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DNA-Dependent RNA Polymerase Subunits Encoded within the Vaccinia Virus Genome

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Antiserum to a multisubunit DNA-dependent RNA polymerase from vaccinia virions was prepared to carry out genetic studies. This antiserum selectively inhibited the activity of the viral polymerase but had no effect on calf thymus RNA polymerase II. The specificity of the antiserum was further demonstrated by immunoprecipitation of RNA polymerase subunits from dissociated virus particles. The presence in vaccinia virus-infected cells of mRNA that encodes the polymerase subunits was determined by in vitro translation. Immunoprecipitable polypeptides with M_rs of about 135,000, 128,000, 36,000, 34,000, 31,000, 23,000, 21,000, 20,000, and 17,000 were made when early mRNA was added to reticulocyte extracts. The subunits were encoded within the vaccinia virus genome, as demonstrated by translation of early mRNA that hybridized to vaccinia virus DNA. The locations of the subunit genes were determined initially by hybridization of RNA to a series of overlapping 40-kilobase-pair DNA fragments that were cloned in a cosmid vector. Further mapping was achieved with cloned *Hin*dIII restriction fragments. Results of these studies indicated that RNA polymerase subunit genes are transcribed early in infection and are distributed within the highly conserved central portion of the poxvirus genome in *Hin*dIII fragments E, J, H, D, and A.

Eucaryotic RNA polymerases are complex enzymes containing multiple subunits (6, 34). Class II RNA polymerases, which are responsible for the synthesis of mRNA and are isolated from diverse groups of eucaryotes including mammals (18, 22, 36), amphibians (33), insects (12), higher plants (15), and yeast (9), display strikingly similar subunit compositions. Typically, there are two large subunits that are greater than 100,000 daltons and eight or more small ones ranging in size from 10,000 to 40,000 daltons. Immunological cross-reactivity between subunits of different species (13, 14, 19, 20, 37, 39) suggests the evolutionary conservation of structure and function.

Although DNA viruses generally use host RNA polymerase for transcription, poxviruses may be notable exceptions. A DNA-dependent RNA polymerase is present in vaccinia virus particles and the cytoplasm of infected cells (17, 27). The purified enzyme is similar in overall size to eucaryotic RNA polymerase and also contains numerous subunits (2, 38). Two large polypeptides of 130,000 to 140,000 daltons and at least seven smaller ones ranging in size from 13,000 to 36,000 daltons have been described. An additional polypeptide of 77,000 daltons was associated with the RNA polymerase that was isolated from infected cells (28). Functional as well as structural similarities exist between vaccinia virus-associated and eucaryotic class II RNA polymerases. Neither purified enzyme can transcribe double-stranded DNA efficiently, and both require single-stranded DNA and Mn^{2+} for optimal activity (2, 28, 38). In each case, the correct initiation of transcription occurs only in crude systems or when the polymerase is supplemented with protein factors (10, 11, 31, 35).

A central question with regard to the biology of poxviruses concerns the nature of the RNA polymerase that is packaged within the virus particle. The virus-associated polymerase differs from that of the host in chromatographic properties, electrophoretic mobilities of the component polypeptides, and resistance to α-amanitin (2, 28, 38). These differences could result from the virus-induced modification of the cellular polymerase that are analogous to those that occur after infection with bacteriophage T4 (5). Alternatively, some or all of the subunits could be virus-specific. In this study we undertook to determine which of the polypeptides associated with purified vaccinia virus RNA polymerase are virus-encoded. In this report we demonstrate that genes for at least eight subunits are located within the central, highly conserved region of the poxvirus genome. Elsewhere (4), we have reported that the largest RNA polymerase subunit of vaccinia virus has considerable sequence homology with the corresponding RNA polymerase subunits of *Escherichia coli*, *Saccharomyces cerevisiae*, and *Drosophila melanogaster*.

MATERIALS AND METHODS

Preparation of antiserum. RNA polymerase was purified from vaccinia virus particles as described previously (2) by using successive columns of DEAE-cellulose, DEAE-Bio-Gel (Bio-Rad Laboratories, Richmond, Calif.), phosphocellulose, and aminopentyl agarose. Approximately 100 μg of purified enzyme was emulsified with complete Freund adjuvant and injected into a rabbit. Thirty days later, the animal received the first of three weekly booster inoculations of 50 μg of purified enzyme in incomplete Freund adjuvant. Bleedings were carried out 7 days after each booster inoculation.

Preparation of [^{35}S]methionine-labeled RNA polymerase. Approximately 2.5×10^9 HeLa cells were infected with 30 PFU of vaccinia virus per cell in 500 ml of Eagle medium containing 5% horse serum. After 1 h the cells were diluted 10-fold into methionine-free medium containing 5% dialyzed

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horse serum and 5 mCi of [^{35}S]methionine (600 Ci/mmol; Amersham-Searle). The cells were incubated for a further 23 h at 37°C and then collected by centrifugation. Virus was purified by sedimentation through a sucrose cushion and two successive sucrose gradients (26). A soluble deoxycholate extract was prepared, and RNA polymerase was purified as described previously (2), except that the enzyme was step eluted with 0.75 M NaCl from the phosphocellulose column, the aminopentyl agarose column was omitted, and glycerol gradient centrifugation was added.

Construction of cosmids. A cosmid library of vaccinia virus DNA was prepared in the vector pHC79 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Vaccinia virus DNA was partially digested with *Sau*3A and fractionated by sucrose gradient sedimentation. Each fraction was assayed by agarose gel electrophoresis, and fragments of approximately 40 kilobase pairs were ligated to *Bam*HI-digested and alkaline phosphatase-treated pHC79, as described by Maniatis et al. (23). Colonies were transferred to nitrocellulose, and cosmids containing viral DNA were identified by hybridization to ^{32}P-labeled vaccinia virus DNA. Initial characterization of positive colonies was performed by agarose gel electrophoresis of *Hin*dIII- and *Sal*I-digested cosmid DNA. Subsequently, the cosmid DNA was labeled in vitro with ^{32}P and hybridized to strips of nitrocellulose containing electrophoretically resolved *Hin*dIII or *Sal*I fragments of vaccinia virus DNA. In this manner, the segment of the vaccinia virus genome within each cosmid was determined. A representative set of cosmids that spanned the genome was used for this study.

Hybrid selection and cell-free translation of RNA. HeLa cells were treated with cycloheximide (100 μg/ml) to block protein synthesis and after 10 min were infected with 30 PFU of purified vaccinia virus per cell. Cycloheximide was maintained in the medium, and the cells were harvested after 4 h at 37°C. Cytoplasmic RNA was purified by CsCl centrifugation (8). Plasmid DNA (25 μg) was transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) (32) and hybridized to RNA as described by Jones and Moss (16). After the filters were washed, RNA was eluted and translated in a micrococcal nuclease-treated reticulocyte lysate (7, 30). Following translation, samples were incubated with antiserum as described below and prepared for polyacrylamide gel electrophoresis.

Immunoprecipitation and polyacrylamide gel electrophoresis. Solubilized virus proteins or translation mixtures were incubated with preimmune rabbit serum at 4°C for 4 h, and antigen-antibody complexes were bound to *Staphylococcus aureus* protein A (10% solution; Pansorbin; Calbiochem-Behring, La Jolla, Calif.; or protein A Sepharose CL-4B; Sigma Chemical Co., St. Louis, Mo., or Pharmacia Fine Chemicals, Piscataway, N.J.). The supernatant that remained after sedimentation in a microcentrifuge was transferred to a fresh tube and incubated overnight at 4°C with antiserum. Staphylococcal protein A was added to bind the immune complexes. The beads were washed twice with 0.05 M Tris hydrochloride (pH 7.5)–0.15 M NaCl–0.1% sodium deoxycholate and twice with 2 M urea–0.4 M LiCl–0.01 M Tris hydrochloride (pH 8.0). The beads were boiled in sample buffer just before electrophoresis. Protein samples also were routinely analyzed without immunoprecipitation. In that case, the translation mixture, soluble virus extract, or purified RNA polymerase was diluted with several volumes of sample buffer and boiled for 5 min. Samples were applied to 10% polyacrylamide gels containing sodium dodecyl sulfate, and electrophoresis was continued until the heme reached the bottom of the gel. Separation of the two largest subunits was achieved by electrophoresis in 5% polyacrylamide gels. The gels were then fixed, soaked in En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried under vacuum, and exposed to X-Omat XAR-5 film (Eastman Kodak Co., Rochester, N.Y.).

TABLE 1. Inhibition of vaccinia virus RNA polymerase activity

Sample	RNA polymerase activity as:	
	[^{32}P]UMP incorporated (cpm)	% Activity remaining
Vaccinia RNA polymerase with:		
Preimmune serum	43,814	
Antiserum to vaccinia virus RNA polymerase	2,517	6
Calf thymus RNA polymerase II with:		
Preimmune serum	17,007	
Antiserum to vaccinia virus RNA polymerase	17,473	103

RNA polymerase assay. RNA polymerase activity was measured by using a single-stranded DNA template as described by Baroudy and Moss (2), except that [α-^{32}P]UTP was used. Samples were then monitored by Cerenkov counting. Calf thymus RNA polymerase was assayed under the same conditions.

RESULTS

Inhibition of vaccinia virus RNA polymerase activity with antiserum. DNA-dependent RNA polymerase that was purified from vaccinia virions was previously shown to have a molecular weight of approximately 500,000 and to be dissociable into polypeptides with molecular weights of approximately 140,000, 137,000, 37,000, 35,000, 31,000, 22,000, and 17,000 (2, 38). Antiserum, which was prepared from a rabbit that received multiple inoculations of purified vaccinia virus RNA polymerase, inhibited the activity of the RNA polymerase by more than 90%, whereas preimmune serum had little or no effect (Table 1). The antiserum was not directed toward conserved epitopes in viral and cellular RNA polymerases because calf thymus RNA polymerase II activity was not inhibited (Table 1). This antiserum also inhibited transcription of early vaccinia virus genes that were added to extracts of vaccinia virus-infected cells (31).

Immunoprecipitation of vaccinia virus RNA polymerase subunits. The specificity of the antiserum was tested by immunoprecipitation of vaccinia virus proteins. [^{35}S]methionine-labeled polypeptides, which were present in a soluble extract of virus particles or purified RNA polymerase, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1, lanes 1 and 4). Under these electrophoretic conditions, the two large polymerase subunits were not resolved, and they migrated with a molecular weight of approximately 135,000. Prominent 36,000-M_r (36K), 34K, 23K, 20K, and 17K polypeptides and faint 31K and 21K polypeptides in the RNA polymerase preparation were similar in size to those described previously (2). (The 17K polypeptide migrated off the bottom of the gel and is not seen in Fig. 1.) The prominent 70K polypeptide and minor 55K to 200K polypeptides in the RNA polymerase preparation (Fig. 1, lane 4) were reduced in amount when the purification was performed as originally described (2), which was the procedure used to obtain RNA polymerase for immunization.

When the partially purified polymerase was incubated with immune serum, at least eight polypeptides were specifically precipitated (Fig. 1, lanes 5 and 6). Because the polypeptides were physically associated in a complex, this type of analysis did not reveal whether the antiserum was directed against each individual polypeptide or against only a few. The absence from the immunoprecipitate of the prominent 70K polypeptide as well as the minor ones greater than 50,000 daltons, however, indicates that they are neither recognized by the antiserum nor tightly associated with the RNA polymerase in the triple detergent buffer used for antibody binding. Some additional polypeptides were precipitated from the crude virus extract (Fig. 1, lane 3); however, this was largely nonspecific because most were also seen after incubation with preimmune serum (Fig. 1, lane 2).

Immunoprecipitation of vaccinia virus RNA polymerase subunits synthesized in vitro. The following experiments were designed to determine whether mRNA for RNA polymerase subunits was present in the cytoplasm of vaccinia virus-infected cells and could be translated in vitro. Results of preliminary control experiments indicated that specific immunoprecipitable polypeptides were not made when RNA from uninfected cells was added to micrococcal nuclease-treated reticulocyte lysates. An autoradiograph of the total [^{35}S]methionine-labeled translation products produced in such a lysate programmed with RNA from vaccinia virus-infected cells is shown in Fig. 1, lane 7. As usual, large-sized translation products are underrepresented. The antiserum specifically precipitated 36K, 34K, 31K, 23K, and 20K polypeptides (Fig. 1, lane 8). Although not clearly visible in Fig. 1, immunoprecipitable 135K, 21K, and 17K polypeptides also were made and will be shown in subsequent figures.

The RNA that was used to program the reticulocyte lysate

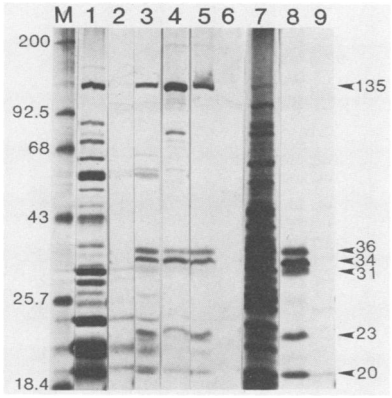

FIG. 1. Immunoprecipitation of RNA polymerase subunits synthesized in vivo and in vitro. Autoradiographs of radioactively labeled proteins heated at 100°C with sodium dodecyl sulfate and mercaptoethanol and subjected to polyacrylamide gel electrophoresis are shown. Lane M, ^{14}C-labeled standard protein markers; lanes 1 to 3, proteins extracted with deoxycholate from [^{35}S]methionine-labeled purified virions; lanes 4 to 6, [^{35}S]methionine-labeled purified vaccinia virus RNA polymerase; lanes 7 to 9, [^{35}S]methionine-labeled reticulocyte cell-free translation products of total cytoplasmic RNA from HeLa cells treated with cycloheximide and infected with vaccinia virus for 4 h. Lanes 1, 4, and 7 contain total protein; lanes 3, 5, and 8 contain proteins that were immunoprecipitated with antiserum to vaccinia RNA polymerase; lanes 2, 6, and 9 contain proteins that were immunoprecipitated with preimmune serum. Molecular weights (10^3) are indicated to the left and right of the gels.

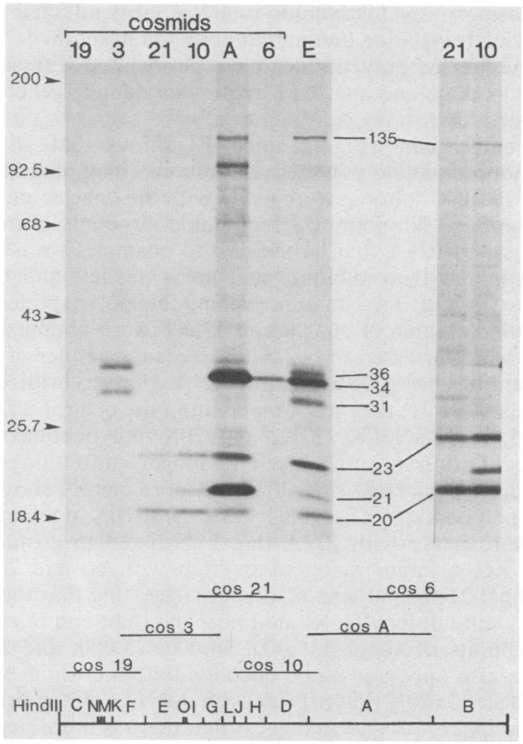

FIG. 2. Mapping of RNA polymerase subunit genes by translation of mRNA selected by hybridization to a cosmid library. A schematic representation of the vaccinia virus DNA contained within 6 cosmid (cos) clones is shown at the bottom. The letters, which refer to *Hin*dIII fragments, are shown for reference. Cytoplasmic RNA, which was obtained from HeLa cells that were treated with cycloheximide and infected with vaccinia virus for 4 h, was hybridized to cosmids that were immobilized on nitrocelluose filters. The specifically bound RNA was eluted and translated in a micrococcal nuclease-treated reticulocyte cell-free system containing [^{35}S]methionine. Labeled proteins were incubated with vaccinia virus RNA polymerase antiserum, bound to *S. aureus* protein A, and dissociated with sodium dodecyl sulfate for polyacrylamide gel electrophoresis. Autoradiographs are shown. In lanes 19, 3, 21, 10, A, and 6, the reticulocyte lysates were programmed with mRNA that was selected by hybridization to the indicated cosmid. The two lanes at the extreme right are longer autoradiographic exposures. Lane E contains immunoprecipitated translation products of total cytoplasmic early RNA. Molecular weights (10^3) of marker proteins are indicated.

was obtained from cells that were infected with vaccinia virus in the presence of cycloheximide. With this protein synthesis inhibitor, early RNA is made in large amounts and DNA replication and the switch to late transcription is prevented. When similar experiments were carried out with RNA that was obtained at 6 h after infection in the absence of inhibitors, much lower amounts of immunoprecipitable polypeptides were obtained (data not shown). All subsequent experiments therefore were carried out with RNA that was made in the presence of cycloheximide.

Hybridization of RNA to vaccinia virus DNA fragments in a cosmid library. To determine whether the RNA polymerase subunits are virus encoded and to locate their map positions, we translated mRNA that hybridized to vaccinia virus DNA segments. A set of six cosmids containing overlapping 40-kilobase-pair vaccinia virus genome fragments (Fig. 2) were immobilized on nitrocellulose membrane filters and used to hybrid select RNA that was purified from the

cytoplasm of cycloheximide-treated, virus-infected cells. Following translation and immunoprecipitation, the [^{35}S]methionine-labeled polypeptides were dissociated with sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis.

A control lane (Fig. 2, lane E) shows that all size-immunoprecipitable polymerase subunits, including at least one of 135,000 daltons, were made with the unselected RNA preparations. No immunoprecipitable products were obtained when RNA that hybridized to cosmid 19 was used, suggesting that no subunits map near the left end of the genome (Fig. 2). Two immunoprecipitable polypeptides with molecular weights of 36,000 and 31,000 were obtained with RNA that hybridized to cosmid 3. Because neither of these polypeptides was made with RNA that hybridized to cosmids 19 or 21, they must map within the central region of cosmid 3. The 135K, 23K, and 20K polypeptides were mapped within cosmid 21. The longer autoradiographic exposure on the right side of Fig. 2 more clearly shows the 135K polypeptide. The 135K, 23K, and 20K polypeptides also were made with RNA that hybridized to cosmid 10, which has a large region of overlap with cosmid 21. An additional 21K band was resolved, suggesting that the gene for this subunit may be located near the right end of cosmid 10. Subunits of about 135,000, 34,000, 23,000, and 20,000 daltons also appeared to be encoded within cosmid A. The formation of a 23K polypeptide with mRNAs that hybridized to cosmids 21, 10, and A suggest that there is more than one polymerase subunit of this size or that there are repetitive DNA sequences. Additional polypeptides such as the prominent one of 95,000 daltons and minor ones below it might result from premature termination or other artifacts of in vitro translation (see below). The synthesis of a 34K polypeptide with RNA that hybridized to cosmid 6 suggests that the gene is located within the overlapping right half of cosmid A.

Further mapping. Cosmid A contains the largest of the 15 fragments that were obtained by digestion of vaccinia virus DNA with *Hin*dIII. Except for the two terminal *Hin*dIII fragments, the rest have been cloned in pBR322 previously (3). The 13 available *Hin*dIII fragments were used for hybrid selection, and the mRNAs were translated as described above. Only fragments E, J, H, D, and A hybrid selected mRNA for one or more polypeptides that were recognized by antiserum to RNA polymerase (Fig. 3). *Hin*dIII-E, which is entirely contained within cosmid 3, encoded prominent polypeptides of 36,000 and 31,000 daltons. Because of the overexposure of the autoradiograph, additional smaller polypeptides, some of which may represent premature termination products of the larger ones, were detected (see below). Two polypeptides that comigrated with RNA polymerase subunits of 135,000 and 23,000 daltons were encoded within the *Hin*dIII J fragment. Because the larger polypeptide also was synthesized from RNA that hybridized to the adjacent *Hin*dIII H fragment, this gene evidently spans the *Hin*dIII-J and -H junction. Previous studies showing that a long transcript crosses that *Hin*dIII site (1, 21, 40) and nucleotide sequence data (4) support this interpretation.

mRNA for the 20K polypeptide was selected most efficiently by *Hin*dIII-D. We are not sure whether minor 20K bands synthesized with mRNA that hybridized to DNA fragments other than *Hin*dIII-D represent additional RNA polymerase subunits or are simply artifacts of in vitro translation. The failure of *Hin*dIII-D to hybrid select mRNA for a large subunit is significant because it implies that the large subunits that map to the left and right are encoded by separate genes. The data (Fig. 3) obtained by hybrid selection with the *Hin*dIII A fragment are similar to those shown in Fig. 2. Because of the shorter electrophoresis time, however, the 17K polypeptide was retained on the gel and could be mapped within *Hin*dIII-A.

Resolution of the two high-molecular-weight RNA polymerase subunits. To better characterize the two large subunits, efforts were made to improve their electrophoretic separation. mRNA was hybrid selected to *Hin*dIII-J, *Hin*dIII-H, and a *Bam*HI subclone of cosmid A (which has been shown to encode one of the large subunits; unpublished data). Translation and immunoprecipitation were performed as described above, but equal portions of the immunoprecipitates were electrophoresed on 10 and 5% polyacrylamide gels. Under our standard electrophoresis conditions (10% gel), the cosmid A subclone A62 and the *Hin*dIII J and H fragments hybrid selected mRNA encoding 135K proteins that comigrated (Fig. 4.) In the 5% gel, however, the large polypeptides from *Hin*dIII-A and from the J-H junction were clearly distinguishable (Fig. 4). The molecular weight of the former was estimated to be 128,000 and of the latter was 135,000. Additional translation products from mRNA that was hybrid selected to the subclone of *Hin*dIII-A and from *Hin*dIII-J and -H may represent premature terminations.

A summary of the data is presented in Fig. 5. One of the high-molecular-weight polypeptides (135K) mapped at the *Hin*dIII-J and -H junction, and another (128K) mapped within the large *Hin*dIII A fragment. An RNA polymerase subunit of 36,000 daltons and an additional polypeptide of 31,000 daltons mapped within *Hin*dIII-E. The 34K subunit mapped within *Hin*dIII-A, although a faint 34K band also

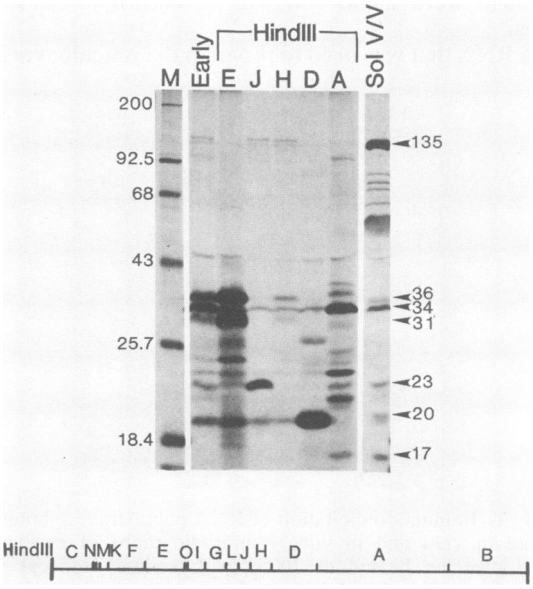

FIG. 3. Further mapping of RNA polymerase subunits. Hybrid selection and cell-free translation were carried out as described in the legend to Fig. 2, except that plasmids containing vaccinia virus DNA *Hin*dIII E, J, H, and D fragments were immobilized on filters. The *Hin*dIII A fragment was cloned in a cosmid. Lane M contains ^{14}C-labeled marker proteins. Other lanes contain immunoprecipitated translation products of total early cytoplasmic RNA (Early) or RNA that was selected by hybridization to the indicated *Hin*dIII DNA fragment. The lane marked Sol V/V contains soluble proteins that were extracted from [^{35}S]methionine-labeled vaccinia virions with deoxycholate and immunoprecipitated. Molecular weights (10^3) are indicated to the left and right of the gels.

was produced by using RNA that hybridized to *Hin*dIII-E. Two 23K polypeptides, one mapping to *Hin*dIII-J and the other to *Hin*dIII-A, were found. In addition, a major 20K polypeptide mapped to *Hin*dIII-D, and 21K and 17K polypeptides mapped to *Hin*dIII-A.

DISCUSSION

The subunit structure of the vaccinia virus-associated DNA-dependent RNA polymerase conforms to that of eucaryotic RNA polymerases in general. Results of the experiments reported here indicate, however, that many or perhaps all of the subunits are virus-encoded. Although the polyvalent antiserum that we prepared to the viral enzyme did not inhibit the activity of calf thymus RNA polymerase II, this does not mean that the viral and cellular polymerases are unrelated. One might expect that the most immunogenic regions of the viral polymerase are those that are most distinct from cellular polymerase. Morrison and Moyer (25) have prepared a monoclonal antibody that reacts with the large subunits of cellular RNA polymerase II and rabbitpox RNA polymerase. In addition, analysis of the predicted amino acid sequence of the large vaccinia virus RNA polymerase subunit revealed extensive homology with the large RNA polymerase subunits of *E. coli*, *S. cerevisiae*, and *D. melanogaster* (4). Other vaccinia virus-encoded RNA polymerase subunits are also likely to share some homology with RNA polymerases from procaryotes and eucaryotes because

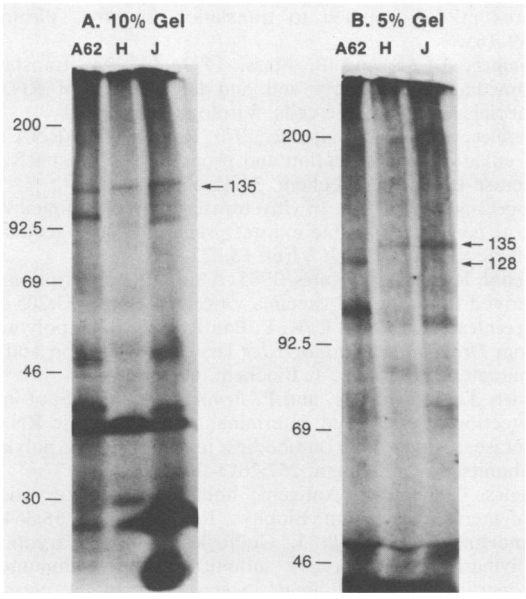

FIG. 4. Separation of the two large molecular weight subunits of vaccinia RNA polymerase. Hybrid selection to plasmids containing the *Hin*dIII J and H fragments or to plasmid A62, a *Bam*HI subclone of *Hin*dIII A (A62), was performed as described in the text. The translation products were immunoprecipitated with antiserum to the viral RNA polymerase, and equal amounts of the immunoprecipitated material were loaded onto 5 and 10% polyacrylamide gels. Autoradiographs of the immunoprecipitates that electrophoresed in a 10 and 5% gel are shown in panels A and B, respectively. Lane M contains ^{14}C-labeled marker proteins. The lanes designated A62, H, or J show immunoprecipitates of translation products of mRNA that was selected by hybridization to plasmids containing a *Bam*HI subclone of *Hin*dIII-A or the *Hin*dIII H or *Hin*dIII J fragments, respectively. Molecular weights of marker proteins and immunoprecipitable RNA polymerase subunits are shown on the left and right of the gels, respectively.

FIG. 5. Locations of RNA polymerase subunit genes. Letters indicate the *Hin*dIII fragments.

immunological relatedness has been demonstrated for both large and small subunits from widely different organisms (13, 14, 19, 20, 37, 39).

Because our antiserum was made against extensively purified RNA polymerase, it was directed against polypeptides that were tightly associated with the enzyme. Whether each of these polypeptides was a functional subunit, however, cannot be determined at this time. Conversely, the vigorous purification procedure may have led to the dissociation of important accessory polypeptides. The participation of additional polypeptides in transcription is likely because highly purified enzyme is unable to initiate correct transcription on a double-stranded viral DNA template (2, 38). These problems of interpretation are not specific for vaccinia virus, however, but are general ones for eucaryotic RNA polymerases.

Our basic approach to mapping the RNA polymerase subunits was to immunoprecipitate reticulocyte cell-free translation products of mRNA that were selected by hybridization to cloned vaccinia virus genome fragments. The major technical problem that was encountered in this project related to the difficulty in translating mRNAs for the high-molecular-weight subunits. In some experiments, the autoradiographic bands were too faint to permit positive identification. This difficulty may have resulted from both degradation of mRNA and premature termination. Indeed, discrete immunoprecipitable polypeptide bands below those of full size were frequently noted and caused some difficulties in interpretation. Similar problems arose when the vaccinia virus DNA polymerase gene was mapped previously (16).

It is not surprising that all of the RNA polymerase subunits mapped within the central highly conserved region of the poxvirus genome and not within the more variable ends. Nevertheless, the wide dispersion of the subunits within the central region was unanticipated. Although further mapping is required for most of the RNA polymerase subunits, detailed transcriptional analysis and DNA sequence data have been reported for several regions in which polymerase subunits appear to be encoded. Within the *Hin*dIII J fragment, five early mRNAs (590, 1,070, 1,790, 2,380, and 3,840 nucleotides long) were mapped by blot hybridization and S1 nuclease analysis (1, 4, 40). The 590- and 2,380-nucleotide species were identified as major and readthrough forms of the thymidine kinase message, respectively. The 1,790-nucleotide RNA is coterminal with the longer thymidine kinase message and encodes a 41K early protein of unknown function. The 1,070-nucleotide RNA, which also is coterminal with the 1,790-nucleotide mRNA, encodes a 21K polypeptide that is almost certainly the 23K RNA polymerase subunit described here (4, 21, 40). The DNA sequence encoding a long transcript of greater than 3,800 nucleotides is bisected by the *Hin*dIII site separating fragments J and H. The translational product of this long mRNA was reported previously to be 110,000 daltons (3); however, our reappraisal of the original autoradiograph indicated that this value was an underestimate and that the

size of the polypeptide predicted by the open reading frame was 147,000 daltons (4). Thus, the true size of the 135K RNA polymerase subunit is probably 147,000 daltons. The large subunit of rabbitpox RNA polymerase complex has also been mapped to the same region (24, 25).

Although it has not yet been transcriptionally mapped, the entire sequence of the *Hin*dIII D fragment has been published (29). Inspection of this sequence revealed 13 open reading frames encoding proteins of greater than 145 amino acids. Based on this analysis, there are four open reading frames in the *Hin*dIII D fragment that encode proteins with molecular weights in the range of the 20K RNA polymerase subunit. Because temperature-sensitive mutants have been localized to some of these open reading frames (29), identification of the 20K subunit gene is of immediate interest.

Neither detailed transcriptional analysis nor extensive DNA sequencing has been reported for either the *Hin*dIII A or E fragments. However, Morrison et al. (24) mapped a 34K RNA polymerase subunit within *Hin*dIII-A, which is consistent with our results.

Attempts at determining molar ratios for the vaccinia virus RNA polymerase subunits previously led to the suggestion of multiple copies of 34K and 23K polypeptides (2). Data from the results of this study indicate that for the 23K, and possibly the 34K, polypeptide more than one gene exists that presumably codes for different polypeptides of similar size. On the other hand, results of preliminary peptide analyses suggest that the 36K and 31K polypeptides that map to the *Hin*dIII E fragment may be derived from one gene (unpublished data). The low amount of the 31K polypeptide in RNA polymerase raises further doubts about its significance. We estimate, therefore, that there are at least nine viral genes that encode polypeptides that are physically associated with the vaccinia virus RNA polymerase. Association of the large subunit of cellular RNA polymerase with rabbitpox virus RNA polymerase also has been suggested (25).

The ability to synthesize all of the polymerase subunits from cells that are infected with vaccinia virus in the presence of cycloheximide indicates that these genes are expressed early in infection. Other data, however, suggest that some subunits may be expressed at late times in the virus life cycle as well. Mahr and Roberts (21) observed mRNA species that hybridized to the *Hin*dIII J fragment and which could encode the 23K and 135K subunits in preparations of late RNA (8 h after infection). Results of S1 nuclease analysis also suggest the possibility of a late RNA start site upstream of the early one encoding the large subunit (4). Morrison and co-workers (24, 25) found by immunoblot analysis of rabbitpox-infected cells that accumulation of the large subunit is detected only after viral DNA replication occurs.

Mapping of the RNA polymerase subunits opens the way for a variety of new experiments. Nucleotide sequencing of two of the subunit genes is completed, and for one of these genes homology with procaryotic and eucaryotic RNA polymerase subunits has been demonstrated (4). Efforts to fine map and sequence the other RNA polymerase subunit genes are in progress. This structural information will aid in the preparation of specific antiserum, either by using expression vectors to generate large amounts of individual polypeptides or by oligopeptide synthesis. The production of large amounts of individual subunits by recombinant DNA methods also should be useful in defining their catalytic roles. Additionally, the map and sequence information will facilitate the generation and analysis of RNA polymerase mutations.

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