, were identified, and the deduced protein sequences indicated homology to the corresponding large subunits of eukaryotic and prokaryotic DNA-dependent RNA polymerases (5-7). Thus far, the genes for three of the small subunits, *rpo30*, *rpo22*, and *rpo18*, have been found (5, 8-11). In contrast to the large subunits, the small ones identified thus far are not closely related to reported prokaryotic or eukaryotic small subunit genes, although homology between *rpo30* and eukaryotic transcription elongation factor SII was suggested (9).

The identification of the remaining RNA polymerase subunit genes is a prerequisite for studying the assembly and structure-function relationships of this important and complex enzyme. We have taken another step toward this goal by identifying and characterizing the gene encoding an RNA polymerase subunit of M_r 35,000. This gene was mapped to the 45-kbp¹ *Hind*III A fragment of the vaccinia virus strain WR genome (4), and analyses described here localized it to a 2.3-kbp *Sst*I-*Sall* subsegment. Four complete ORFs, of which only one could encode a protein close to the size of the M_r 35,000 subunit, were found upon sequencing the fragment. Further studies correlated the latter ORF with the RNA polymerase subunit gene, which we named *rpo35*.

EXPERIMENTAL PROCEDURES

Virus and Cells—Vaccinia virus (WR strain) stocks were prepared in HeLa spinner cells that were propagated in minimum essential spinner medium (Quality Biologics Inc.) supplemented with 5% horse serum.

Plasmid Constructions—Standard methods (12) were used for isolation and construction of plasmids; restriction endonucleases and other DNA-modifying enzymes were usually from Boehringer Mannheim. Generally, the reaction conditions of the vendor were used for restriction endonuclease digestions. The pUC19-derived plasmids pA34, pA25, pA81, pA62, pA64, pA13, pA821, and pA32 contain segments derived by *Bam*HI digestion of the *Hind*III A fragment of the vaccinia virus (strain WR) genome (7). The 14.2-kbp *Bam*HI insert of pA64 was digested with *Sall*, and the 6.0-kbp subfragment was ligated to *Sall*-cleaved pGEM3zf(+) from Promega Biotec to generate pBA6s. Plasmids pBA6s/b and pBA1.4s/b were constructed by insertion of *Sall/Bam*HI fragments of pA64 (7) into the *Sall* and *Bam*HI sites of pUC19. The 6.6-kbp vaccinia virus DNA

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M61187.

¹ The abbreviations used are: kbp, kilobase pair; AraC, cytosine arabinoside; bp, base pair; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; WR, Western Reserve.

insert of pBA6.6s/b was digested with either *Sst*I or *Sst*I and *Sal*I and then cloned into the corresponding sites of pGEM3zf(+) to produce pBA2s/sst and pBA4sst. The plasmids pBA1.4s/b, pBA6s, pBA6.6s/b, pBA2s/sst, and pBA4sst were used for hybridization and selection of viral mRNA.

For expression of *rpo35* in *Escherichia coli*, the plasmid pET3c35 was constructed. Polymerase chain reaction was used to place *Nde*I and *Bam*HI restriction sites at the 5'- and the 3'-ends of the *rpo35* ORF, respectively (13), and the amplified DNA was ligated with the *Nde*- and *Bam*HI-digested pET3c vector (14).

Plasmid pBA0.5s/h, used for preparation of an RNA probe for nuclease S1 analysis, was constructed as follows. Two oligonucleotides, RP1 (GTCGACTTTTGGAACGATTG) and RP2 (AAGCTT GTTAGCCATTCCCTCATTGACAGCGG), containing restriction endonuclease *Sal*I and *Hind*III sites, respectively, were used as polymerase chain reaction primers to amplify a 500-bp fragment. The amplified DNA product was digested with *Sal*I and *Hind*III restriction endonucleases and then cloned into a pGEM3zf(+) vector.

DNA Sequencing—Overlapping fragments of DNA were inserted into the phagemid vector pGEM3zf (Promega Biotec) in both orientations for complete sequencing of the two DNA strands. Single-stranded phage DNA templates were prepared and sequenced by the dideoxynucleotide chain termination method (15), using synthetic oligonucleotide primers and Sequenase kits purchased from U. S. Biochemical Corp. Microgenie (Beckman) and FASTA (16) computer programs were used for manipulations of the DNA sequence and protein homology searches, respectively.

Selection and in Vitro Translation of mRNA—Cytoplasmic RNA from infected cells was prepared by CsCl centrifugation and hybridized to DNA immobilized on filters as described (17). Early RNA was obtained at 4 h after infection of HeLa cells with 15 plaque-forming units/cell of vaccinia virus in the presence of 100 µg of cycloheximide/ml. Late RNA was isolated at 7 h after infection in the absence of drug. The RNAs selected by hybridization were translated in micrococcal nuclease-treated rabbit reticulocyte lysates (Promega Biotec) containing [³⁵S]methionine. The translation products were incubated with either preimmune serum or antiserum to vaccinia virus RNA polymerase (4) and then with staphylococcal A protein attached to Sepharose beads. The proteins were dissociated from the beads by boiling with SDS, and were then analyzed by PAGE.

Expression of the 35-kDa Protein in E. coli and Preparation of Antiserum—Single colonies, from fresh plates containing *E. coli* strain BL21 (DE3) that had been transformed with the pET3c plasmid vector (14) or with pET3c35, the vector containing the ORF encoding the 35-kDa protein, were inoculated into 1 ml of L broth containing ampicillin, and after 2 h at 37 °C, isopropyl-β-D-thiogalactoside was added. The bacteria were incubated for an additional 2 h, collected by centrifugation, resuspended in 100 µl of lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), and boiled for 5 min. The samples were then centrifuged for 5 min, and 20 µl of the supernatant proteins were resolved by SDS-PAGE on a 12% gel. For purification of the 35-kDa protein, larger preparations were made and the appropriate gel band was cut out. The protein was eluted by extracting the gel slices twice with 25 ml of sterile distilled water and then concentrated to 1 ml. The protein was injected into New Zealand White rabbits in Freund's complete adjuvant. A boost with antigen in incomplete adjuvant was given 2 weeks after the first injection.

Purification of RNA Polymerase from Vaccinia Virions—Vaccinia virions were purified twice by sucrose density gradient centrifugation, and a soluble extract containing RNA polymerase and other virion proteins was prepared essentially as described (3). The RNA polymerase was further purified by successive chromatography on columns of DEAE-cellulose, heparin agarose, single-strand DNA agarose, and phosphocellulose. The enzyme activity was assayed using single-stranded DNA of bacteriophage M13 mp18 as template (9). The purity of the enzyme, examined by SDS-PAGE and silver-staining of the proteins, was comparable with that previously described (3). A 10-µg sample of pure enzyme was subjected to glycerol density gradient centrifugation, and the resultant fractions were used for immunoblot analysis of the *rpo35* protein, as described (9). For the preparation of enzyme used in protein sequence analysis, columns of heparin agarose and single-strand DNA agarose were replaced by DEAE-cellulose and Bio-Gel A-1.5m.

Protein Sequence Analysis—Purified vaccinia virus RNA polymerase (200 pmol, approximately 100 µg) was resolved into subunits by SDS-PAGE. The polypeptides were transferred to nitrocellulose and visualized by staining with 0.1% Ponceau S as described by Aebersold

et al. (18). The strip containing the band of approximately *M_r* 35,000 was excised for internal protein sequencing under the direction of William Lane of the Harvard Microchemistry Facility. The excised band was subjected to *in situ* tryptic digestion, and the resulting peptides were separated by microbore reverse-phase high pressure liquid chromatography. The peptide-containing fractions were collected based on the UV absorption at 210 nm, and appropriate fractions were subjected to a gas-phase protein sequencer (ABI model 470A) connected with the 120A on-line phenylthiohydantoin-derivative analyzer.

Analysis of Proteins from Vaccinia Virus-infected Cells—HeLa cells were infected with vaccinia virus at a multiplicity of 15 plaque-forming units/cell. The cells were harvested at various times after infection, resuspended in 4 volumes of 100 mM Tris-HCl, pH 8, 100 mM NaCl, 0.5% Nonidet P-40 buffer. The suspensions were mixed by vortexing and were centrifuged in a microcentrifuge. The clear supernatants were heated with SDS and mercaptoethanol and resolved by PAGE. The proteins were transferred onto a nitrocellulose membrane and incubated with antiserum, followed by ¹²⁵I-labeled protein A.

RNA Analyses—RNA, from cells infected for 4 h in the presence of cycloheximide or 7 h in the presence or absence of AraC, was isolated by the RNAzol (Cinna/Biotex) method. For primer extension analysis, 20 pmol of the primer was 5'-end-labeled with polynucleotide kinase and [γ -³²P]ATP and annealed to 20 µg of either early or late RNA, followed by extension with avian myeloblastosis virus reverse transcriptase, as described (12). Internally labeled [α -³²P]UTP complementary RNAs were synthesized and used for nuclease S1 analysis, as described (34). The plasmid pBA0.5s/h was cleaved at the *Sal*I site and transcribed using SP6 RNA polymerase in the presence of [α -³²P]UTP to produce a 500-nucleotide transcript that was used as an RNA probe for 5'-end mapping. Protected segments were then analyzed by PAGE on a 6% gel.

RESULTS

Localization of the RNA Polymerase *M_r* 35,000 Subunit Gene—The gene encoding an RNA polymerase subunit of approximately *M_r* 35,000 was previously mapped to the 45-kbp *Hind*IIIA fragment of the vaccinia virus genome (4). The localization was achieved using antiserum, raised to purified vaccinia virus RNA polymerase, for immunoprecipitation of *in vitro* translation products of early viral mRNAs that were selected by hybridization to immobilized, cloned genomic DNA fragments. To further localize the gene encoding this polypeptide, mRNA from cells infected with vaccinia virus in the presence of cycloheximide, a protein synthesis inhibitor that enhances early transcription, was hybridized to a panel of cloned restriction endonuclease fragments derived from the *Hind*IIIA segment (Fig. 1B). Following previous procedures (4), the hybridized mRNA was isolated and then translated in a micrococcal nuclease-treated reticulocyte lysate containing [³⁵S]methionine. The labeled proteins were incubated with antiserum to RNA polymerase, bound to staphylococcal A protein attached to beads, and analyzed by PAGE. As shown by the autoradiograph in Fig. 1A, an immunoprecipitated polypeptide of about *M_r* 35,000 was made only in lysates that were programmed with mRNAs that hybridized to plasmid pA64, which contained a 14.2-kbp *Bam*HI genome fragment.

Additional mRNA hybridizations and cell-free translations, using smaller DNA fragments, were needed to further localize the RNA polymerase gene. The 14.2-kbp *Bam*HI fragment was subdivided by cleavage with *Sal*I, and the estimated 6.6-kbp *Bam*HI/*Sal*I, 6.0-kbp *Sal*I, and 1.4-kbp *Sal*I/*Bam*HI segments were cloned (Fig. 2C). The mRNAs that hybridized to these plasmids were translated *in vitro*, and the labeled proteins were immunoprecipitated and analyzed by PAGE. Synthesis of the *M_r* 35,000 polypeptide was programmed by RNA that hybridized to the 6.6-kbp fragment (Fig. 2A). Next, the 4.0-kbp *Sst*I and the 2.3-kbp *Sst*I/*Sal*I subclones of the 6.6-kbp fragment (Fig. 2C) were used to select early mRNAs. The immunoprecipitable *M_r* 35,000 polypeptide was synthesized

Vaccinia Virus RNA Polymerase Subunit

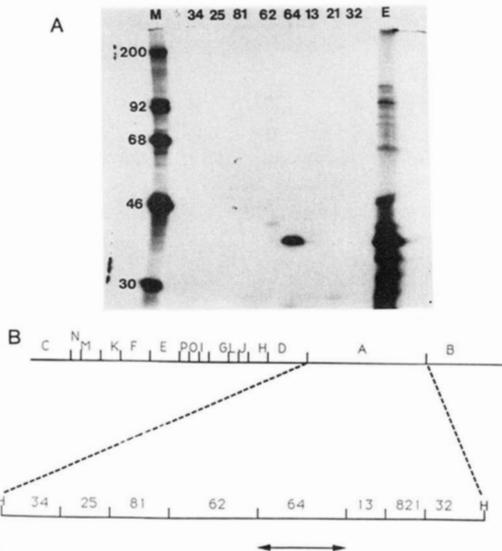


FIG. 1. Localization of the gene encoding the M_r 35,000 subunit of vaccinia virus RNA polymerase. *A*, cytoplasmic RNA, from HeLa cells that were infected with vaccinia virus in the presence of cycloheximide, was hybridized to plasmids containing cloned vaccinia virus DNA fragments immobilized on nitrocellulose filters. The RNA was eluted and then translated in a micrococcal nuclease-treated rabbit reticulocyte lysate in the presence of [35 S]methionine. The translation products were incubated successively with antiserum to purified vaccinia virus RNA polymerase and beads containing staphylococcal A protein. The bound proteins were eluted with SDS and resolved by electrophoresis on a 10% polyacrylamide gel. An autoradiograph is shown. Lane *M* contains 14 C-labeled protein markers with kilodaltons indicated on the left. Lane *E* contains the immunoprecipitated translation products of the total early RNA from the cytoplasm of infected cells. The remaining lanes contain immunoprecipitated translation products of RNA that hybridized to plasmids pA34, pA25, pA81, pA62, pA64, pA13, pA821, and pA32. The p has been omitted and 821 has been shortened to 21 to save space. *B*, the linear order and relative sizes of the HindIII fragments and the BamHI subclones of the HindIIIA fragment are indicated. The unlabeled vertical lines indicate BamHI sites, and those labeled *H* refer to HindIII sites. The double-headed arrow marks the region that hybridized to mRNA encoding an immunoreactive M_r 35,000 polypeptide.

only in the reticulocyte lysate that had been programmed with RNA that hybridized to the 2.3-kbp fragment (Fig. 2B).

Nucleotide Sequence of the 2.3-kbp DNA Fragment—The DNA sequence of the SstI-Sall vaccinia genome fragment and the predicted amino acid sequence of a 35.4-kDa ORF are shown in Fig. 3. Additional nonoverlapping ORFs, not depicted in Fig. 3, could encode polypeptides of 8.7, 16.3, and 12.3 kDa. In addition, the SstI site interrupts an ORF of 25 kDa (data not shown). The 12.3-kDa ORF differed by 5 nucleotides from a previously identified vaccinia virus WR fusion protein gene (19). Subsequent to our collection of the above data for the WR strain of vaccinia virus, the genomic sequence of the Copenhagen strain of vaccinia virus was reported (20), allowing us to make a comparison. An alignment with the region described here indicated the same open reading frames with 99% identity for the 8.7- and 35.4-kDa ORFs and 97 and 96% identity for the 16.3- and 12.3-kDa ORFs, respectively. *Capripoxvirus* sequences corresponding to a part of the 35.4-kDa ORF and the complete 16.3- and 12.3-kDa ORFs (21) were 63, 50, and 37% identical with that of vaccinia WR, respectively, which is consistent with the relationships between members of different poxvirus genera.

Searches of GenBank (volume 65) revealed no similarities of polypeptides encoded within the 2.3-kbp fragment of vaccinia virus DNA with previously sequenced RNA polymerase subunits of prokaryotes or eukaryotes. Based on size, however,

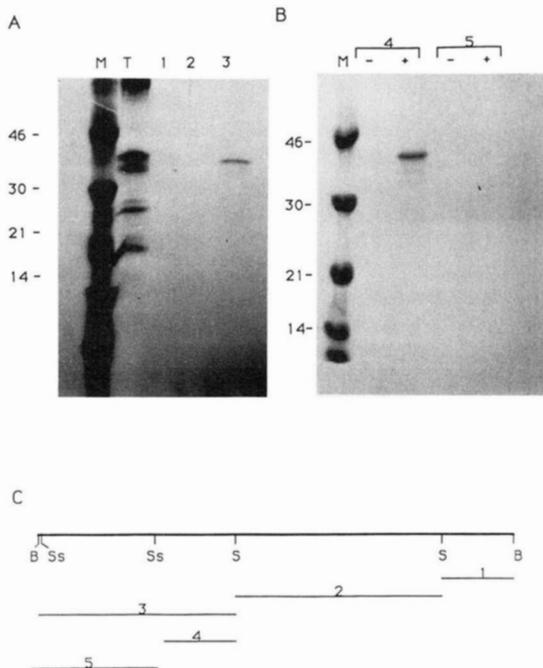


FIG. 2. Fine mapping of the RNA polymerase subunit gene. *A* and *B*, the translation products of early vaccinia virus mRNAs that hybridized to filters containing cloned subfragments of pA64 were bound to RNA polymerase antiserum and analyzed by PAGE using procedures similar to those described in the legend to Fig. 1, except that the gel was 15% polyacrylamide. Autoradiographs are shown. *M*, 14 C-labeled marker proteins in kDa; *T*, translation products of total early RNA from the cytoplasm of infected cells. *Lanes 1–5* refer to the DNA fragment number, indicated in panel *C*, used to select mRNAs by hybridization. In *B*, – and + refer to the use of preimmune and immune antisera, respectively. *C*, a partial restriction map of the 14.2-kbp *Bam*HI fragment from pA64. The numbered bars indicate the cloned fragments present in plasmids that were bound to filters and used to select mRNA by hybridization. Restriction endonuclease sites are abbreviated: *B*, *Bam*HI; *Ss*, *Sst*I; *S*, *Sal*I.

the ORF that was calculated to produce a polypeptide of 35.4 kDa appeared likely to encode the M_r 35,000 RNA polymerase subunit.

Evidence That the Product of the 35.4-kDa ORF Is an RNA Polymerase Subunit—The protein, encoded by the 35.4-kDa ORF, was expressed in bacteria in order to prepare a specific antibody that could be used to provide an alternative approach to the identification of the RNA polymerase subunit gene, as well as a reagent for expression studies. *E. coli* BL21(DE3) was transformed with plasmid pET3c35, which was made by inserting the 35.4-kDa ORF next to the bacteriophage T7 ϕ 10 promoter in the vector pET3c (14). Bacteria transformed with the vector alone also were isolated to serve as a control. A prominent M_r 35,000 band was resolved by SDS-PAGE and detected by Coomassie Blue staining of proteins from pET3c35- but not pET3c-transformed *E. coli*. After transfer to a nitrocellulose membrane, the polypeptides bound antibody raised to the RNA polymerase purified from vaccinia virions. The recombinant M_r 35,000 protein was therefore purified by PAGE and used to immunize a rabbit. A preliminary assessment of the antiserum was made by its reactivity to SDS-PAGE blots of recombinant protein made by the *E. coli* pET3c35 transformants.

A highly purified preparation of RNA polymerase, isolated from vaccinia virions, was then sedimented through a glycerol gradient (Fig. 4*A*), and the proteins of individual fractions were subjected to SDS-PAGE and transferred to nitrocellulose membranes (9). The autoradiograph of a blot, incubated with antibody to the whole RNA polymerase, revealed the

FIG. 3. Sequence of a 2.3-kbp segment of the vaccinia virus genome. The sequence corresponds to the *Sst*I-*Sal*I segment designated 4 in Fig. 2C. Numbering of the nucleotide sequence starts from the *Sal*I site and is in the opposite orientation of the viral genome as displayed in Fig. 1B. The predicted amino acid sequence of the 35.4-kDa ORF is indicated in the single-letter amino acid code above the DNA sequence. The asterisk represents a translational termination codon. Two RNA start sites that map upstream of the 35.4-kDa ORF are underlined. Two copies of the early transcription termination motif (TTTTTNT) are underlined by dashes.

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GTCGACTTTTGAAAGATTGATAAAAAGCTGGTTTATTTATGTTTACGGACAAGTGTGTAATGCGCTTACTAGTAAAAAATCTATCCATAAAAGACGAATAGTTACAAAC 120
ACAATGTTATATAATTTAAATGCAAGGCTTACGAGGCAACTTICCACTTATGATAATTAATCTAATAAAGATATGATGCCAATACGGCTCATCATTCCGTTG 240
M Q H P R E E
TACATGAATGCTATCGCTATAAATTTAAATATTAATAAACAAAAGTCGAAAAGAATCCAAATATCGCACACATCCGCTGTTCATGCGCATCCGGGGAGAG 360
N S I V V E L E P S L A T F I K Q G F N N L V K W P L L N I G I V L S N T S T A
AATTCATCGCTGTTGAACTCGAACCTCATGGCTACTTCAACAAAGGATTAAATCTGTAATGGCCUTTGTAACATGGAAATGTTTGCTAATACATCTACCGCT 480
V N E W L T A V E H I P T M K I F Y K H I K R I L T R E M G F L V Y L K R S Q
GTCATGAGGAATGGCTACTCGGTAGAGCATATCCAACCATGAAAGATTTACAAACATACATACAAAGATACTTACAGAGAAATGGGTTTTAGTCTATTGAAAGATCCAA 600
S E R D N Y I T L Y D F D Y Y I D K D T N S V T M V D K P T E L K E T L L H V
TCTGAACGGGATATTATATTAACCTTATACGATTGTAATTATATAGATAAGGATACRAATTCGTAACTATGGTAGATAACCCGACCGAGTTAAAGGAACATTTGTTACGTA 720
F Q E Y R L K S S Q T I E L I A F S S G T V I N E D I V S K L T F L D V E V F N
TTTCAGAATATCGTTAAAGGTTCTCAAACATAGCGCTATAGCGTTAGTCAGGTTACGGTAATAACAGAACATAGTCTTAAACATTAACTTATAGATGIGGAGGTATTAAAT 840
R E Y N N V K T I I D P D F V F R S P F I V I S P M G K L T F F V E V Y S W F D
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F K S C L K D I I D F L E G A L I A N I H N H M I K V G N C D E T V S S Y N P E
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S G M L F V N D L M T M N I V N F F G C N S R L E S Y H R F D M T K V D V E L F
TCTGAATGTTGTTGTAATGACTTAAGCATAGTCACACTTTCGGATGTAATTCTAGGTTAGNATCATACATCGGTTGATATGACAAAGTAGATGTTGAACTT 1200
I K A L S D A C K K I L S A S N R L *
ATTAAACGATCTGCTGATGCTGTTAAAGAAATTTGCACTCTCTATAGATATAAIGAACCTCTATCAATTTTTTATGIGGTAAGCCACGGCTGGGTTGTTACTTTTATC 1320
CAGGGTACTCAATATTAAGAAATATGCGAATTAAGGAATTANTCTACTCTGCGACATCGAATATCAAATCTATGGGAAACCCGCAATTAGATAGGAGACTTCAGAT 1440
GTOAACGACACAAACTTCTGATGTAACCGGAAAGTGTGTTTATCCAGGAAACGGGTTGTAACCGCTTCCATATTTGGATTITCAGGCAAGGTTGGACCAAATAATCTAGA 1560
TCCATGAGAAATTTAACACGATGCAACATGATGACTTACATTTCTGATGTTATTAACATCAATTTATAATCATGTTGTAATGIGCAGATGTTGCAAAATAAAACGCAGATGTCAGTT 1680
CTAAACCTGCTATTAAAGCGGACACTCTTCTCCGGAGATGCGATCTTCGCAACTGATTTTCTACAAAGGCTGTAAAACCCAGATCGGAAACGGGACAC 1800
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TGAGAATAGAAATCTCCATTAGGAGTC -2309

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large subunits that were unresolved from each other under the conditions of PAGE, as well as several small subunits (Fig. 4B). The autoradiograph of a replicate blot, incubated with antibody to the recombinant product of the 35.4-kDa ORF, specifically revealed the M_r 35,000 subunit (Fig. 4C). The gradient fractions that were most reactive with these antibodies contained the peak RNA polymerase activity.

Although the above experiment demonstrated that highly purified RNA polymerase retained detectable amounts of M_r 35,000 polypeptide, the possibility remained that only a small percentage of the total virion-derived polypeptide was associated with polymerase. To investigate this question, the soluble extract of vaccinia virions was applied directly to a glycerol gradient, and the fractions obtained after sedimentation were analyzed by immunoblotting. Immunoreactive 35-kDa polypeptide was detected only in association with other RNA polymerase subunits, whether antiserum to the whole RNA polymerase (Fig. 5A) or to the product of the 35.4-kDa ORF (Fig. 5B) was used. Based on these results, and those described in the previous section, we gave the name *rpo35* to the gene containing the 35.4-kDa ORF.

Amino Acid Sequence of Tryptic Peptides—The electrophoretic mobility of the RNA polymerase subunit that reacted with the antibody to the *rpo35* ORF was similar to that of the previously described *rpo30* subunit, which maps to the *Hind*III-E fragment of the vaccinia virus genome and migrates anomalously slowly on PAGE (9, 11). It seemed likely that the two different subunits were simply unresolved by SDS-PAGE. Another possibility, however, was that there is only one subunit to which the different antibodies cross-reacted. Proof that both polypeptides are subunits was obtained by protein sequencing. A preparation of highly purified RNA polymerase was resolved by SDS-PAGE, and the polypeptides were transferred to a nitrocellulose membrane. The proteins in the band of approximately M_r 35,000 were digested *in situ*

with trypsin, and peptides were purified by reverse-phase liquid chromatography and sequenced. The amino acid sequences of peptides from four chromatographic peaks (Fig. 6) were determined. Based on the number of amino acids released during each sequencing cycle, two of the peaks contained single peptides, one contained two peptides, and one contained three peptides. Of the seven peptides sequenced in this manner, four matched *rpo35* and three matched *rpo30* (Table I). Thus, *rpo30* and *rpo35* encode RNA polymerase subunits with similar mobilities, as determined by SDS-PAGE.

Regulation of Expression of *rpo35*—We used the specific antiserum prepared to the *rpo35* product to study the time of synthesis of the RNA polymerase subunit in vaccinia virus-infected cells. Cytoplasmic proteins, obtained at various times after infection, were resolved by SDS-PAGE and transferred to a nitrocellulose membrane that was incubated with the antiserum. An immunoreactive M_r 35,000 polypeptide was first detected at 2 h after infection, increased markedly between 4 and 6 h, and continued to accumulate for several more hours (Fig. 7). This time course was consistent with expression from an early class promoter or a compound promoter containing both early and late promoter elements.

Transcriptional Analysis of the *rpo35* Gene—Early RNA was obtained from cells infected with vaccinia virus in the presence of cycloheximide or AraC, inhibitors of protein synthesis and DNA replication, respectively. Late RNA was obtained at 7 h after infection in the absence of drugs. The 5'-end of the *rpo35* transcript was analyzed by both nuclease S1 protection and primer extension techniques. For the former analysis, a 500-nucleotide [α -³²P]UTP uniformly labeled RNA probe made *in vitro* using bacteriophage SP6 RNA polymerase was hybridized to the three sets of RNAs. The unhybridized, single-stranded RNA probe was digested with nuclease S1, and the resistant material was resolved by PAGE

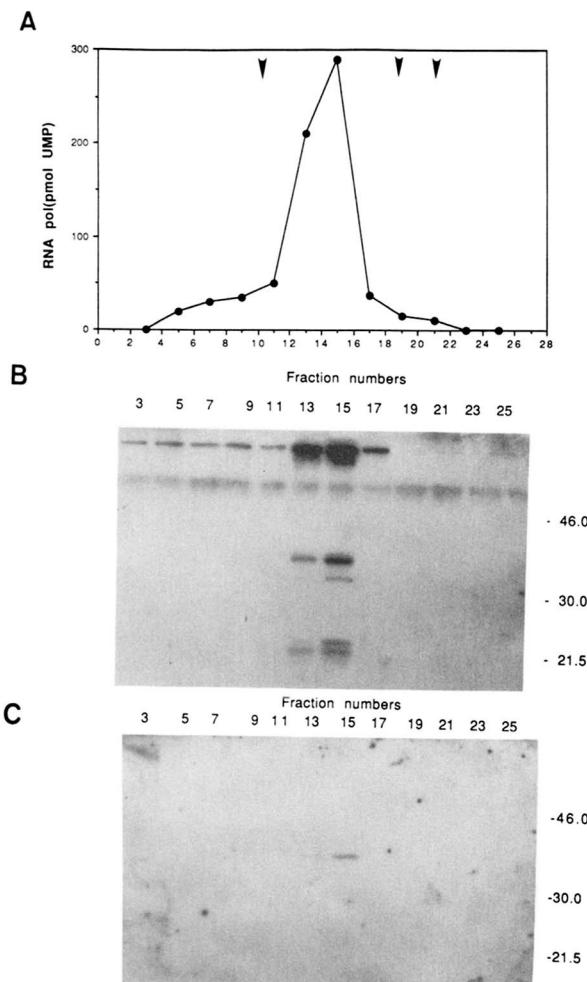


FIG. 4. Co-sedimentation of the product of the 35.4-kDa ORF with purified vaccinia virus RNA polymerase. Highly purified RNA polymerase (*RNA pol*) from vaccinia virions, was applied to a 15–35% linear glycerol gradient and sedimented as described (9). The fraction numbers are in increasing order from the bottom to the top of the tube. *A*, RNA polymerase activity was measured in alternate fractions. Arrowheads indicate the positions of three protein markers (thyroglobulin, M_r 690,000; bovine serum albumin, M_r 66,000; carbonic anhydrase, M_r 29,000) sedimented in a parallel gradient. *B*, proteins in alternate fractions were treated with SDS, resolved by PAGE on a 15% gel, transferred to nitrocellulose, and incubated with antisera to vaccinia virus RNA polymerase and ^{125}I -staphylococcal protein A. An autoradiograph is shown. *C*, autoradiograph of a replica blot incubated with antisera to the product of the 35.4-kDa ORF made in *E. coli*. The numbers on the right indicate the positions of protein markers in kilodaltons.

(Fig. 8A). With early RNA, made with either cycloheximide or AraC, a band of approximately 185 nucleotides was resolved. This size corresponded to a transcript that starts 25 bp upstream of the translation initiation codon of the 35.4-kDa ORF. With late RNA, the 185 nucleotide band was not detected, but instead, there was a prominent 360 nucleotide band. The latter corresponded to a transcript that starts near the conserved late promoter TAAAT motif of a short 77-amino acid (8.7-kDa) ORF that maps upstream of and partially overlaps *rpo35* (Fig. 3, not translated). A 360 nucleotide band was barely detected in cells infected in the presence of AraC and not at all when cycloheximide was used to block late transcription (Fig. 8A).

The location of the 5'-end of the early *rpo35* transcript, as determined by primer extension analysis (Fig. 8B), was consistent with that obtained by the nuclease S1 method. Analy-

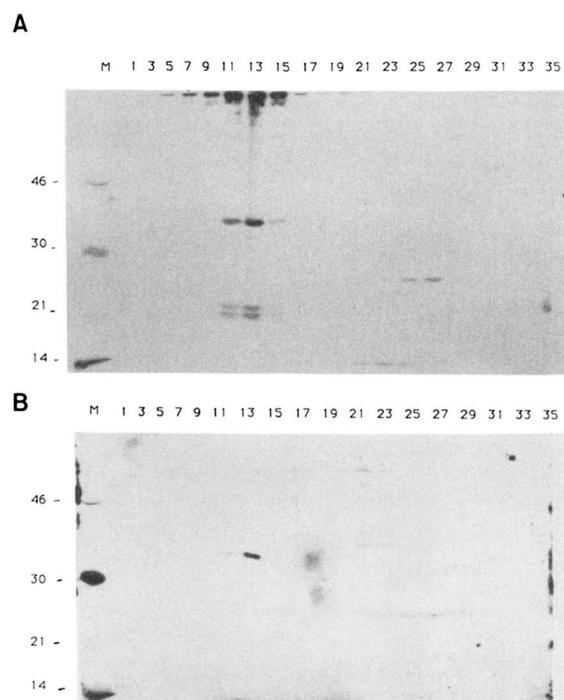


FIG. 5. Sedimentation of the immunoreactive M_r 35,000 polypeptide from a total virion extract. Purified vaccinia virus was disrupted with deoxycholate and dithiothreitol, and the soluble fraction was applied to a glycerol gradient and sedimented and analyzed as described in the legend to Fig. 4. The immunoblots were prepared using antibody to total RNA polymerase (*A*) or the product of the 35.4-kDa ORF made in *E. coli* (*B*). Lane *M* contains protein molecular weight markers, the sizes of which are indicated in kilodaltons on the left.

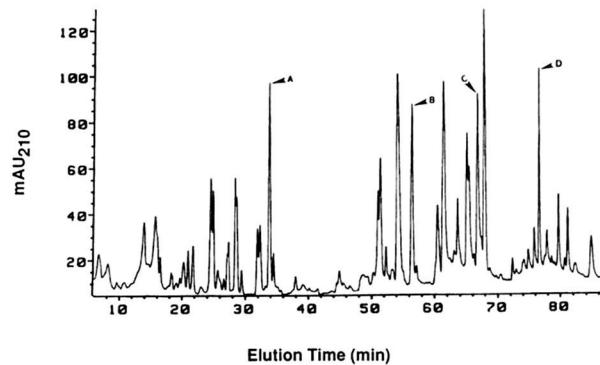


FIG. 6. Chromatography of a tryptic digest of polypeptides with M_r of 35,000 from purified vaccinia virus RNA polymerase. The purification, membrane transfer, and *in situ* trypsin digestions are described under "Experimental Procedures." Peptides were separated on a microbore reverse-phase high pressure liquid chromatography column. Absorption of the column effluent was measured at 210 nm as a function of elution time. *mAU*, milliabsorbancy unit.

TABLE I
Peptide sequences

Peak	Sequence	Match
A	QGFNNLVK	<i>rpo35</i>
	NKPSIATNLE	<i>rpo30</i>
	KILSAS	<i>rpo35</i>
	TIIDPDFVFR	<i>rpo35</i>
B	YLLFGIK	<i>rpo30</i>
	DNYITLYDFD	<i>rpo35</i>
D	ELLSQYVDDA	<i>rpo30</i>

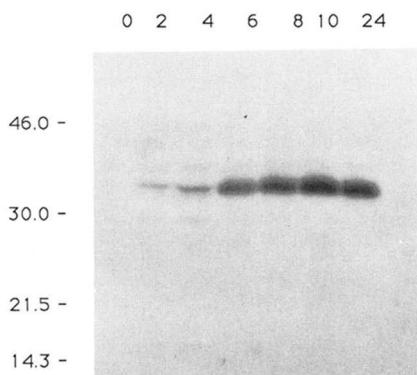


FIG. 7. Time course of synthesis of the RNA polymerase M_r 35,000 subunit. Lysates of vaccinia virus-infected HeLa cells were analyzed by SDS-PAGE. The resolved polypeptides were transferred onto a nitrocellulose membrane and incubated with antiserum to the product of the 35.4-kDa ORF made in *E. coli*. The positions of protein markers in kilodaltons are indicated on left; lane 0, mock infection; lanes 2 through 24, hours after infection that cells were harvested. An autoradiograph is shown.

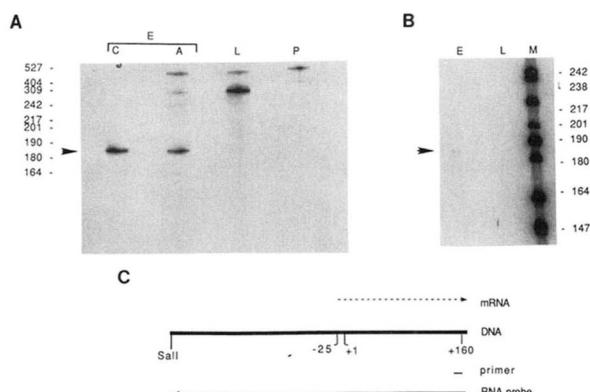


FIG. 8. Analysis of the 5'-ends of the *rpo35* transcript. *A*, RNA preparations, from cells at 4 h after vaccinia virus infection in the presence of cycloheximide, 7 h after infection in the presence of AraC, or 7 h after infection in the absence of drugs, were hybridized to a 500-nucleotide [α -³²P]UTP internally labeled complementary RNA probe. Single-stranded RNA was digested with nuclease S1, and the resistant material was resolved by PAGE in 7 M urea. The sizes in bases of marker DNA fragments are indicated on the left. Lane E, early RNA made in the presence of cycloheximide (C) or AraC (A); lane L, late RNA made in absence of drugs; lane P, untreated RNA probe. The arrowheads point to the bands obtained with early RNAs. *B*, primer extension analysis of early and late RNAs. Lane M, labeled DNA markers with sizes in bases on the right; lane E, early RNA made in the presence of cycloheximide; lane L, late RNA made in absence of drugs. *C*, the heavy bar represents the portion of the vaccinia virus genome used to make the complementary RNA probe. The initiation codon of the *rpo35* gene is indicated as +1, and the 5'-end of the early transcript as -25. The *Sall* site marks the 3'-end of the RNA probe.

sis of the late RNA using the same primer produced no distinct band (Fig. 8B). The significance of this negative result is uncertain, however, since primer extension is less sensitive than nuclease S1 for analysis of late RNAs. The possible reasons for the insensitivity include the heterogeneity of 5'-ends due to variable length 5'-poly(A) leaders (22–25) and competition for primer binding by anti-sense RNA (26, 27).

DISCUSSION

Our initial studies indicated that an M_r 35,000 subunit of the vaccinia virus RNA polymerase was encoded within a 2.3-kbp segment near the center of the vaccinia virus genome. Sequencing of this DNA segment revealed four complete

ORFs. Identification of the one that comprised *rpo35* was based on several criteria: 1) the ORF predicted a polypeptide of 35.4 kDa, 2) RNA, from vaccinia virus-infected cells, that hybridized to the DNA of that ORF programmed the *in vitro* synthesis of a M_r 35,000 polypeptide that bound to antibody produced to purified RNA polymerase from vaccinia virions, 3) expression of the ORF in *E. coli* led to production of a M_r 35,000 polypeptide that also reacted with the RNA polymerase antiserum, 4) antibody to the recombinant protein made in *E. coli* bound specifically to a M_r 35,000 polypeptide that was associated with RNA polymerase purified from vaccinia viroids by successive column chromatography and glycerol gradient centrifugation, 5) all of the immunoreactive M_r 35,000 polypeptide extracted from virions co-sedimented with RNA polymerase, and 6) the amino acid sequence of tryptic peptides from the purified RNA polymerase subunit matched the ORF. Like the other three previously identified small subunit genes of vaccinia virus RNA polymerase, the translated *rpo35* ORF exhibited no homology to any of the reported RNA polymerase genes of prokaryotic or eukaryotic organisms. Indeed, *rpo35* is not obviously related to any known nonpoxvirus gene, nor does it have any recognizable sequence motifs. In contrast, the two large subunits of vaccinia virus and cowpox virus RNA polymerases are homologous to the corresponding prokaryotic and eukaryotic subunits (5–7), and *rpo30* is homologous to a eukaryotic transcription elongation factor (9).

All of the vaccinia virus RNA polymerase genes identified thus far have early promoters and are expressed prior to DNA replication. Some of them also have late promoter elements providing a mechanism for their continued synthesis throughout the life cycle of the virus. *rpo35* is transcribed under conditions that block intermediate and late gene expression, indicating that it has an early promoter. The 5'-end of the mRNA was mapped 25 bp upstream of the translation initiation codon, and the sequence AAAAGTCGAAAAAA from -13 to -26 relative to the RNA start site closely resembles in sequence and location the early promoter consensus AAAATGAAAAAAA (28). Two forms, TTTTTT and TTTTTAT, of the vaccinia virus early transcriptional termination motif, TTTTTNT (29), occur 18 and 51 bp past the translation termination codon of *rpo35*. Consistent with this feature, the 3'-end of the RNA maps about 60 bp downstream of the stop codon.² The presence of two tandem transcription termination motifs is unusual but not unprecedented; it also occurs in the vaccinia virus growth factor gene (30). Immunoblotting studies indicated that synthesis of the M_r 35,000 RNA polymerase subunit begins within 2 h after infection, which also is consistent with early regulation of the gene. The polypeptide increases in amount between 2 and 6 h after infection but continues to accumulate for at least several more hours. Just upstream of the *rpo35* gene is a 77-amino acid ORF that is preceded by a conserved TAAAT late promoter motif (31, 32) and is transcribed at late times after infection. Since this late RNA does not terminate before *rpo35*, it might serve as a polycistronic message for continued synthesis of the M_r 35,000 polypeptide.

In conclusion, the discovery of *rpo35* increases the number of reported vaccinia virus RNA polymerase subunit genes to six. An additional subunit gene, *rpo19*, is located within another region of the *Hind*III A³ fragment. Thus, the complexity of the vaccinia virus RNA polymerase is approaching that of eukaryotic RNA polymerase II, for which 10 subunits have been identified (33).

² B. Amegadzie, unpublished data.

³ B.-Y. Ahn, manuscript in preparation.

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