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Characterization of a 7-Kilodalton Subunit of Vaccinia Virus DNA-Dependent RNA Polymerase with Structural Similarities to the Smallest Subunit of Eukaryotic RNA Polymerase II

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A previously unrecognized 7-kDa polypeptide copurified with the DNA-dependent RNA polymerase of vaccinia virus virions. Internal amino acid sequences of the small protein matched a viral genomic open reading frame of 63 codons. Antipeptide antiserum was used to confirm the specific and complete association of the 7-kDa protein with RNA polymerase. The amino acid sequence predicted from the viral gene, named *rpo7*, was 23% identical to that of the smallest subunit of *Saccharomyces cerevisiae* RNA polymerase II, and a metal-binding motif, Cys-X-X-Cys-Gly, was located at precisely the same location near the N terminus in the two proteins. RNA analyses demonstrated early transcriptional initiation and termination signals in the *rpo7* gene sequence. The viral RNA polymerase subunit was synthesized during the early phase of infection and continued to accumulate during the late phase.

The poxviruses, of which vaccinia virus is the prototype, are unique among DNA viruses in that they replicate in the cytoplasm of the cell and encode their own multisubunit DNA-dependent RNA polymerase which is packaged in virus particles (reviewed in references 27 and 28). The RNA polymerase from purified virions contains two large subunits of 147 and 132 kDa and several smaller ones (10). The amino acid sequences deduced from poxvirus *rpo147* and *rpo132* genes indicated homology with the corresponding large subunits of eukaryotic and prokaryotic DNA-dependent RNA polymerases (7, 13, 29). Five genes encoding small subunits, *rpo35*, *rpo30*, *rpo22*, *rpo19*, and *rpo18*, have been identified (3–6, 13, 31). Unlike the two large subunits, the small ones identified thus far are not closely related in sequence to prokaryotic or eukaryotic RNA polymerase small subunit genes, although homology between *rpo30* and eukaryotic transcription elongation factor SII was suggested (3). The vaccinia virus RNA polymerase, like its eukaryotic counterpart, is incapable of specific transcription of double-stranded DNA templates without additional proteins. Different virus-encoded factors are required for transcription of early and late genes (12, 14, 19, 23, 36).

The characterization of all the RNA polymerase subunits is a prerequisite for studying the assembly and structure-function relationships of this important and complex enzyme. In the present report, we describe the discovery and genetic identification of the smallest known vaccinia virus DNA-dependent RNA polymerase subunit and its structural resemblance to the *Saccharomyces cerevisiae* RNA polymerase II *RPB10* subunit.

MATERIALS AND METHODS

Virus and cells. Vaccinia virus (WR strain [ATCC VR 1354]) stocks were prepared in HeLa spinner cells that were propagated in minimum essential spinner medium (Quality Biologics Inc.) supplemented with 5% horse serum at 37°C.

Protein sequence analysis. For protein sequence analysis, the DNA-dependent RNA polymerase from vaccinia virus virions was purified on columns of DEAE-cellulose, phosphocellulose, and Bio-Gel A1.5m (Bio-Rad) as previously described (5). Approximately 100 μg of enzyme, equivalent to 200 pmol, was denatured with sodium dodecyl sulfate (SDS) and mercaptoethanol and resolved into individual subunits by polyacrylamide gel electrophoresis (PAGE) in a 5 to 15% polyacrylamide gradient gel. The polypeptides were electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell) which was stained with 0.1% Ponceau S (Sigma), and the membrane strip containing the appropriate band was sent to William Lane (Harvard Microchemistry Laboratory) for peptide sequencing. After in situ digestion with trypsin (2), the resultant peptides were separated by reverse-phase high-pressure liquid chromatography, and some were then analyzed on a gas-phase protein sequencer (Applied Biosystems Inc. model 470A) connected to an on-line phenylthiohydantoin-amino acid analyzer.

DNA sequencing. Two oligonucleotide primers, 7KF (5′-GGGCTGTAACACGATAGTATTCGGCAT-3′) and 7KR (5′-GGGATTCAAATGACTTGTTAATGA-3′) derived from the DNA sequence of the Copenhagen strain of vaccinia virus (20), were used in a polymerase chain reaction (PCR) with the vaccinia virus WR strain *Hin*dIII G DNA fragment as a template. The resulting 420-bp DNA fragment was cloned into phagemid vector pGEM3zf (Promega Biotec) in both orientations. Templates, prepared from each strand of phage DNA, were sequenced by the dideoxynucleotide chain termination method using synthetic oligonucleotide primers as described in the instructions for the Sequenase kit (U.S. Biochemical Corporation). Microgenie (Beckman) and TFASTA (30) computer programs were used for manipulations of the DNA sequence and protein homology searches, respectively.

Preparation of antiserum. A 24-residue peptide representing the predicted C terminus of the product of the G5.5R open reading frame (ORF) was chemically synthesized. Approximately 2 mg of the peptide was coupled to keyhole limpet hemocyanin by using an Imject Activated Conjugation Kit (Pierce) as described by the vendor. The conjugated proteins were purified and then injected in Freund's complete adjuvant into a New Zealand White rabbit. Booster

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injections with the antigen in incomplete Freund's adjuvant were given 2 and 6 weeks after the first injection.

Analysis of proteins from vaccinia virus-infected cells. CV-1 cells were infected with 15 PFU of vaccinia virus per cell, harvested at various times after infection at 37°C, and resuspended in 4 volumes of ice-cold extraction buffer (250 mM KCl, 100 mM Tris-HCl [pH 8.4], 0.1 mM EDTA, 10 mM dithiothreitol, 0.2% deoxycholate). The suspension was mixed, homogenized, and then clarified by centrifugation for 2 min at 4°C in a microcentrifuge. The supernatant was adjusted to contain 2% SDS and 10% (vol/vol) 2-mercaptoethanol and heated at 100°C for 5 min prior to electrophoresis in a Tricine-SDS-polyacrylamide (16.5% polyacrylamide separation and 10% polyacrylamide spacer) gel as described elsewhere (33). The proteins were electrophoretically transferred to a nitrocellulose membrane and incubated at room temperature with solution A (phosphate-buffered saline containing 5% nonfat dried milk and 0.1% [vol/vol] Tween 20) for 1 h to prevent subsequent nonspecific antibody binding and then with anti-*rpo7* peptide serum (diluted 1:200 in solution A) for 16 h. The membrane was washed with solution A and incubated with ^{125}I-labeled protein A (0.1 μCi/ml; Amersham) in solution A for 2 h. Autoradiographs were made with X-ray film, and the radioactivity of the bands was quantitated with a PhosphorImager (model 400; Molecular Dynamics, Inc.).

In vivo labeling of virus proteins. HeLa cell monolayers were infected with 15 PFU of vaccinia virus per cell at 37°C. At various times after infection, the medium was removed, and 2.5×10^6 cells were incubated in 1.5 ml of cysteine-free medium supplemented with 10% dialyzed fetal calf serum and 50 μCi of [^{35}S]cysteine (600 Ci/mmol; Amersham) per ml for 30 min. When appropriate, 40 μg of cytosine arabinoside (araC) per ml was added to the medium after the 30-min virus inoculation period. The cells were harvested, resuspended in 0.5 ml of extraction buffer, homogenized, and clarified as described above. Portions (0.1 ml) of the appropriate samples were adjusted to contain 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 0.1% (vol/vol) Triton X-100, 0.1% (vol/vol) Nonidet P-40, 0.1% SDS, 0.2% bovine serum albumin, 0.2 mM phenylmethane sulfonyl fluoride, and 0.01% aprotinin (Boehringer Mannheim Biochemicals) in a total volume of 0.25 ml. The samples were then incubated successively with 20 μl of preimmune serum and 50 μl of protein A-agarose beads (50% [vol/vol] suspension in phosphate-buffered saline) for 2 h at 4°C with constant agitation. The beads were removed by centrifugation, and the samples were incubated with 20 μl of anti-*rpo7* peptide serum and 50 μl of protein A-agarose beads for 7 h. The beads were washed three times with Triton X buffer (300 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% [vol/vol] Triton X-100) and once with DOC buffer (300 mM NaCl, 50 mM Tris-HCl [pH 7.5], 0.1% SDS, 0.1% deoxycholate). The proteins were dissociated from the beads by boiling the beads in 2% SDS–10% mercaptoethanol and were analyzed by PAGE in an SDS-Tricine buffer (33).

RNA analysis. RNA was isolated by the RNAzol method as described by the supplier (Cinna/Biotecx) at various times after infection in the presence or absence of metabolic inhibitors as specified. For Northern blot analysis, 30 μg of RNA per lane was subjected to electrophoresis in a 1.5% agarose gel in 6% formaldehyde–20 mM morpholinepropanesulfonic acid–50 mM sodium acetate–10 mM EDTA (pH 7.0). The RNA was then transferred to a nylon membrane and probed with an internally ^{32}P-labeled cRNA containing the *rpo7* gene as described elsewhere (9).

The hybridization probe for 5′-end analysis was prepared by using two oppositely oriented oligonucleotide primers. Primer 7KR2 (nucleotides 331 to 361 [see Fig. 2]) was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase and was used together with primer 7KF2 (nucleotides 1 to 24) to synthesize a 360-bp double-stranded DNA probe from the vaccinia virus *Hin*dIII G fragment template by 30 PCR cycles. The resulting product was loaded onto a 5% DNA sequencing gel, and the position of the probe was determined by a brief autoradiographic exposure at the ambient temperature. The desired band was electroeluted from a gel slice into a dialysis membrane, and the DNA was ethanol precipitated. The labeled DNA probe was then used for hybridization to RNA, and the segments resistant to digestion with S1 nuclease were analyzed by electrophoresis in a 6% polyacrylamide gel as described elsewhere (7). For primer extension analysis, 20 pmol of the oligonucleotide primer TTCTT TACACTGACGAGTACATCC was 5′-end labeled with polynucleotide kinase and [γ-^{32}P]ATP, annealed to 20 μg of RNA, and then incubated with avian myeloblastosis virus reverse transcriptase in the presence of deoxynucleotides as described elsewhere (6).

Nucleotide sequence accession number. The *rpo7* gene sequence in Fig. 2 has been deposited in GenBank under accession number M85279.

RESULTS

Identification of a 7-kDa polypeptide associated with the DNA-dependent RNA polymerase from vaccinia virus virions. Previous studies described polypeptides ranging in size from approximately 17 to 140 kDa associated with purified vaccinia virus RNA polymerase (10, 22). Silver staining of the small subunits resolved on high-percentage polyacrylamide gels, however, demonstrated an additional polypeptide of approximately 7 kDa (Fig. 1, inset). In order to further characterize this polypeptide, it was transferred to nitrocellulose and digested in situ with trypsin. The resulting peptides were resolved by reverse-phase liquid chromatography (Fig. 1), and the N-terminal amino acid sequences of two were determined.

The TFASTA computer program (30) was used to compare the two peptide sequences, LSTQIEPQ and NLTVQPLLDI, with all six possible reading frame translations of the entire DNA sequence of the Copenhagen strain of vaccinia virus (20). Both peptide sequences perfectly matched parts of an ORF that was located within the vaccinia virus *Hin*dIII G fragment. This 63-codon ORF, located between ORFs G5R and G6R, had been ignored because of its small size. Since the DNA sequence of the *Hin*dIII G fragment of the vaccinia virus strain WR genome used in this study was unavailable at the time, two oligonucleotide primers based on the Copenhagen strain sequence were designed. These primers were used in a PCR with vaccinia virus strain WR cloned DNA as the template. The predicted 420-bp DNA fragment was amplified and cloned. Sequencing of the DNA revealed a 63-codon ORF that predicted a polypeptide of 7,288 Da (Fig. 2A). Comparison of the ORFs of the vaccinia virus WR and Copenhagen strains showed only two silent nucleotide substitutions. The same vaccinia virus WR DNA sequence was recently reported by Meis and Condit (26), and the ORF was named G5.5R. We shall refer to the gene as *rpo7* for consistency with other vaccinia virus RNA polymerase genes which have been named according to the predicted molecular masses (10^3 kDa) of their protein products.

The *rpo7* ORF was subcloned into a vector containing a T7 promoter, and in vitro-synthesized RNA was translated in a

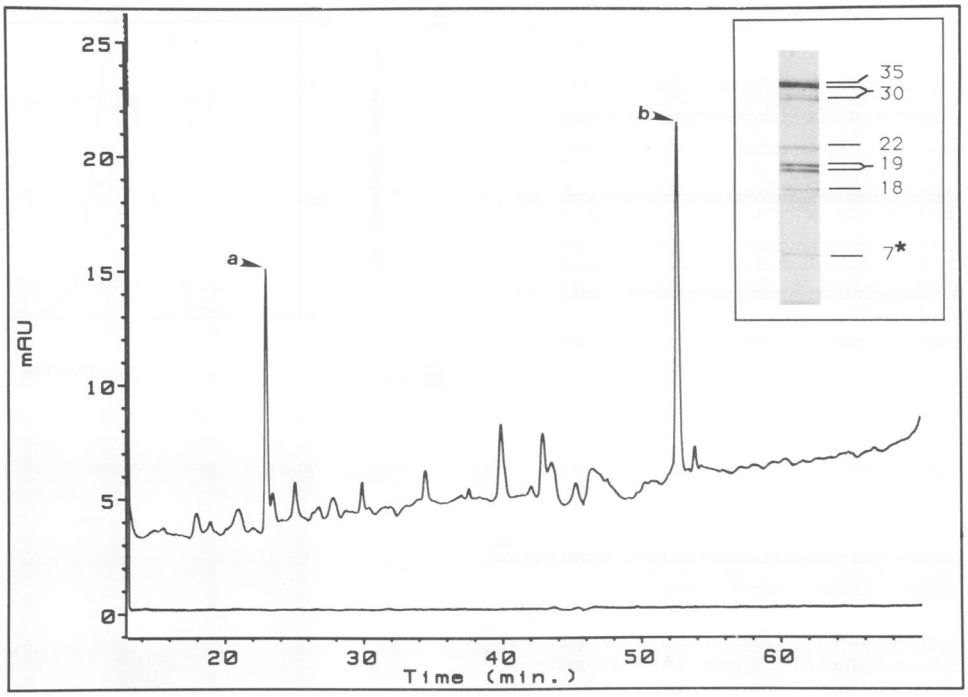

FIG. 1. Detection of a 7-kDa polypeptide associated with purified vaccinia virus RNA polymerase. The multisubunit vaccinia virus virion DNA-dependent RNA polymerase was purified as described in Materials and Methods and then resolved by SDS-PAGE. The 8 to 18% polyacrylamide gradient gel containing the RNA polymerase subunits was then silver stained (inset). The large subunits were overstained and not well resolved, so only the small ones are shown. Subunit sizes in kilodaltons are indicated. The 7-kDa polypeptide is marked with an asterisk. Evidence for multiple electrophoretic forms of the *rpo19* and *rpo30* subunits was presented previously (3, 5). For protein sequencing, the RNA polymerase subunits from a preparative gel were transferred to a nitrocellulose membrane, and the 7-kDa band was digested with trypsin. The peptides were separated by reverse-phase high-pressure liquid chromatography, and the N-terminal sequences of peptides a and b were determined to be LSTQIEPQ and NLTVQPLLDI, respectively. mAU, milliabsorbancy units.

micrococcal nuclease-treated reticulocyte lysate and analyzed by polyacrylamide gel electrophoresis. A polypeptide of approximately 7 kDa corresponding in size to the RNA polymerase subunit was detected (data not shown).

Sequence similarities between *rpo7* and a eukaryotic RNA polymerase II subunit. A computer search of the GenBank data base using the TFASTA program failed to find significant similarities between *rpo7* and non-vaccinia virus genes. Alignment of the *rpo7* ORF with that of the smallest subunit of the *S. cerevisiae* RNA polymerase II, *RPB10* (35), however, revealed similarities as shown in Fig. 2B. The overall percent identity was 23%, which increased to 48% when conservative changes were included. Furthermore, the potential metal-binding domain CXXCG (1), where X can represent any amino acid residue, was located at exactly the same position near the N terminus in the yeast and viral proteins.

Association of the *rpo7* subunit with RNA polymerase. To produce an antibody reagent for further studies, a synthetic peptide containing 24 amino acids from the C terminus of the protein predicted by the *rpo7* ORF was coupled to a carrier and used to immunize a rabbit. The efficacy of the resultant antiserum was demonstrated by its reactivity with the 7-kDa protein on SDS-PAGE blots of virion extracts (data not shown). When the extract was applied directly to a glycerol gradient, the immunoreactive 7-kDa polypeptide (Fig. 3B, fractions 10 to 14) cosedimented with RNA polymerase activity (Fig. 3A). The absence of the 7-kDa band in other fractions suggested that all of the 7-kDa protein that could be extracted from the virus was associated with the polymerase. There was some cross-reactive material that was not associated with the polymerase and sedimented at a rate that corresponded to its molecular weight (Fig. 3B, fractions 22 to 28). When the blot was reprobed with antiserum raised to the entire vaccinia virus RNA polymerase, the incompletely resolved 18- to 22-kDa and 30- to 35-kDa small subunit bands were detected in the same fractions as the 7-kDa protein (Fig. 3C).

The tight association of the 7-kDa immunoreactive polypeptide with RNA polymerase was further demonstrated by an immunoblot of glycerol gradient fractions of enzyme that had been purified on successive columns of DEAE-cellulose, heparin-agarose, single-stranded DNA–agarose, and phosphocellulose (Fig. 4). Cross-reactive proteins were no longer present in the RNA polymerase preparation.

Transcriptional analysis. Total RNA from uninfected cells or from cells at various times after infection was resolved by denaturing agarose gel electrophoresis and transferred to a nylon membrane. The immobilized RNA was then hybridized to a ^{32}P-labeled RNA probe obtained by transcribing a DNA fragment containing part of the *rpo7* coding region with bacteriophage T7 RNA polymerase. A band of about 300 nucleotides was detected in the lanes containing RNAs isolated at 2 and 4 h after infection (Fig. 5). This band was less intense at 6 h and was difficult to resolve at later times. The 300-nucleotide RNA was made in the presence of araC and was overexpressed in the presence of cycloheximide, as expected for an early transcript (Fig. 5). The long heterogeneous RNAs that appeared at 4 h in the absence of inhibitors and became more abundant thereafter may represent read-

A

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CTGTAACACGATAGTATTCGGCATCTATAAGGATAATTTACTACTGAGAATAAATAATTC  60
ATTTTACTTTAACGATAGTCTGTTAATAACCAATACTAAAAGTGATAATATAATAAATAT 120
AGGTTACTAGATTAAAAATGGTGTTCCAACTCGTGTGCTCTACGTGCGGCAAAGATATTT 180
                  M  V  F  Q  L  V  C  S  T  C  G  K  D  I  S
CTCACGAACGATATAAATTGATTATACGAAAAAAATCATTAAAGGATGTACTCGTCAGTG 240
 H  E  R  Y  K  L  I  I  R  K  K  S  L  K  D  V  L  V  S  V
TAAAGAACGAATGTTGTAGGTTAAAATTATCTACACAAATAGAACCTCAACGTAACTTAA 300
 K  N  E  C  C  R  L  K  L  S  T  Q  I  E  P  Q  R  N  L  T
AGTGCAACCTCTATTGGATATAAACTAATATGGATCCGGTTAATTTTATCAAGACATAT 360
 V  Q  P  L  L  D  I  N  *
GCGCCTAGAGGTTCTATTATTTTTATTAATTATACCATGTCATTAACAAGTCATTTGAAT 420
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B

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          1                                                  63
rpo7      MVFQLVCSTCGKDISHER--YKLIIRKKSLKDVLVSVKNECCRLKLSTQIEPQRNLTVQPILL
          X:..: X .XXX ::..:  X  ::....X.: .. XX  : :: X
RPB10     MIVPVRCFSCGKVVGDKWESYLNLLQEDELDERYCIVKIRSKKILL
          1    CXXCG                                 46
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FIG. 2. Sequence analysis of the vaccinia virus *rpo7* gene and comparison with the *S. cerevisiae RPB10* gene. (A) The numbering of the nucleotide sequence starts from the left and is in the conventional orientation of the vaccinia virus genome. The DNA sequences corresponding to the RNA start site and early termination motif (TTTTTAT) are underlined with solid and dashed lines, respectively. The predicted amino acid sequence of the ORF is indicated in the single-letter code below the DNA sequence. The asterisk represents a translational termination codon, and the amino acid sequences matching the tryptic peptides are underlined. (B) The predicted 63- and 46-amino-acid sequences of *rpo7* and *S. cerevisiae RPB10*, respectively, are aligned; the metal-binding motif, CXXCG, is shown below and underlined. The alignment, made by computer with the GAP program (17), indicated 23% identity in the 46-amino-acid overlap. In the line between the *rpo7* and *RPB10* sequences, X denotes identical amino acid residues. The conserved and acceptable amino acid substitutions are indicated by double and single dots, respectively. The dashed line indicates a gap introduced into the sequence of *rpo7* for optimized alignment.

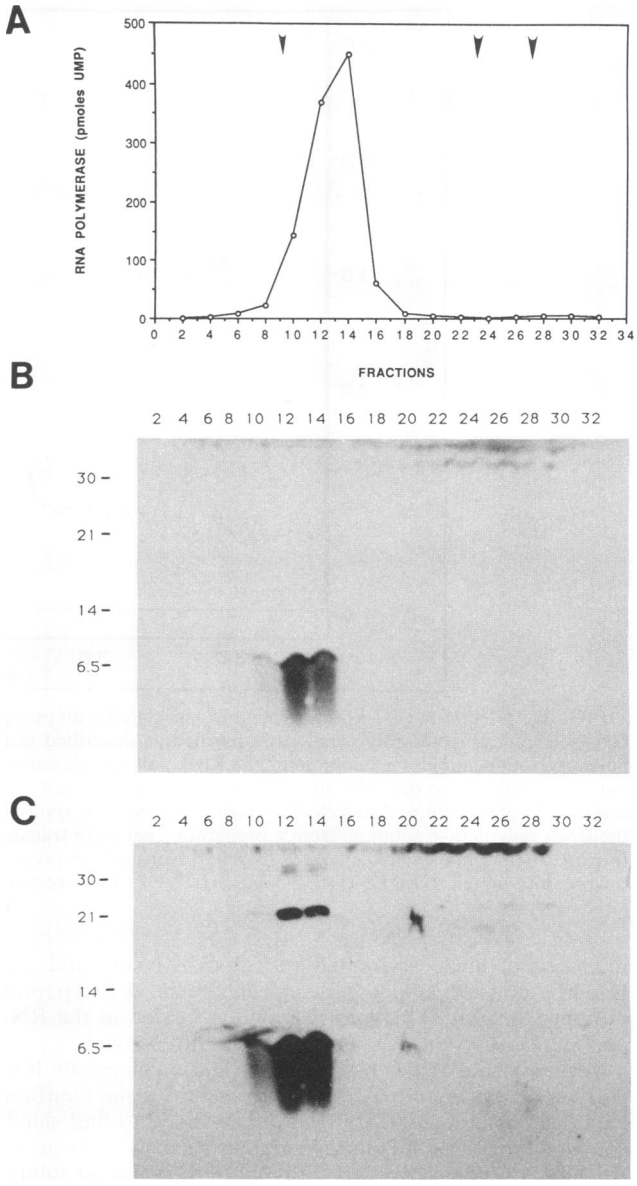

FIG. 3. Sedimentation of the immunoreactive 7-kDa polypeptide from a virion extract. Purified vaccinia virus virions were disrupted with deoxycholate and dithiothreitol, and the soluble fraction was applied to a 15 to 35% linear glycerol gradient and centrifuged as described elsewhere (3). The fractions were collected from the bottom 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], and carbonic anhydrase [M_r, 29,000]) sedimented in a parallel gradient. (B) Proteins in alternate fractions were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with antibody to a peptide of the *rpo7* ORF and ^{125}I-staphylococcal protein A. An autoradiograph is shown. (C) The blot from panel B was reprobed with an antibody to total RNA polymerase. The positions of protein molecular mass markers are shown in kilodaltons on the left.

through transcripts which characteristically form late in infection (15, 25).

For 5′-end analysis of the *rpo7* transcript, a 360-bp labeled DNA probe generated by PCR was hybridized to either early or late RNA from cells infected for 7 h in the presence or absence of araC, respectively. The S1 nuclease-resistant material was then resolved by PAGE (Fig. 6A and C). A band of approximately 240 nucleotides was detected in the lane containing early RNA made in the presence of araC (Fig. 6A, lane E), whereas only the fully protected probe was detected in the lane containing late RNA (lane L). The 240-nucleotide size corresponded to a transcript that starts about 20 nucleotides upstream of the translation initiation codon of the *rpo7* ORF. The RNA start site occurring within the sequence TATAG consistent with the nuclease protection data is underlined in Fig. 2A. We noted, however, that the sequence immediately upstream of this region is extremely AT rich. Had RNAs initiated within the AT-rich region, it would have seemed possible that S1 nuclease nibbled the ends of the labeled probe and moved the apparent RNA start site downstream.

Primer extension was used as an alternative method of defining the early RNA start site that would not be affected by S1 nuclease nibbling. The complimentary oligonucleotide primer, from nucleotide 223 to 247 (Fig. 2A), was hybridized to early RNA and extended with reverse transcriptase. The size of the extended product (Fig. 6B and C) was in agree-

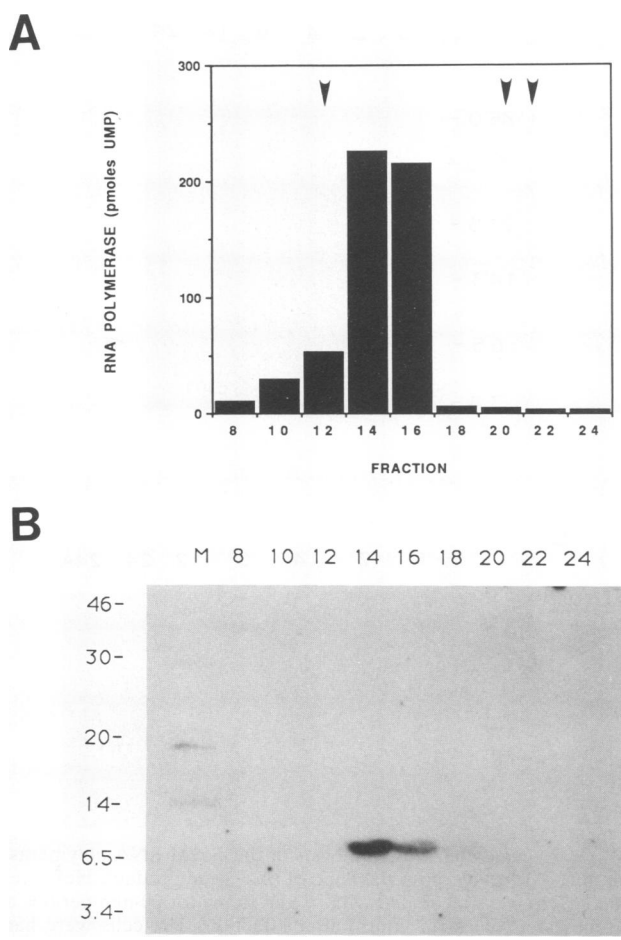

FIG. 4. Cosedimentation of the product of the *rpo7* gene with highly purified RNA polymerase. Purified RNA polymerase from vaccinia virus virions was applied to a 15 to 35% linear glycerol gradient. After centrifugation, the fractions were collected from the bottom and analyzed for RNA polymerase activity (A) or by Western blotting (immunoblotting) (B) as in the experiment whose results are illustrated in Fig. 3. The arrowheads in panel indicate the positions of three protein markers (thyroglobulin, bovine serum albumin, and carbonic anhydrase). (B) Lane M, molecular mass markers (indicated in kilodaltons on the left).

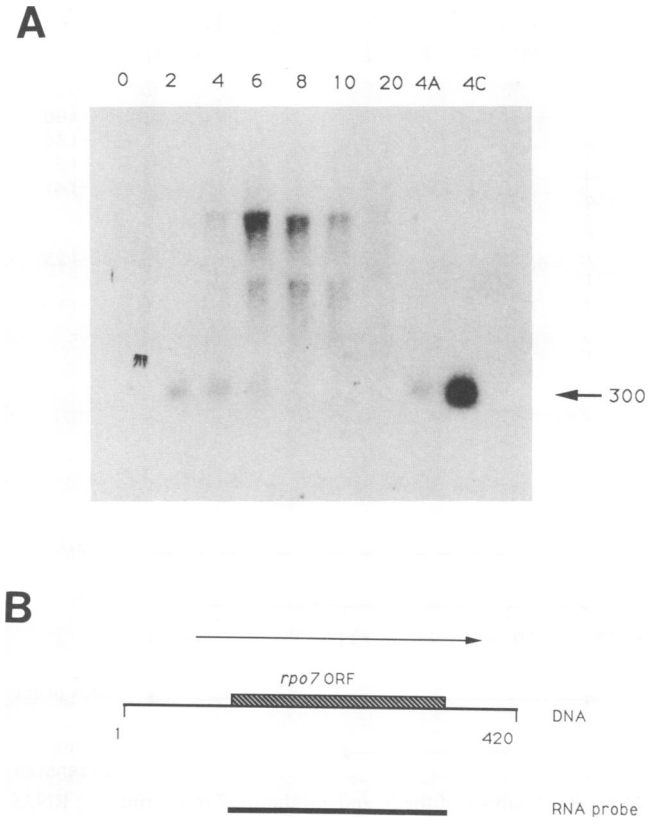

FIG. 5. Northern blot analysis of the *rpo7* transcript. (A) RNA samples (30 μg) isolated from cells at various times after infection were analyzed by electrophoresis on a 1.5% denaturing agarose gel. The RNA was transferred to a nylon membrane and hybridized to an internally ^{32}P-labeled complementary RNA probe. RNA was isolated from cells that were mock infected (lane 0), infected for 2 to 20 h (lanes 2 to 20), infected for 4 h in the presence of araC (lane 4A), or infected for 4 h in the presence of cycloheximide (lane 4C). The arrow points to the 300-nucleotide *rpo7* transcript. (B) Representation of the DNA region shown in Fig. 2A. The thin line represents the DNA sequence from nucleotide 1 to 420. The hatched box represents the *rpo7* ORF. The arrow shows the direction of the *rpo7* mRNA, and the thick line represents the labeled cRNA used to probe the blot.

ment with the result obtained by S1 nuclease analysis. It is noteworthy that we did not see evidence of a 5′ poly(A) leader that might have formed by RNA polymerase slippage had transcription initiation occurred within the TAAAT sequence a few nucleotides upstream. Such heterogeneous poly(A) leaders were found on early transcripts encoding some other RNA polymerase subunits (4, 5).

Synthesis of the *rpo7* polypeptide. To determine the time of synthesis and accumulation of the 7-kDa polypeptide, both pulse-labeling with a radioactive amino acid and immunoblotting were used. Since the *rpo7* ORF predicted four cysteines and only a single N-terminal methionine, the former amino acid was chosen for labeling the 7-kDa polypeptide. Vaccinia virus-infected cells were pulse-labeled for 30 min with [^{35}S]cysteine at various times after infection. The cells were washed and then lysed with a buffer capable of extracting active RNA polymerase from virions (10). The soluble proteins were incubated with the anti-C-terminal *rpo7* peptide antiserum, resolved by SDS-PAGE, and detected by autoradiography. The predominant band was the 7-kDa polypeptide; some additional minor bands could represent proteins specifically or nonspecifically associated with the RNA polymerase subunit. The labeled 7-kDa polypeptide could be detected at all time points, but the largest amounts were immunoprecipitated at 2 and 4 h after infection (Fig. 7A). No additional labeled 7-kDa polypeptide was obtained when five times more antiserum was used for the immunoprecipitation, indicating that the antibody was not limiting. Thus, the predominant early synthesis of the 7-kDa polypeptide as determined by pulse-labeling was consistent with the synthesis of the *rpo7* mRNA determined by Northern blotting (Fig. 5).

For immunoblotting studies, the cells were infected, harvested, and lysed as described above except that no labeled amino acids were used. The soluble proteins were treated with SDS and mercaptoethanol, separated by electrophoresis, transferred to a nitrocellulose membrane, and incubated with the anti-C-terminal peptide antiserum. Synthesis of the 7-kDa polypeptide was detected at 2 h after infection, but

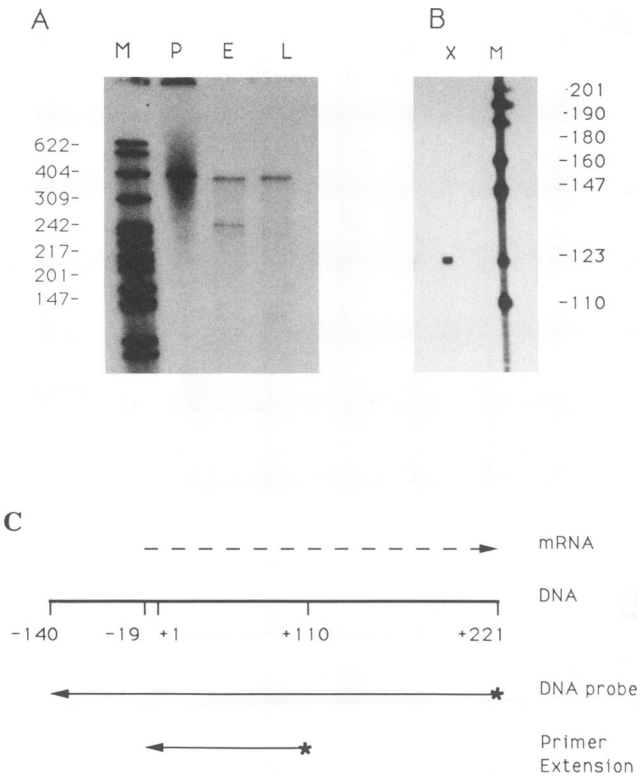

FIG. 6. Analysis of the 5′ ends of the *rpo7* transcript. (A) RNAs isolated 7 h after vaccinia virus infection in the presence or absence of araC were hybridized to a ^{32}P-labeled 360-base denatured DNA probe. The RNA-DNA hybrid was then treated with S1 nuclease, and the resistant material was resolved by PAGE in a 7 M urea–6% polyacrylamide gel. Lanes: M, marker DNA with sizes in bases on left; P, untreated DNA probe; E, protected early RNA made in the presence of araC; L, protected late RNA made in the absence of the drug. (B) Primer extension analysis of early mRNA. Lanes: X, extensions of the 5′-end-labeled primer in the presence of all four deoxynucleotides; M, labeled DNA markers with sizes in bases on the right. (C) The thick bar represents the portion of the vaccinia virus genome used to make the DNA probe. Arrows: dashed, *rpo7* mRNA; long, full-length DNA probe; short, full length of cDNA derived from the primer extension. Asterisks indicate the ^{32}P-labeled ends of the probe and primer. The initiation codon of the *rpo7* gene is indicated as +1, and the 5′ end of the early transcript is indicated as −19.

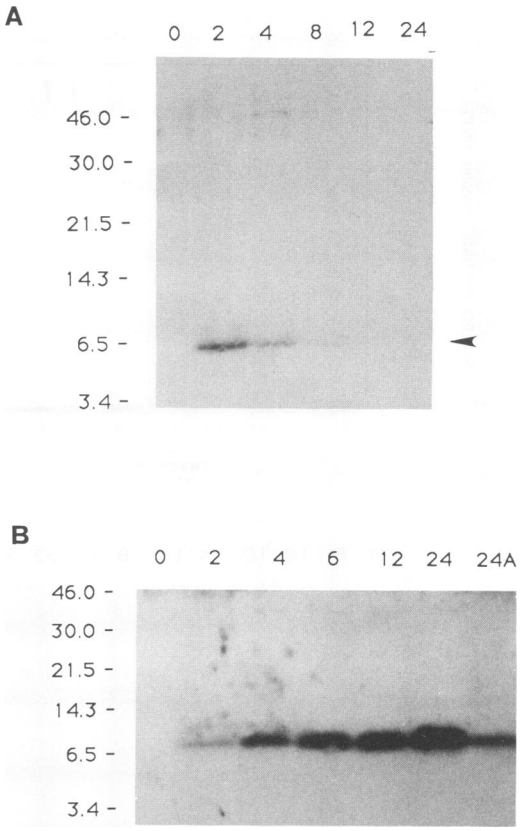

FIG. 7. Time course of synthesis of the 7-kDa RNA polymerase subunit. (A) In vivo pulse-labeling of *rpo7* gene product. HeLa cell monolayers were incubated with [^{35}S]cysteine for 30-min periods at the indicated times (in hours) after infection. The cells were harvested and lysed, and the labeled proteins were immunoprecipitated with *rpo7* antipeptide serum and analyzed by SDS-PAGE. An autoradiograph is shown, and the sizes of markers in kilodaltons are indicated on the left. The arrowhead points to the *rpo7* polypeptide. (B) Lysates of vaccinia virus-infected CV-1 cells were analyzed by SDS-PAGE. The resolved polypeptides were transferred onto a nitrocellulose membrane and incubated with a peptide antiserum to *rpo7*. The sizes of protein markers in kilodaltons are indicated on the left. The numbers above the lanes indicate the times (in hours) after infection at which the cells were harvested. For lane 24A, the cells were infected in the presence of araC.

substantial amounts of the protein continued to accumulate for more than 12 h (Fig. 7B). Quantitation of the amounts of radioactive material on the blots indicated that the rate of accumulation of the 7-kDa polypeptide was greatest during the first 6 h of infection; by 6 h after infection, nearly 40% of the 24-h value was obtained. However, the prolonged synthesis of the 7-kDa polypeptide and the finding that less of it accumulated in the presence than in the absence of araC (Fig. 7B) suggested that the regulation of expression of the *rpo7* gene may be more complex than anticipated from Northern blotting and amino acid pulse-labeling studies. Some possible explanations for these differences are discussed below.

DISCUSSION

We identified a small subunit of the vaccinia virus DNA-dependent RNA polymerase that is derived from the G5.5R ORF, which has the potential to encode a polypeptide of 63 amino acids. The polypeptide was previously overlooked because of its small size and failure to react with a polyclonal antiserum raised to the purified RNA polymerase (22). Evidence that the small polypeptide is a bona fide RNA polymerase subunit was based on the following observations: (i) the 7-kDa polypeptide was associated with virion-associated RNA polymerase that had been purified by column chromatography and gel filtration steps; (ii) the amino acid sequence of two tryptic peptides precisely matched the G5.5R ORF; (iii) the deduced amino acid sequence of the G5.5R ORF had structural similarities to the smallest subunit, *RPB10*, of yeast RNA polymerase II; (iv) an antibody raised to a carboxy-terminal peptide, the sequence of which was predicted by the G5.5R ORF, bound specifically to a 7-kDa polypeptide that was associated with RNA polymerase purified from vaccinia virus virions by successive column chromatography and glycerol gradient centrifugation

steps; and (v) all of the immunoreactive 7-kDa polypeptide extracted from virions cosedimented with RNA polymerase. On the basis of these findings, we named the gene encoding this small RNA polymerase subunit *rpo7*.

Although the general subunit structure of the vaccinia virus RNA polymerase resembles that of eukaryotic RNA polymerases, significant homology had previously been found only for the two large subunits (7, 13, 29). Of the five previously identified vaccinia virus RNA polymerase small subunits, one resembled eukaryotic elongation factor SII (3), but none seemed to be obviously related to the small subunits of eukaryotic RNA polymerase. It was of interest, therefore, to align the smallest subunit of the vaccinia virus RNA polymerase with RPB10, a 46-amino-acid subunit of *S. cerevisiae* RNA polymerase II, and find a 23% amino acid identity that increased to 48% if conservative amino acid changes were included. Most intriguing was the presence of the Cys-X-X-Cys-Gly metal-binding motif at precisely the same location near the N terminus in the two proteins. Only one of these motifs is present in the vaccinia virus and yeast RNA polymerase small subunits, even though studies of the iron-binding protein rubredoxin indicated that two of these sequence motifs are involved in the binding of one Fe^{2+} ion (1). Although an analysis of the stoichiometry of the yeast RNA polymerase was consistent with a single molecule of RPB10 (24), the Cys-X-X-Cys-Gly sequence is also present near the amino terminus of RPB1, perhaps providing the other half of the binding motif. The stoichiometry of the vaccinia virus *rpo7* subunit has not been determined; however, the vaccinia virus RNA polymerase large subunit also has the Cys-X-X-Cys-Gly motif. The binding of zinc to RPB10 has been demonstrated by a blotting technique (34).

The *rpo7* gene, like those of the previously identified vaccinia virus RNA polymerase subunits, was transcribed immediately after infection by the transcription system packaged within the virion. A 300-nucleotide transcript was detected between 2 and 4 h after infection as well as in the presence of inhibitors of translation and DNA replication. The observed overexpression of the *rpo7* transcript in the presence of a translational inhibitor is a hallmark of early genes. The RNA start site was mapped about 10 nucleotides downstream of a sequence closely matching the early promoter consensus AAAAATGAAAAAAAA (16). In addition, the early termination signal TTTTTNT (37) occurs about 50 bp downstream of the translational termination codon, consistent with the length of the early *rpo7* transcript. Although a late RNA start site consensus motif, TAAAT (21, 32), occurred a few nucleotides upstream of the early RNA start site, no evidence of specific late RNAs originating from this site was obtained. There was, however, evidence of long read-through transcripts containing *rpo7* sequences.

Consistent with the transcriptional studies, synthesis of the 7-kDa polypeptide was detected by immunological methods soon after infection. Radioactive amino acid pulse-labeling studies indicated that maximal rates of synthesis occurred between 2 and 4 h after virus addition. The continued accumulation of the RNA polymerase subunit beyond 6 h was more evident by immunoblotting than by immunoprecipitation of pulse-labeled polypeptides. This difference could reflect less-efficient immunoprecipitation of the 7-kDa polypeptide after assembly into RNA polymerase or a greater stability of the subunit when it is synthesized during the period of virion maturation. The possibility that translation of long polycistronic mRNAs accounts for late expression of some RNA polymerase subunits was discussed previously (5, 6). The coordinated synthesis and assembly of the vaccinia virus multisubunit RNA polymerase are obviously a complex topic.

We attempted to inactivate the *rpo7* gene by insertion of the gene for xanthine guanine phosphoribosyltransferase, an antibiotic-selectable marker (11, 18), into the *rpo7* ORF. The progeny viruses, after four successive plaque purifications with antibiotic selection, retained an intact copy of the *rpo7* gene, suggesting that it is essential for viral replication (8). Similarly, *RPB10* is essential for viability of *S. cerevisiae* (35).

The identification of *rpo7* brings the number of known vaccinia virus RNA polymerase subunit genes to eight. To further understand the structure of the RNA polymerase, we now need to determine the number of copies of each small subunit per molecule and the physical relationship of the subunits to each other.

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