

# Transcriptional Mapping of the DNA Polymerase Gene of Vaccinia Virus

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Vaccinia virus DNA polymerase, a single-subunit enzyme of 110,000 molecular weight, is induced early after infection. Genetic analysis suggests that the gene encoding the enzyme maps within a 15-kilobase *Hind*III fragment located 45 kilobases from the left-hand end of the genome. We identified the in vitro translation product with these properties and mapped the transcript by hybrid selection, RNA filter hybridization, and S1 nuclease mapping. Two mRNAs from this region, 3.4 and 3.9 kilobases in size, could be translated in vitro to yield a 110K polypeptide. The two RNAs shared a common 5' terminus and had staggered 3' ends. Sequences mapping entirely within this gene were shown to be biologically active in rescuing mutants with temperature-sensitive or drug-resistant polymerase activity to the wild-type phenotype.

Vaccinia virus, a poxvirus, is an extraordinarily large and complex virus. The virion is 200 nm in diameter, contains a genome of 190 kilobases (kb) of double-stranded DNA, and is thought to contain >100 different polypeptides. Upon entry into susceptible cells, the virus begins a rapid and productive infectious cycle which is unusually autonomous of host cell functions. Viral replication and transcription occur entirely within the cytoplasm, which is reorganized around the viral "factories." The cycle progresses through distinct stages from uncoating to assembly. It has been known for some years that virions specifically encapsidate a number of enzymatic activities required for the synthesis of mature RNA: nicking-closing activity, RNA polymerase, "capping enzyme," and poly A polymerase (for review see references 9 and 15). Moreover, during early infection, a number of DNA synthetic enzymes are induced: thymidine kinase, DNA polymerase, DNA ligase, etc. (9, 15). Recently, proof has been obtained that at least some of these enzymes are indeed virally encoded (11, 16, 22a, 25). The identification of more of the genes encoding these enzymes will facilitate the use of genetic and biochemical approaches to examine, dissect, and reconstitute the processes of replication and transcription.

Toward this end, we have been studying the viral DNA polymerase, which is comprised of a single polypeptide of 110,000 molecular weight. It is most active in vitro as a gap-filling enzyme and is similar in many respects to host alpha polymerases. However, by physical characteristics, optimization studies, and immunological evidence, the viral enzyme has been shown to be unique (5, 7, 8, 14).

With the known characteristics of the enzyme in mind, we sought to identify the homologous polypeptide among the products synthesized when RNA isolated soon after viral infection ("early RNA") was translated in a cell-free system. In addition to monitoring molecular weight and the kinetics of the polypeptide's appearance, we took advantage of the known affinity of the viral DNA polymerase for DNA coupled to a solid matrix (7). It has been shown (L. Cohen, personal communication; J. Morgan, A. Mahr, P. Traktman, and B. E. Roberts, manuscript in preparation) that in vitro

translation products often retain the affinity shown by their in vivo counterparts for such a matrix. Among the in vitro translation products directed by early RNA, two viral polypeptides with molecular weights in the 100,000 range were shown to have affinity for DNA-cellulose. To locate the viral sequences encoding these two polypeptides on the viral genome, fragments of vaccinia virus DNA were used to select their complementary RNAs (2, 3, 20). These RNAs were translated in vitro, and the cell-free products were chromatographed on DNA-cellulose. The polypeptides of 100,000 molecular weight with affinity for DNA were shown to be encoded by two viral fragments designated *Hind*III-E and -H. Simultaneously, viral mutants defective in DNA replication were isolated and, upon assaying extracts of infected cells, were shown to have altered DNA polymerase activity (16, 22a). These mutants were mapped by genetic analysis to the *Hind*III-E fragment (22a); this fragment was thus the focus of our analysis.

Within the *Hind*III-E fragment we have located and determined the organization of the transcripts encoding a 110K polypeptide with affinity for DNA-cellulose. These sequences have been shown to be sufficient to restore the wild-type phenotype when introduced by marker rescue into the genomes of viral mutants with altered polymerase activity. Hence, we feel we have precisely located the sequences encoding the DNA polymerase on the vaccinia virus genome.

## MATERIALS AND METHODS

**Cells and virus.** Vaccinia virus (WR strain) was a gift from J. Kates. Plaque-purified virus was propagated in mouse L cells, grown as monolayers in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum. For the purpose of preparing RNA from infected cells, monolayers were infected at a multiplicity of infection varying from 15 to 30 PFU per cell. For the purpose of isolating early RNA, infections were performed in the presence of cycloheximide at 100 µg/ml, as indicated. This treatment has been shown to elevate the levels of early viral RNAs without disturbing the pattern of transcription (for review, see reference 15).

**Isolation of RNA from infected cells.** Cultures were rinsed

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with ice-cold phosphate-buffered saline and then lysed in 4 M guanidinium thiocyanate at  $2 \times 10^7$  cells per ml. The extract was passed through a 23-gauge needle five times to reduce viscosity and then centrifuged through a cushion of 5.7 M CsCl. The RNA pellet was rinsed with ethanol and resuspended at 3 to 10 mg/ml in distilled water.

**Plasmids.** A library of *Hind*III fragments D to O of the vaccinia virus genome, cloned in pBR322, was kindly supplied by B. Moss (3). These plasmids and those constructed for the experiments reported here were grown in the C600 strain of *Escherichia coli* and purified by the alkaline lysis procedure (12).

**Marker rescue.** Marker rescue studies were performed as previously described (8a). Monolayers of BSC40 cells were infected at a multiplicity of infection of 0.3 PFU per cell with stocks of either *ts42* or NG26, viral mutants which have been previously described (8a, 22a). Shortly after infection, 10  $\mu$ g of the indicated plasmid DNA, digested with the appropriate restriction endonuclease to excise the vaccinia virus insert, was applied to the cells as a calcium phosphate precipitate. After 4 days of growth under nonpermissive conditions, the monolayers were processed in one of two ways. For the one-step assay (8a, 18), the monolayers were stained with crystal violet and evaluated qualitatively for evidence of widespread viral growth. For the two-step assay (25), the monolayers were scraped and prepared appropriately for titration in a plaque assay. The latter method yields a quantitative measure of the extent of viral growth during the marker rescue. Plaque assays of serially diluted stock were performed on BSC40 cells at 31 or 40°C; phosphonoacetic acid at 200  $\mu$ g/ml was sometimes present, as indicated.

**RNA filter hybridization.** RNA filters were prepared by two methods. In one, polyadenylated RNA was electrophoresed through an agarose gel in the presence of methyl mercuric hydroxide and then transferred to diazobenzylmethyl paper (1). The diazobenzylmethyl filter used in Fig. 3A to D was kindly provided by Jeffrey Morgan in our laboratory. In the second method, total RNA was electrophoresed through an agarose gel in the presence of formaldehyde and then transferred to nitrocellulose (22). Nick-translated probes were prepared as described before (21). Hybridization was performed at 42°C in 50% formamide.

**Hybrid selection.** DNA samples were denatured and immobilized on diazobenzylmethyl paper as previously described (2). Filters were hybridized with 40  $\mu$ g of total RNA for 3 to 4 h at 37°C in 50% formamide. After extensive washing, bound RNA was eluted in 90% formamide at 65°C, precipitated, and translated in a cell-free system as described below.

**S1 nuclease analysis.** A modification of S1 nuclease analysis (4, 24) was performed with fragments of DNA labeled at the 5' terminus with polynucleotide kinase in the presence of [ $\gamma$ -<sup>32</sup>P]ATP or at the 3' terminus by the Klenow fragment of DNA polymerase I in the presence of one or more  $\alpha$ -<sup>32</sup>P-labeled deoxynucleotide triphosphates. DNA was often subdigested with another restriction endonuclease to yield fragments radiolabeled at only one position. DNA was mixed with 35  $\mu$ g of total RNA, denatured, and then hybridized for 3 h at 42°C in 80% formamide. Samples were then diluted 10-fold into buffer containing S1 nuclease and digested for 30 min at 45°C. After precipitation, samples were analyzed on alkaline agarose gels.

**In vitro translation and polypeptide analysis.** RNA was translated in an mRNA-dependent reticulocyte system (19) (lysate was obtained from Green Hectares, Oregon, Wis.). [<sup>35</sup>S]methionine-labeled polypeptides were fractionated by

electrophoresis through 10% sodium dodecyl sulfate-polyacrylamide gels (13). Gels were prepared for fluorography and exposed to Kodak XAR-5 film at -70°C (6).

## RESULTS

**Localization of the sequences encoding the 110K polypeptide by hybrid selection.** The *Hind*III-E fragment is 15 kb in size and thus clearly contains sequences other than those within the DNA polymerase gene. Indeed, early RNA hybrid selected to *Hind*III-E directed the translation of four other major polypeptides (67K, 31K, 25K, and 19K; Fig. 1A and B). Mapping experiments were undertaken to localize the 110K polypeptide; a summary of the restriction mapping and the subcloning strategy is shown in Fig. 2. The right and left halves of the E fragment generated by digestion with *Bam*HI were subcloned into pBR322 and designated E1 and E2, respectively. Hybrid selection studies revealed that the 110K polypeptide mapped exclusively within E2, as shown in Fig. 1A and B (lanes 2 and 3). *Cla*I was found to cleave E2 into three fragments which were subcloned and designated E21, E22, and E23. All three subclones hybrid selected the mRNA for the 110K polypeptide (Fig. 1B, lanes 4, 5, 6). E23 also hybrid selected the mRNA for an abundant 67K protein. A fragment representing the right two-thirds of E23 (E23 R-Bg; see Fig. 2) was prepared and used for hybrid selection. This fragment did not select the mRNA for the 110K polypeptide, but only that for the 67K polypeptide (Fig. 1C). The data suggest that the 67K polypeptide is most likely the one previously reported and mapped to *Hind*III-E and the adjacent fragment, *Hind*III-O (3).

**Identification and mapping of transcripts by RNA filter hybridization.** A filter of early RNA probed with nick-translated E2 revealed three RNA bands: 3.9, 3.4, and 2.2 kb (Fig. 3A). The same filter hybridized with E21, E22, or E23 revealed the presence of the 3.9- and 3.4-kb bands (Fig. 3B, C, and D). Only an E23 probe revealed the 2.2-kb RNA, consistent with its being the mRNA for the 67K polypeptide. A filter probed with the *Eco*RI-*Bgl*II fragment of E23 (see Fig. 2) revealed the 2.2-kb RNA but no longer showed the 3.4- and 3.9-kb transcripts (Fig. 3E, lane 2), in keeping with the hybrid selection data described above. The 3.4- and 3.9-kb RNAs, mapping within E21, E22, and E23, showed the same arrangement as the 110K polypeptide, and presumably one or both serves as its mRNA. The data in Fig. 3E suggest that both of these RNAs terminate within 1,100 base pairs of the *Cla*I boundary of E23 (i.e., before the *Eco*RI restriction site). A filter probed with the *Bgl*II-*Hpa*II fragment of E21 (see Fig. 2) revealed the 3.9-kb but not the 3.4-kb RNA; apparently the 3.9-kb RNA extends to the left of the *Hpa*II site, but the 3.4-kb transcript terminates between the *Hpa*II site and the *Cla*I site (Fig. 3E, lane 3).

**Determination of transcript location and orientation by S1 nuclease analysis.** To further map the termini of the 3.4- and 3.9-kb messages, and to determine their orientation, S1 nuclease analysis was performed. All experiments used early RNA to protect fragments of DNA labeled at the 5' or 3' terminus on one strand. Protection of a fragment by the RNA indicated, first, that the radiolabeled strand was the template for transcription, defining the transcript's polarity. Second, the size of the protected fragment reflected the distance from the terminus of the RNA to the end label.

The scheme for the S1 nuclease analysis and selected data are presented in Fig. 4. To correlate the transcript orientation directly with the physical map of the vaccinia virus genome, we observed the following convention: the DNA

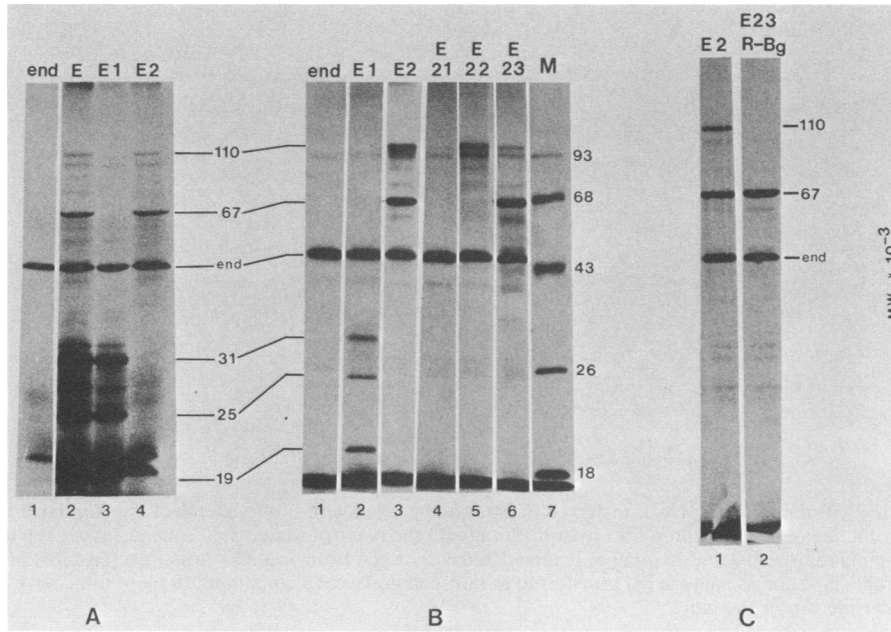


FIG. 1. Gel electrophoresis of the in vitro translation products of RNA hybrid selected to parts of the *Hind*III-E fragment. Early RNA from infected cells was hybrid selected to the clones or fragments listed at the top of each lane and then translated in a cell-free system. Lanes A1 and B1 represent the products of an in vitro translation reaction performed in the absence of exogenously added RNA. The polypeptide band labeled "end" is endogenous to all translation reactions. Lane B7 represents molecular weight standards. All designations and positions for the DNA fragments are described in the legend to Fig. 2.

strand whose 5' to 3' polarity is left to right on the physical map is designated "top," and its complement is designated "bottom." Since the 3.4- and 3.9-kb messages span all of E22 and extend into E21 and E23, only the latter clones were used to map the termini of the messages. RNA challenged with E23 labeled at the 5' position at the *Cla*I site (top strand) protected a single fragment of 570 bases (Fig. 4A, probe 2). When challenged with E21 labeled at the 3'

position at the *Cla*I site (the same strand as above), early RNA protected two fragments of 720 and 300 bases (Fig. 4B, probe 3). Likewise, by using a larger probe labeled at the 3' terminus on the same strand, shown as probe 4 in Fig. 4B, two fragments of 2.5 and 2.9 kb were protected. The conclusion (Fig. 4C) is that the two RNAs originate at a common 5' terminus in E23 and are transcribed leftward, terminating at staggered positions in E21. By this analysis,

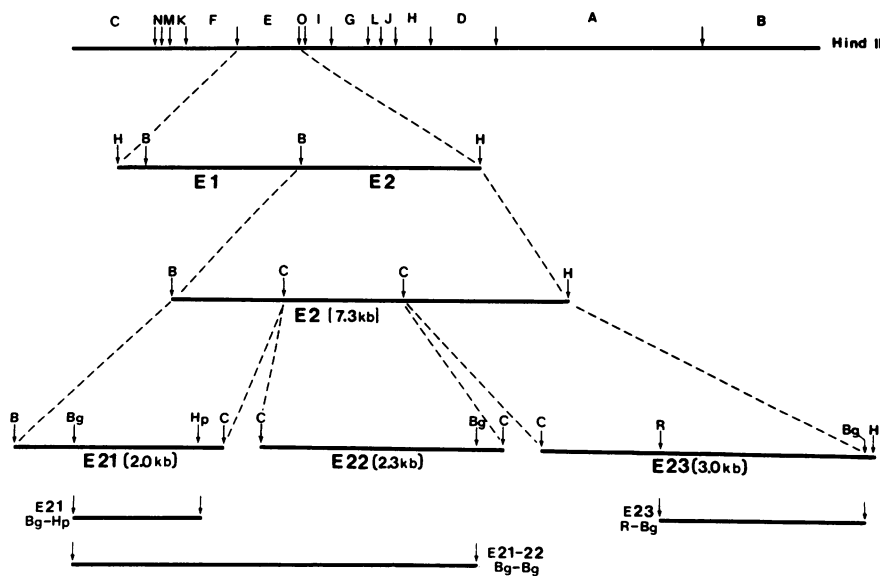


FIG. 2. *Hind*III restriction map of vaccinia virus genome and partial restriction map and subcloning strategy for the *Hind*III-E fragment. Only those sites within E2 referred to in the figures and text are shown. Subgenomic fragments shown at the bottom were prepared for use in experiments described in this study. Abbreviations: H, *Hind*III; B, *Bam*HI; C, *Cla*I; Bg, *Bgl*II; Hp, *Hpa*II, R, *Eco*RI.

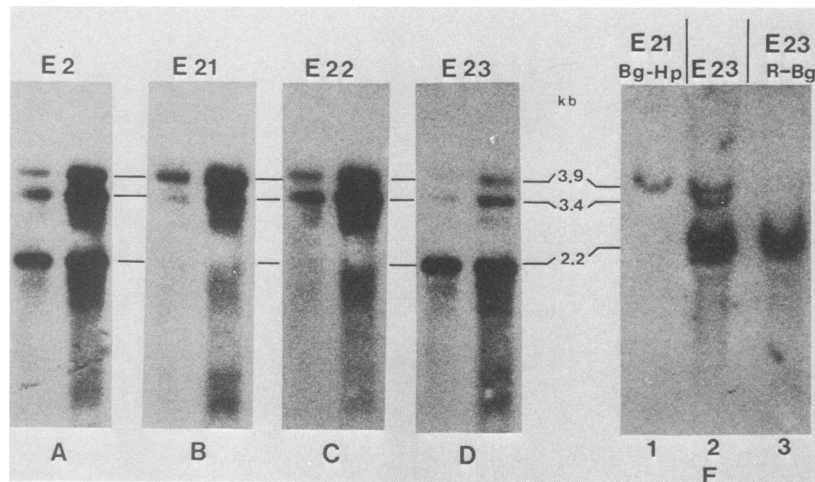


FIG. 3. Electrophoretically fractionated RNA, transferred to filters and probed with subfragments of the *HindIII*-E fragment. A, B, C, and D represent the same diazobenzoyloxymethyl filter after hybridization with the nick-translated probe named at the top of each panel. The first and second lanes in each panel represent 2 and 10  $\mu$ g of polyadenylated early RNA from vaccinia virus-infected cells, respectively. Lanes 1, 2, and 3 of (E) represent parallel lanes of an agarose gel transferred to nitrocellulose, each containing 10  $\mu$ g of total early RNA and each probed with the fragment shown at the top of the lane.

the calculated sizes of the RNA transcripts are 3.6 and 3.2 kb, which are within experimental error of the 3.9- and 3.4-kb estimates derived from filter hybridization studies.

An E21 fragment labeled at the 5' position at the *ClaI* site (bottom strand) did not yield any labeled, protected fragments when hybridized to early RNA (Fig. 4A, probe 1). Likewise, an E23 fragment labeled at the *ClaI* site at the 3' position (bottom strand) yielded no radiolabeled fragments (Fig. 4B, probe 5). Thus, the bottom strand is transcriptionally inactive in this region.

Finally, a fragment of E23 labeled at the *BglII* site at the 3' position (top strand) revealed an mRNA whose 3' terminus maps 1,600 bases from the *BglII* site. This must be the terminus of the 2.2-kb mRNA, which thus originates 475 bases into *HindIII*-O and terminates within E23. It is transcribed in the same direction as the 3.4- and 3.9-kb transcripts and terminates approximately 700 bases from their common initiation site.

**Identification of the mRNA for the 110K polypeptide.** To determine which of the 3.4- or 3.9-kb transcripts serves as the mRNA for the 110K polypeptide, early RNA was size fractionated on an agarose gel containing methyl mercuric hydroxide. Fractions of the gel containing RNA from 3 to 5 kb in size were kindly provided by Anna Mahr in our laboratory. RNA was extracted from these gel slices; three-quarters of the RNA in the various fractions was electrophoresed through an agarose gel in the presence of formaldehyde and transferred to nitrocellulose. The filter, when probed with E2, revealed that the 3.9- and 3.4-kb transcripts had been separated in the methyl mercury gel and so were present in different lanes of the filter (data not shown). The remaining quarter of each fractionated RNA sample was translated in a cell-free system, and the resulting polypeptides were examined on a sodium dodecyl sulfate-polyacrylamide gel (data not shown). RNA in fractions containing either the 3.9- or the 3.4-kb RNA directed the translation of the 110K polypeptide, suggesting that both transcripts may be authentic mRNAs.

**Confirmation of the 110K polypeptide as the vaccinia virus DNA polymerase.** To confirm that this 110K polypeptide, encoded by the 3.4- and 3.9-kb mRNAs, was indeed the DNA polymerase, marker rescue studies were undertaken (8a, 18, 25). Two previously described mutants were used (8a, 22a). NG26 is a double mutant, with two very closely linked lesions. One renders the virus temperature sensitive for DNA replication; the other confers resistance to phosphonoacetic acid. Phosphonoacetic acid has been previously shown to affect the viral DNA polymerase (16); assays of partially purified extracts from NG26-infected cells have shown that the polymerase activity in this mutant is both drug resistant and temperature sensitive (22a). *ts42* is another mutant with a temperature sensitivity in DNA replication; it is in the same complementation group as NG26, and both have been mapped to the *HindIII*-E fragment (8a, 22a).

Marker rescue of both mutants was attempted with the E2, E21, E22, and E23 plasmids. The data are shown in Table 1. As indicated, rescue with E2 was clearly successful with both mutants in a one-step assay. A two-step assay was performed to quantitate the rescue with each of the plasmids and to characterize the rescued virus. E2 and E22 were shown to rescue both mutants, yielding high titers of virus displaying wild-type behavior with respect to temperature and drug sensitivity (data for wild-type virus are not shown). The data in Table 1 indicate that neither E21 nor E23 can rescue these mutants. Since E22 lies entirely within the gene for the 110K polypeptide, it seems certain that the 110K polypeptide is the viral DNA polymerase.

The data in Table 1 also indicate that rescue with E22 is somewhat less efficient than with E2. Since marker rescue has been shown to involve the insertion of the added sequences by recombination into the infecting genome (18, 25), it follows that rescue frequency varies with the length of the DNA available for recombination. Since the 2.3-kb E22 sequence, within which the mutant lesions lie, is found in the middle of the 7.3-kb E2 fragment, there is greater likelihood that successful recombination between the termi-

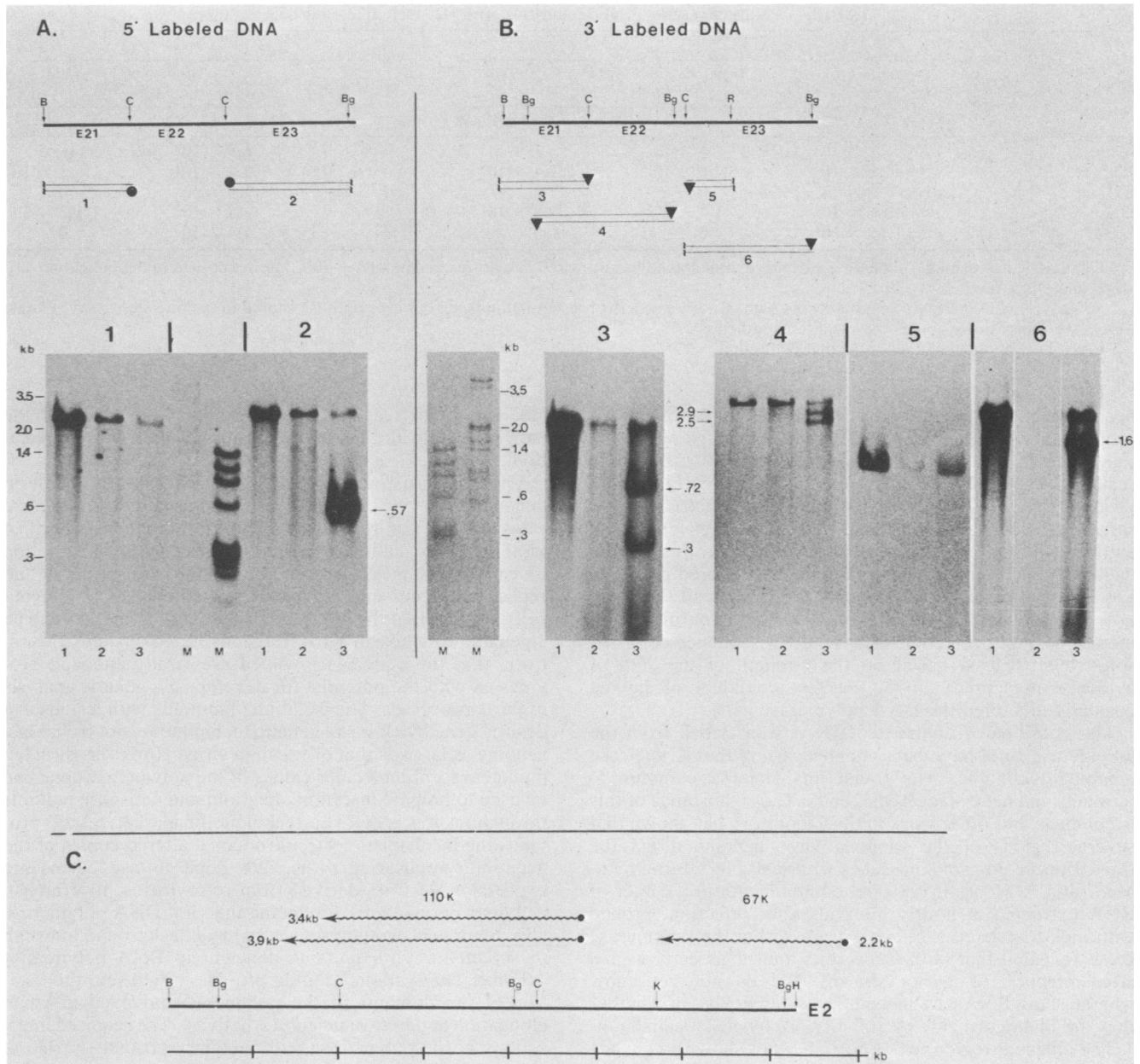


FIG. 4. S1 nuclease analysis of the transcripts mapping within E2. (A) Mapping the 5' termini of RNAs, using DNA fragments radiolabeled at the 5' position on one strand. Symbols: ●, position of the radiolabel; §, unlabeled terminus of the fragment generated by restriction endonuclease digestion. Probe 1, *Bam*HI-*Cl*aI fragment of E21; probe 2, *Cl*aI-*Bg*II fragment of E23. Autoradiogram represents alkaline agarose electrophoresis of products of S1 nuclease mapping. The DNA fragment used is indicated at the top of each panel. In each case, lane 1 represents the starting DNA fragment; lane 2, the DNA fragment alone after S1 nuclease treatment; and lane 3, the DNA fragment hybridized with early RNA, after S1 nuclease treatment. The molecular weight markers are the products of *Hae*III digestion of  $\phi$ X DNA and *Eco*RI and *Hind*III double digestion of  $\lambda$  DNA. (B) Mapping the 3' termini of RNAs, using DNA fragments radiolabeled at the 3' terminus. Symbols: ▼, position of the radiolabel; §, unlabeled terminus of the fragment generated by restriction endonuclease digestion. Probe 3, *Bam*HI-*Cl*aI fragment of E21 (hybridization of early RNA with probe 3 was performed at 34°C); probe 4, *Bg*II fragment of E2 (this fragment was not subdigested); probe 5, *Cl*aI-*Eco*RI fragment of E23; probe 6, *Cl*aI-*Bg*II fragment of E23. Autoradiogram represents alkaline agarose electrophoresis of the products of S1 nuclease mapping. All conventions are as described for (A). (C) Schematic drawing of arrangements of mRNAs within E2. For (A), (B), and (C), abbreviations are as follows: B, *Bam*HI; Bg, *Bg*II; C, *Cl*aI; H, *Hind*III; R, *Eco*RI.

nus of the added DNA fragment and the mutant lesion can occur when E2 is used. Moreover, since two recombinational events are required for successful marker rescue, the effect of decreasing fragment size on rescue efficiency will be especially pronounced.

## DISCUSSION

Vaccinia virus, a cytoplasmic DNA virus, has been shown to encode its own DNA polymerase. This enzyme, composed of a single subunit of 110,000 molecular weight, has

TABLE 1. Marker rescue of NG26 and *ts42* with E2, E21, E22, and E23

DNA	NG26				One step	<i>ts42</i>	
	One step <sup>a</sup>	Titration of 40°C yield <sup>b</sup> (PFU/ml)				Titration of 40°C yield (PFU/ml)	
		31°C	40°C	40°C + PAA <sup>c</sup>		31°C	40°C
None	–	8 × 10 <sup>2</sup>	1 × 10 <sup>3</sup>	1 × 10 <sup>3</sup>	–	6.8 × 10 <sup>3</sup>	0
E2	+	8 × 10 <sup>8</sup>	4.6 × 10 <sup>7</sup>	2.4 × 10 <sup>5</sup>	+	2.4 × 10 <sup>7</sup>	1 × 10 <sup>7</sup>
E21		2 × 10 <sup>2</sup>	0	0		1.4 × 10 <sup>3</sup>	0
E22		6 × 10 <sup>6</sup>	1.4 × 10 <sup>5</sup>	9.8 × 10 <sup>3</sup>		8 × 10 <sup>5</sup>	1.2 × 10 <sup>5</sup>
E23		8 × 10 <sup>2</sup>	0	0		1.4 × 10 <sup>2</sup>	0

<sup>a</sup> Qualitative assessment of whether extensive viral spread has occurred in cultures maintained at 40°C after exposure to the indicated viral stock and DNA fragment.

<sup>b</sup> As described in the text, this represents a quantitation of virus present in duplicate cultures of those scored in the one-step assay. Plaque assays were performed under the conditions indicated.

<sup>c</sup> PAA, Phosphonoacetic acid, used at 200 µg/ml.

been previously purified and characterized. In this paper we propose that the gene encoding the enzyme maps within the right half of the *HindIII*-E fragment. Two mRNAs from this region, 3.4 and 3.9 kb in size, can be translated in vitro to yield a 110K polypeptide with affinity for DNA. Marker rescue studies have confirmed that sequences contained entirely within these transcripts are sufficient to restore the wild-type phenotype to viral mutants with altered polymerase activity. We acknowledge that the identity of the 110K polypeptide encoded within *HindIII*-E and the purified polymerase has not been confirmed by direct sequence comparison. Nevertheless, based on the strength of the sum of evidence presented above, we feel confident of having correctly identified the DNA polymerase gene.

The coordinates of the two RNAs transcribed from the polymerase gene have been mapped, using the S1 nuclease mapping technique. The transcripts share a common 5' terminus and have staggered 3' ends. The significance of this is not clear, but other work in this laboratory has shown that several regions of the vaccinia virus genome direct the transcription of nested messages with multiple 3' termini (A. Mahr and J. Morgan, personal communication). A filter of RNA extracted at hourly intervals after infection, probed with nick-translated sequences from within the polymerase gene, revealed that both transcripts appear as early as 1 h after infection (data not shown). We do not yet know whether both RNAs are indeed translated in vivo or whether they are distinguishable by stability or physical localization.

The adjacent regions of *HindIII*-E were shown to encode the transcripts for at least four other early polypeptides and three late polypeptides (data not shown). Transcriptional mapping of these genes, and some effort to characterize the polypeptides they encode, will be undertaken. It will be of interest to determine whether the genes flanking the DNA polymerase gene also encode proteins involved in DNA replication.

The mapping of the viral DNA polymerase, surely an essential function for viral replication, opens a variety of possibilities. We hope to examine the 5'- and 3'-terminal regions of the gene to distinguish promoter and terminator regions and to see whether expression of the gene can be altered by manipulating these sequences. Although the features distinguishing the early and late genes of vaccinia virus are not yet understood, it seems likely that some insight will be gained from examining mRNA structure. Furthermore, it is now possible to insert the coding sequences of the gene into a procaryotic expression vector and thus to obtain sufficient material to prepare polymerase-specific antibodies

(10, 23). Such reagents would facilitate the study of the enzyme within the infected cell during viral DNA replication.

Vaccinia virus, and poxviruses in general, are unique in encoding a broad spectrum of enzymes involved in viral transcription and replication. The knowledge that purified viral cores are sufficient to direct the accurate transcription of early viral messages, and that viral transcription and replication occur entirely within the cytoplasm of infected cells, implies that the biochemistry of these processes will be amenable to study in vitro in reconstituted systems. Moreover, that the enzymes involved are virally encoded provides us with the potential for developing a genetic analysis of these processes. The isolation of mutants with lesions in a desired gene by classical genetic techniques is not trivial in a genome as large as that of vaccinia virus. However, directed mutagenesis of molecular clones of the polymerase gene can be used to prepare insertion, deletion, and nonsense mutants throughout the gene. The technique of marker rescue provides the mechanism for reintroducing altered copies of the gene into replicating virus. We hope to use eucaryotic expression vectors, derived from retroviruses, to establish cell lines permanently producing the viral DNA polymerase (17). Such lines would provide an ideal background in which to screen for viral mutants deficient in DNA polymerase activity. These studies should provide insight into the location of the domains of the polymerase involved in chain elongation and in exonucleolytic activity. The required interactions of the polymerase with auxiliary proteins is also of interest. In the future, when in vitro replication systems may well have been developed, the availability of viral mutants with a spectrum of lesions in the DNA polymerase will be a strong asset. Analysis of the polymerase by these diverse methods should provide a unique opportunity to study the structure and function of a DNA polymerase capable of replicating linear duplexes in eucaryotic cells. Furthermore, these studies may help to define the intimate relationship between the initiation of viral DNA replication and the dramatic shift from early to late gene expression that characterizes vaccinia infection.

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