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Mapping of the Vaccinia Virus DNA Polymerase Gene by Marker Rescue and Cell-Free Translation of Selected RNA

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The previous demonstration that a phosphonoacetate (PAA)-resistant (PAA^r) vaccinia virus mutant synthesized an altered DNA polymerase provided the key to mapping this gene. Marker rescue was performed in cells infected with wild-type PAA-sensitive (PAA^s) vaccinia by transfecting with calcium phosphate-precipitated DNA from a PAA^r mutant virus. Formation of PAA^r recombinants was measured by plaque assay in the presence of PAA. Of the 12 *Hin*dIII fragments cloned in plasmid or cosmid vectors, only fragment E conferred the PAA^r phenotype. Successive subcloning of the 15-kilobase *Hin*dIII fragment E localized the marker within a 7.5-kilobase *Bam*HI-*Hin*dIII fragment and then within a 2.9-kilobase *Eco*RI fragment. When the latter was digested with *Cla*I, marker rescue was not detected, suggesting that the PAA^r mutation mapped near a *Cla*I site. The sensitive *Cla*I site was identified by cloning partial *Cla*I-*Eco*RI fragments and testing them in the marker rescue assay. The location of the DNA polymerase gene, about 57 kilobases from the left end of the genome, was confirmed by cell-free translation of mRNA selected by hybridization to plasmids containing regions of PAA^r vaccinia DNA active in marker rescue. A 100,000-dalton polypeptide that comigrated with authentic DNA polymerase was synthesized. Correspondence of the in vitro translation product with purified vaccinia DNA polymerase was established by peptide mapping.

Vaccinia virus, a member of the poxvirus family, is a large, double-stranded DNA virus that packages a complete transcription system and replicates in the cytoplasm of infected cells (26). Recent studies of the physical structure of the 180-kilobase (kb) genome demonstrated that both ends of vaccinia DNA are hairpin loops (2, 17) and contain a 10-kb inverted repetition (16, 38). Within this terminal repetition are sets of tandemly repeating units and the coding sequences for three early genes (1, 2, 40). The vaccinia virus genome may code for about 180 polypeptides, of which approximately 75 early and 40 late have been mapped by cell-free translation of mRNA selected by hybridization to purified (8, 13) or cloned restriction fragments (3, 14). However, the location of genes with known functions has just begun; thymidine kinase is the only vaccinia enzyme that has been physically mapped on the viral genome (20, 36). The purpose of the present investigation was to map a second viral enzyme, the DNA polymerase.

A DNA polymerase with a molecular weight of 110,000 (110K) has been purified from cells infected with vaccinia virus (6). Convincing evidence that this enzyme is virus coded came from studies with phosphonoacetate (PAA). Bolden et al. and Citarella et al. (5, 9) found that the vaccinia DNA polymerase was inhibited by PAA. In addition, Overby et al. (30) observed that high concentrations of PAA partially inhibited virus growth. Subsequently, Condit and Motyczka (11) reported the isolation of phosphonoacetate-resistant (PAA^r) mutants of vaccinia virus. These results led Moss and Cooper (27) to demonstrate that purified DNA polymerase of PAA^r virus is resistant to the drug and therefore virus coded.

In this report, we describe the mapping of the vaccinia DNA polymerase, using the technique of marker rescue (29, 34, 36). Transfection experiments indicated that specific cloned PAA^r mutant DNA fragments could recombine with PAA-sensitive (PAA^s) virus DNA to form PAA^r virus. The genetic location of the DNA polymerase was confirmed by in vitro translation of mRNA selected to the region of the vaccinia genome active in marker rescue.

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MATERIALS AND METHODS

Virus. Vaccinia virus (strain WR) was grown in HeLa cells and purified as described previously (21, 25). The PAA^r mutant virus used in this study has been characterized (27).

Marker rescue. Confluent monolayers of CV-1 cells grown in Dulbecco medium supplemented with 10% fetal bovine serum were infected with PAA^s virus at a multiplicity of 0.05 PFU per cell. Two hours after infection, calcium phosphate-precipitated DNA was added (18, 36). The DNA precipitates contained 1 μg of PAA^r virion DNA or 2 μg of PAA^r recombinant plasmid DNA mixed with 20 μg of carrier DNA per ml. Calf thymus DNA alone or calf thymus and 1 μg of PAA^s virion DNA was used as carrier. At 6 h after infection, fresh medium containing 8% fetal bovine serum was added and cells were harvested at 48 h after infection. Infected cells were disrupted and plaque assayed on CV-1 cells under agar in the absence of PAA to give the total virus titer and in the presence of 300 μg of PAA per ml to measure the PAA^r titer. After 72 h, the plaques were visualized with 0.005% neutral red stain.

Preparation of DNA. Methods for the extraction of DNA from purified vaccinia virus (16), digestion of DNA with restriction enzymes (40), and purification of plasmid recombinants containing fragments of PAA^r mutant DNA (4) have been described.

Recombinant plasmids were prepared in pBR322, pUC9 (a gift of J. Viera and J. Messing), or the cosmid pJB8 (a gift of M. Kuehl). Ligation and packaging of DNA within the cosmid were done essentially by the procedure of Grosveld et al. (19). DNA fragments used in cloning were isolated from agarose gels by electrophoresis onto DEAE-paper (37) or by binding to powdered glass (35).

Hybridization selection and cell-free translation. HeLa cells were treated with cycloheximide (100 μg/ml) and after 10 min were infected with vaccinia virus in the presence of the

drug. Cytoplasmic RNA was then purified by CsCl centrifugation from HeLa cells at 4 h after infection. Intact or linearized PAA^r recombinant plasmid DNA (20 to 25 μg) was denatured, neutralized, and transferred to 2.5-cm-diameter nitrocellulose membranes (Schleicher and Schuell, BA-85) as described by Weir et al. (36). The nitrocellulose was washed thoroughly to remove unbound DNA and baked at 80°C for 2 h.

Portions of the nitrocellulose that contained plasmid DNA were cut into small pieces, put into 1.5-ml microcentrifuge tubes, and hybridized to purified cytoplasmic RNA (350 to 500 μg) from cycloheximide-treated vaccinia-infected cells for 2 h at 50°C by the method of Riccardi et al. (33). Filters were washed stringently, and the specifically bound RNA was eluted and ethanol precipitated at −20°C with 20 μg of calf liver tRNA carrier (33). Before translation, the RNA was pelleted at 15,000 × *g* for 10 min at 4°C, washed twice with 70% ethanol and once with 95% ethanol, and dried under reduced pressure.

Cell-free translation was carried out at 30°C for 2 h in a micrococcal nuclease-treated reticulocyte lysate (32) as described by Cooper and Moss (12). In vitro reactions were prepared for gel electrophoresis by incubation for 15 min at 30°C with 100 μg of RNase A per ml and 10 mM EDTA before adding several volumes of sample buffer (80 mM Tris-hydrochloride [pH 6.8], 1.3 M β-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 10% [wt/vol] glycerol, 0.02% bromophenol blue) followed by boiling the samples for 5 min. Samples labeled in vivo were mixed with unlabeled reticulocyte lysate before analysis so that all samples contained hemoglobin.

Lysates were electrophoresed on 16-cm-long SDS-polyacrylamide gels, using the discontinuous system described by Laemmli (24). The samples were electrophoresed through a 5% stacking gel and 15% resolving gel until the hemoglobin reached the bottom. After electrophoresis, the gels were soaked in En^{3}Hance (New England Nuclear Corp.), dried under vacuum, and exposed to Kodak X-Omat XAR-5 film at −70°C.

Peptide mapping. Peptide mapping was performed essentially by the procedure of Cleveland et al. (10). Bands of interest were excised from Coomassie blue-stained 15% polyacrylamide gels and placed in sample wells of a second SDS gel (20%:0.65% [wt/wt] acrylamide-bisacrylamide). Each slice was overlaid with various concentrations of *Staphylococcus aureus* V8 protease. Digestion occurred directly in the stacking gel during subsequent electrophoresis. After electrophoresis, the gel was prepared for exposure to X-ray film as described above.

Purification of [^{35}S]methionine-labeled vaccinia virus DNA polymerase. The basic procedure reported previously by Moss and Cooper (27) is a modification of that described by Citarella et al. (9). Approximately 10^9 HeLa cells were infected with 30 PFU of vaccinia virus and labeled from 1 to 6 h postinfection with 5 mCi of [^{35}S]methionine in medium otherwise lacking methionine and containing dialyzed horse serum and glutamine. Infected cells were Dounce homogenized and centrifuged to remove nuclei, mitochondria, and ribosomes. The supernatant was applied to a column (1.8 by 18 cm) containing DE-52 (Whatman) cellulose equilibrated with 20 mM potassium phosphate (pH 7.5). A linear 20-to-300 mM potassium phosphate (pH 7.5) gradient in 10% glycerol–0.5 mM dithiothreitol was applied, and the 10-ml fractions collected were assayed for DNA polymerase activity (27). Samples containing peak 1 of DNA polymerase activity were pooled and applied to a phosphocellulose (P-11, Whatman) column (1 by 5 cm) equilibrated with 0.075 M potassium phosphate (pH 7.5)–10% glycerol–1 mM dithiothreitol. After the application of a linear 0.075-to-0.5 M potassium phosphate (pH 7.5) gradient in 10% glycerol–1 mM dithiothreitol, the fractions were assayed for DNA polymerase activity and analyzed by polyacrylamide gel electrophoresis.

Materials. Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. Lysozyme was purchased from Sigma Chemical Co.; calf liver tRNA was from Boehringer Mannheim Corp.; and *S. aureus* V8 protease was from Miles Laboratories. [^{35}S]methionine (600 Ci/mmol) was purchased from Amersham Corp. PAA was kindly supplied by A. O. Geiszler of Abbott Laboratories.

RESULTS

Marker rescue. Initial experiments were designed to determine whether the PAA^r marker could be rescued by adding DNA from PAA^r virus to cells infected with PAA^s virus. A calcium-precipitated mixture of intact PAA^r DNA and calf thymus DNA carrier was added to CV-1 cells at 2 h after infection with PAA^s virus, and the transfected cells were incubated in medium without the selective agent PAA for 48 h. The cells were then harvested, and PAA^r virus titers were measured in medium containing PAA. Spontaneous mutations of PAA^s virus produced a detectable level of PAA^r virus in the absence of added PAA^r DNA. However, the addition of intact PAA^r DNA resulted in nearly a 1,000-fold increase in PAA^r virus titer (Table 1). Similar transfections with PAA^r DNA that was totally digested with the restriction enzyme *Hin*dIII, however, gave marginal results and caused us to search for conditions which optimized rescue of the PAA^r marker from fragmented DNA. When 1 μg of PAA^s vaccinia DNA was coprecipitated with *Hin*dIII-digested PAA^r DNA fragments, the efficiency of marker rescue became equivalent to that observed with intact PAA^r DNA (Table 1). Presumably, the mixture of PAA^s DNA with PAA^r DNA fragments facilitated recombination. This enhancement by coprecipitation with intact viral DNA has been previously described with other markers (34; M. Ensinger, personal communication).

Analysis and cloning of PAA^r mutant DNA. Restriction endonuclease analysis of DNA extracted from the PAA^r mutant revealed that this virus contained a 9-kb deletion covering part of *Hin*dIII fragments C and N. This deletion occurs spontaneously in our virus stock (28, 31) and is unrelated to the PAA^r phenotype of the mutant virus. DNA extracted from several other independently isolated PAA^r mutants did not contain this deletion. To identify which of the 14 *Hin*dIII DNA fragments contained the PAA^r marker, all except the terminal *Hin*dIII fragments B and C were cloned in plasmid vectors for use in marker rescue experiments.

The *Hin*dIII map of the PAA^r mutant used in this study is shown in Fig. 1A and is derived from that of wild-type vaccinia virus (15, 39). The fragments D, E, F, H, I, J, K, L, M, and O from PAA^r DNA were cloned into pUC9, and *Hin*dIII-G was inserted into pBR322. *Hin*dIII-A of vaccinia virus is 45 kb and was not previously cloned in plasmids because of its size. The development of cosmid vectors suitable for replicating DNA inserts of 30 to 45 kb (19) suggested an alternative, and we succeeded in cloning *Hin*dIII-A in the cosmid vector pJB8.

Mapping the DNA polymerase gene with the PAA^r marker. The cloned PAA^r *Hin*dIII fragments were used to identify the one containing the drug resistance marker (Table 2). Transfections were done with 1 μg of intact PAA^r DNA or 2

TABLE 1. Conditions for rescue of the PAA^r marker by PAA^s virus

PAA^r DNA (μg)		PAA^s DNA Carrier (μg)	Titer	
Intact	*Hin*dIII Digest		Total virus (PFU/ml) (10^7)	PAA^r virus (PFU/ml) (10^3)
0	0	0	7.9	2.0
1	0	0	16.0	1,700
0	5	0	4.0	23
0	0	1	11.0	<1.0
1	0	1	4.8	1,900
0	5	1	7.2	1,700

μg of plasmid DNA containing the indicated *Hin*dIII fragment cloned from PAA^r virus. Of the 12 cloned fragments tested, only *Hin*dIII-E contained the PAA^r marker. Indeed, the efficiency of rescue with the *Hin*dIII-E plasmid equaled that observed with intact PAA^r DNA.

To further localize the position of the PAA^r marker, restriction mapping and subcloning of the 15-kb *Hin*dIII-E was performed. Total *Bam*HI digests of both PAA^r DNA and the cloned *Hin*dIII-E DNA were active in marker rescue (Table 3). The *Bam*HI restriction enzyme cleaves *Hin*dIII-E into three fragments of 7.5, 7, and 0.7 kb. These fragments were cloned into pUC9, and their orientation within *Hin*dIII-E was determined as shown in Fig. 1B. Only the 7.5-kb *Bam*HI-*Hin*dIII-A, which mapped on the right end of *Hin*dIII-E, rescued PAA^s virus in the presence of PAA (Fig. 1B, Table 3).

Detailed restriction endonuclease maps of the 7.5-kb *Bam*HI-*Hin*dIII-A segment permitted further localization of the PAA^r marker. Marker rescue was still obtained, using a total *Eco*RI digest of the 7.5-kb *Bam*HI-*Hin*dIII-A clone (Table 4). Seven DNA pieces ranging in size from 2.9 to 0.24 kb generated by *Eco*RI cleavage of *Bam*HI-*Hin*dIII-A were cloned in pUC9. Their arrangement within parental *Bam*HI-*Hin*dIII-A was determined by end labeling and partial endonuclease digestion (Fig. 1C). Only one DNA fragment, the

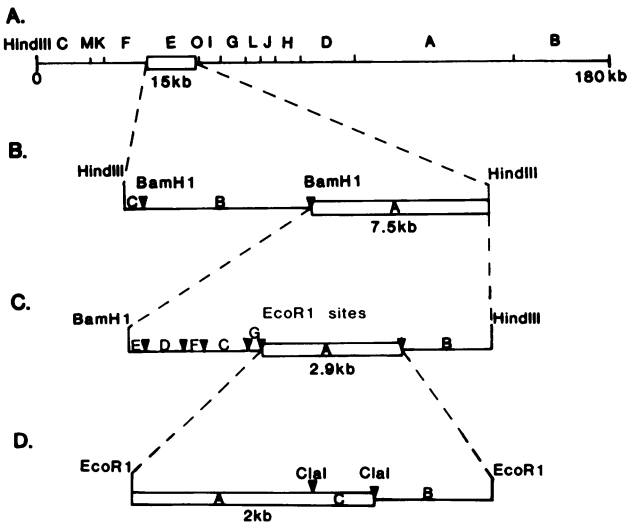

FIG. 1. Schematic diagram of the strategy used for marker rescue of PAA resistance. (A) *Hin*dIII restriction site map of the PAA^r vaccinia virus mutant genome. (B) *Bam*HI map of the 15-kb *Hin*dIII-E. (C) *Eco*RI restriction site map of the 7.5-kb *Bam*HI-*Hin*dIII-A. (D) *Cla*I map of the 2.9-kb *Eco*RI-A. Fragments active in the rescue of the PAA^r marker are indicated as open boxes.

TABLE 2. Rescue of PAA^r marker by PAA^s virus, using cloned *Hin*dIII PAA^r fragments

DNA	Titer	
	Total virus (PFU/ml) (10^8)	PAA^r virus (PFU/ml) (10^4)
None	4.6	2.1
Intact PAA^r	6.1	160
pUC9	5.2	3.2
p*Hin*dIII-A	2.2	3.2
p*Hin*dIII-D	5.0	3.5
p*Hin*dIII-E	6.4	170
p*Hin*dIII-F	4.9	1.7
p*Hin*dIII-G	5.0	2.4
p*Hin*dIII-H	6.9	4.1
p*Hin*dIII-I	4.7	2.8
p*Hin*dIII-J	4.5	2.7
p*Hin*dIII-K	4.5	4.4
p*Hin*dIII-L	3.9	5.1
p*Hin*dIII-M	5.3	2.7
p*Hin*dIII-O	4.2	4.0

2.9-kb *Eco*RI-A, contained the PAA^r marker (Table 4). The PAA^r mutation, then, lies between 1.8 and 4.7 kb from the right end of *Hin*dIII-E.

When the 2.9-kb *Eco*RI fragment was digested with the endonuclease *Cla*I, marker rescue was no longer detected, suggesting that the PAA^r mutation mapped very near to one of the *Cla*I sites (Table 5). *Cla*I cleaves *Eco*RI-A into three subfragments of approximately 1.5, 1.0, and 0.5 kb (Fig. 1D). As expected, pUC9 plasmids containing these fragments were not active in the marker rescue assay. The sensitive *Cla*I site was identified by cloning partially digested *Cla*I-*Eco*RI fragments and testing them. The *Cla*I-*Eco*RI-AC subclone contained the PAA^r marker (Fig. 1D and Table 5). The location of the sensitive *Cla*I site was confirmed by demonstrating that *Cla*I-*Eco*RI-AC lost its rescuable marker upon digestion with *Cla*I. In summary, the PAA^r marker was localized within 2.0 kb of vaccinia DNA, approximately 57 kb from the left end of the 180-kb viral genome (Fig. 1).

Cell-free translation of RNA selected by hybridization. A DNA polymerase with a single polypeptide of about 110K was previously isolated from vaccinia virus-infected cells (6). In agreement with these results, we found a major [^{35}S]methionine-labeled 100K polypeptide that coeluted with DNA polymerase activity (Fig. 2). The 100K polypeptide also cosedimented with active DNA polymerase upon glycerol gradient centrifugation (data not shown).

When RNA from cycloheximide-treated vaccinia virus-infected cells was hybridized to DNA fragments containing

TABLE 3. Marker rescue by PAA^s virus, using cloned subfragments of *Hin*dIII-E

DNA	Titer	
	Total virus (PFU/ml) (10^8)	PAA^r virus (PFU/ml) (10^4)
None	1.0	1.0
Intact PAA^r	3.3	400
p*Hin*dIII-E	3.1	130
p*Hin*dIII-E digested with *Bam*HI and *Hin*dIII	2.2	450
p*Hin*dIII-*Bam*HI-C	7.9	1.1
p*Bam*HI/*Bam*HI-B	1.5	1.1
p*Bam*HI/*Hin*dIII-A	3.9	536

TABLE 4. Marker rescue by PAA^s virus, using cloned subfragments of *Bam*HI-*Hin*dIII-A

DNA	Titer	
	Total virus (PFU/ml) (10^8)	PAA^r virus (PFU/ml) (10^4)
None	1.7	0.1
Intact PAA^r	2.7	108
p*Bam*HI-*Hin*dIII-A	1.0	97
p*Bam*HI-*Hin*dIII-A digested with *Eco*RI	1.6	90
p*Bam*HI-*Eco*RI-E	1.5	1.2
p*Eco*RI-*Eco*RI-C	1.3	0.6
p*Eco*RI-*Eco*RI-G	1.1	0.5
p*Eco*RI-*Eco*RI-D	1.1	1.1
p*Eco*RI-*Eco*RI-F	1.0	0.5
p*Eco*RI-*Eco*RI-A	1.0	51
p*Eco*RI-*Hin*dIII-B	0.7	0.7

the PAA^r marker and then translated in vitro, a polypeptide comigrating with authentic DNA polymerase was obtained (Fig. 3). Smaller polypeptides observed in the lysates programmed with selected mRNA could result from neighboring genes, premature termination of translation of the large DNA polymerase transcript, alternative initiation sites, or overlapping mRNAs. No difference in the polypeptide pattern was observed upon denaturation of the RNA by heating or with methyl mercury immediately before translation.

Peptide mapping. To establish the identity of in vitro-synthesized 100K polypeptide with purified vaccinia DNA polymerase, partial peptide analysis was performed by the method of Cleveland et al. (10). RNA hybrid selected to the 7.5-kb *Bam*HI-*Hin*dIII-A was translated in the cell-free system, and the products were electrophoresed in parallel with purified [^{35}S]methionine-labeled vaccinia DNA polymerase. Gel bands containing the 100K protein synthesized in vitro or purified as DNA polymerase from infected cells were excised and applied directly to a second acrylamide gel in the presence of various concentrations of *S. aureus* V8 protease. The partial peptide map of both proteins is shown in Fig. 4. As expected, protease digestion of this large protein generated numerous peptide fragments. Comparison of the digestion products of the authentic viral DNA polymerase and the cell-free synthesized product revealed identical peptide patterns at all protease concentrations. These results demonstrated that the protein synthesized in vitro is indeed the vaccinia DNA polymerase.

TABLE 5. Marker rescue by PAA^s virus, using cloned subfragments of *Eco*RI-*Eco*RI-A

DNA	Titer	
	Total virus (PFU/ml) (10^8)	PAA^r virus (PFU/ml) (10^4)
None	2.2	1.2
Intact PAA^r	2.7	62
p*Eco*RI-*Eco*RI-A	2.6	13
p*Eco*RI-*Eco*RI-A digested with *Cla*I	2.3	1.2
p*Eco*RI-*Cla*I-B	3.0	.41
p*Cla*I-*Cla*I-C	2.4	.74
p*Cla*I-*Eco*RI-A	3.6	.16
p*Eco*RI-*Cla*I-CB[a]	5.1	1.6
p*Eco*RI-*Cla*I-CB[a] digested with *Cla*I	5.6	.16
p*Cla*I-*Eco*RI-AC[a]	2.0	32
p*Cla*I-*Eco*RI-AC[a] digested with *Cla*I	4.1	.21

[a] *Eco*RI-*Cla*I fragment containing an uncleaved *Cla*I site was purified from a partial restriction endonuclease digest of *Eco*RI-*Eco*RI-A and cloned in pUC9.

DISCUSSION

In this study, we have mapped the vaccinia virus DNA polymerase gene by two independent methods. The first was based on the previous correlation of resistance to PAA by a mutant virus with an altered DNA polymerase (27). The PAA^r DNA polymerase gene provided a selectable marker for transfection experiments. A similar approach was previously used to locate the vaccinia virus thymidine kinase gene (36). Cells infected with PAA^s virus were transfected with cloned fragments of PAA^r DNA, and recombinants were scored by plaque assay in the presence of PAA. Typically, a 50- to 200-fold increase in PAA^r virus was obtained with appropriate cloned DNA fragments. Variations of this method in which PAA selection was performed during transfection were less reliable but gave similar results. By successively subcloning active fragments, PAA^r was located within a 2.9-kb *Eco*RI subfragment of *Hin*dIII-E. Further digestion with *Cla*I, however, led to loss of the PAA^r marker, suggesting that the mutation lay very close to one of the latter restriction sites. By testing cloned partial *Cla*I-*Eco*RI fragments, the sensitive *Cla*I site was identified. Sequencing around that site should reveal the mutated sequence within the DNA polymerase gene.

The DNA polymerase gene was mapped in an independent manner, using cell-free translation of vaccinia RNA selected

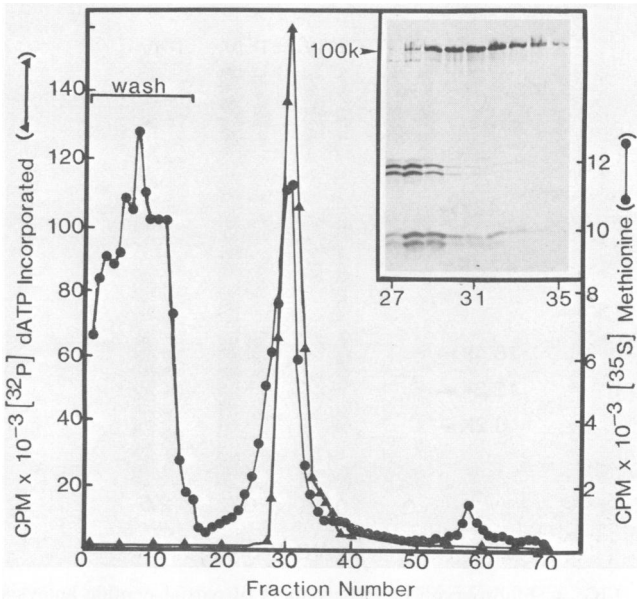

FIG. 2. Phosphocellulose chromatography of pooled [^{35}S]methionine-labeled vaccinia DNA polymerase fractions obtained after DEAE-cellulose chromatography of disrupted infected cells. Each fraction was assayed for DNA polymerase (▲) as described by Moss and Cooper (27) and for [^{35}S]methionine (●). Inset shows SDS-polyacrylamide gel electrophoresis of fractions 27 to 35, which include the peak of DNA polymerase activity.

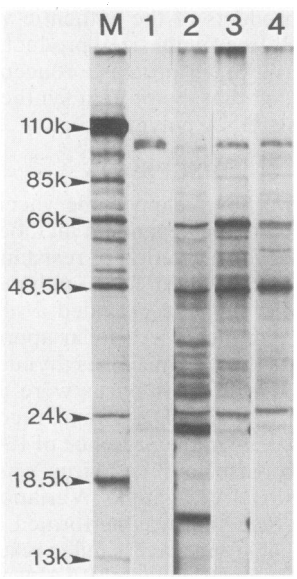

FIG. 3. Cell-free translation products of early mRNAs selected by hybridization to recombinant DNAs active in the marker rescue of PAA^r. Total cytoplasmic RNA was isolated from cells at 4 h after infection in the presence of cycloheximide, translated, and analyzed by electrophoresis on 15% polyacrylamide gels as described in the text. Lane M, [^{35}S]methionine-labeled adenovirus 5 structural proteins used as markers; lane 1, vaccinia DNA polymerase purified from infected cells; lanes 2, 3, and 4, translation products of mRNA selected by hybridization to 15-kb *Hin*dIII-E, 7.5-kb *Bam*HI-*Hin*dIII-A, and 2.9-kb *Eco*RI-A, respectively.

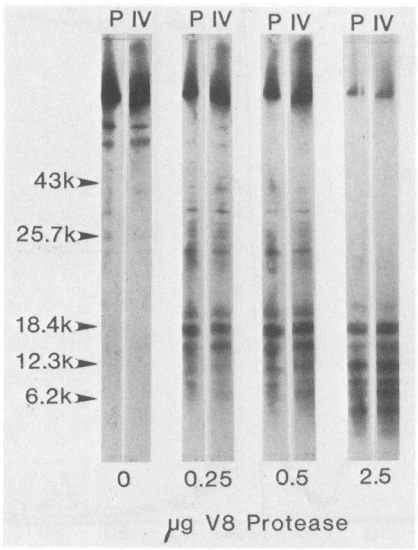

FIG. 4. Fluorograph of the products of partial peptide analysis (10) of purified vaccinia virus DNA polymerase (P) and the 100K protein synthesized in vitro (IV) from mRNA selected by hybridization to the 7.5-kb *Bam*HI-*Hin*dIII-A. DNA polymerase bands were excised from Coomassie blue-stained gels and electrophoresed on a second (20%) polyacrylamide gel with various concentrations of *S. aureus* V8 protease. The arrows indicate positions of molecular weight markers.

by hybridization to those regions of the viral genome active in marker rescue. Vaccinia RNA that hybridized to the 15-kb *Hin*dIII-E, the 7.5-kb *Bam*HI-*Hin*dIII, and the 2.9-kb *Eco*RI DNA fragments coded in vitro for a polypeptide which comigrated with the authentic viral DNA polymerase. Challberg and Englund (6) reported that the vaccinia DNA polymerase is composed of a single 110K polypeptide. Both the product synthesized in vitro and the purified in vivo DNA polymerase migrated as a 100K protein relative to our molecular weight standards. The identity of the in vitro and in vivo DNA polymerases was confirmed by partial peptide mapping. The similarity between the products of the DNA polymerase gene synthesized in vitro and the purified enzyme suggests that the latter undergoes little, if any, post-translational modifications.

Condit and Sridhar (personal communication) have used marker rescue to map a temperature-sensitive mutation within *Hin*dIII-E. This mutant does not synthesize DNA under nonpermissive conditions and is closely linked to a second (PAA^r) mutation. Thus, it seems likely that the temperature-sensitive mutation lies within the DNA polymerase gene.

The mapping of the DNA polymerase gene of vaccinia virus makes a variety of additional studies possible. By sequencing the gene, the primary structure may be determined and eventually compared with eucaryotic and other viral enzymes. Location of the PAA^r mutation may help to identify and understand the active site of the enzyme. Resistance to several drugs including PAA has been mapped within a limited region thought to contain the active site of the herpesvirus DNA polymerase gene (7, 23).

At least three temporal classes of poxvirus genes have been considered: immediate early genes expressed in the absence of de novo protein synthesis, delayed early genes expressed after synthesis of viral proteins but before DNA replication, and late genes expressed only after DNA replication. The majority of vaccinia virus polypeptides appear to be encoded by genes of the first and third classes (3, 12). We found in the present study that mRNA encoding DNA polymerase was synthesized in the presence of cycloheximide, suggesting that this gene falls into the immediate early class. However, previous in vivo experiments suggested that the DNA polymerase of rabbitpox, a virus very closely related to vaccinia, is regulated in a delayed early fashion (22). It is likely that the effects of virus multiplicity, cell type, and a stringency of inhibition of protein synthesis will have to be examined in a systematic fashion to further examine gene regulation. The localization of the DNA polymerase gene will allow the preparation of DNA probes for such studies.

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