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Transcriptional Mapping of the Vaccinia Virus DNA Polymerase Gene

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Transcriptional analysis of the vaccinia virus DNA polymerase gene revealed the presence of overlapping RNAs. Cloned DNA fragments, previously shown to lie within the DNA polymerase gene (E. V. Jones and B. Moss, J. Virol. 49:72–77, 1984) hybridized to early RNA species of ca. 3,400 and 3,700 nucleotides. Nuclease S1 analysis was used to determine the direction of transcription and more precisely map the mRNAs. A single 5′ end and two major and one minor 3′ ends were detected.

The ability of vaccinia virus to replicate within the cytoplasm of eucaryotic cells is correlated with synthesis of a new DNA polymerase, ca. 110,000 daltons in size (2–4). Evidence that this enzyme is virus encoded was obtained by isolation of phosphonoacetate-resistant (PAA^r) and temperature-sensitive DNA polymerases from cells infected with mutant viruses (14, 15). PAA resistance and temperature sensitivity were used in marker rescue experiments to map the vaccinia virus DNA polymerase within the 15-kilobase (kb) fragment *Hin*dIII-E (9, 15). Successive subcloning of fragments active in the marker rescue assay mapped the PAA^r locus within a 2.0-kb *Eco*RI-*Cla*I subfragment of *Hin*dIII-E (9). The location of the DNA polymerase gene was confirmed by cell-free translation of mRNA selected by hybridization to plasmids containing regions of PAA^r vaccinia DNA active in marker rescue (9, 17). Electrophoretic comigration with purified DNA polymerase and partial peptide analysis unambiguously identified the in vitro-synthesized polypeptide (9). As a continuation of these studies, we characterized the transcription products of the DNA polymerase gene and mapped their 5′ and 3′ ends. Work of a similar and consistent nature has recently been described by Traktman et al. (17).

A restriction endonuclease map of the 7.5-kb *Bam*HI-*Hin*dIII fragment which includes the PAA^r locus in shown in Fig. 1A. The 2.0-kb boxed segment, obtained by subcloning a partial *Cla*I digest of the 2.9-kb fragment *Eco*RI-A, includes the DNA polymerase gene as identified by marker rescue of PAA resistance and by cell-free translation of selected mRNA (9). The PAA^r locus must be very close to the internal *Cla*I site of the 2.0-kb fragment, since marker rescue activity was lost upon complete *Cla*I digestion (9).

Hybridization of vaccinia early RNA to ^{32}P-labeled recombinant plasmid containing fragment *Eco*RI-A was used to determine the size of the DNA polymerase messenger species. Purified cytoplasmic RNA from cycloheximide-treated HeLa cells infected with vaccinia virus was denatured with glyoxal and dimethyl sulfoxide, electrophoretically separated, and transferred to nitrocellulose according to the method of Thomas (16). After hybridization to the ^{32}P-labeled plasmid DNA containing the 2.9-kb fragment *Eco*RI-A, two RNA species, ca. 3.7 and 3.4 kb in size, were detected (Fig. 1B, lane A). The adjacent right 1.8-kb fragment *Hin*dIII-*Eco*RI-B hybridized to a 2.0-kb species of vaccinia virus RNA but not to either the 3.4- or 3.7-kb species (Fig. 1B, lane B). However, RNAs of the latter size hybridized to cloned fragments *Eco*RI-G and -C lying left of *Eco*RI-A (Fig. 1, lanes G and C). The 3.7-kb species extended into fragment *Eco*RI-F and possibly into fragment D, which also encoded other RNAs (Fig. 1B, lanes F and D). Thus, there appeared to be two overlapping RNAs produced by transcription of the DNA polymerase gene.

To determine the direction of transcription and accurately locate the 5′ termini of the DNA polymerase mRNAs, nuclease S1 analysis was performed by using purified restriction fragments labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase. First, a 2.0-kb *Bam*HI-*Cla*I fragment from the left portion of the 7.5-kb *Bam*HI-*Hin*dIII segment was 5′ end labeled at the *Cla*I site and used to probe for mRNAs reading left to right (Fig. 2A). When early vaccinia RNA was hybridized to this purified fragment, no protected species were detected (data not shown). To probe for mRNA species reading right to left, the *Cla*I site of the 3.0-kb *Cla*I-*Hin*dIII restriction fragment from the right side of the 7.5-kb *Bam*HI-*Hin*dIII DNA segment was 5′ labeled (Fig. 2A). Controls indicated that no specific bands were resolved when vaccinia virus RNA was absent from the hybridization reaction. However, when early RNA made in the presence of cycloheximide was used, a single protected band of 620 base pairs (bp) was detected (Fig. 2B, lanes 3 and 4). Similar results were observed when a smaller 1.0-kb *Cla*I-*Eco*RI restriction fragment, 5′ end labeled at the same *Cla*I site, was used for hybridization (data not shown). These data define the direction of transcription and suggest that the 5′ ends of both overlapping RNAs map ca. 620 bp upstream of the *Cla*I site.

Mapping of the 3′ termini of the DNA polymerase transcripts was also performed by S1 nuclease analysis. A 2.0-kb *Bam*HI-*Cla*I restriction fragment, isolated from the left end of the putative DNA polymerase-coding region, was 3′ end labeled at the *Cla*I site with [α-^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase (Fig. 3A). When this 3′-end-labeled *Bam*HI-*Cla*I fragment was hybridized to early vaccinia RNA, two major bands of ca. 310 and 780 bp and a minor species of 1,040 bp were protected from S1 nuclease digestion. No protected bands were observed when RNA was omitted (Fig. 3B, lanes 2 and 9). Under conditions where the DNA:RNA hybridization was performed with an excess of RNA, the 780-bp band was predominant (Fig. 3B, lane 3). By contrast, as the ratio of

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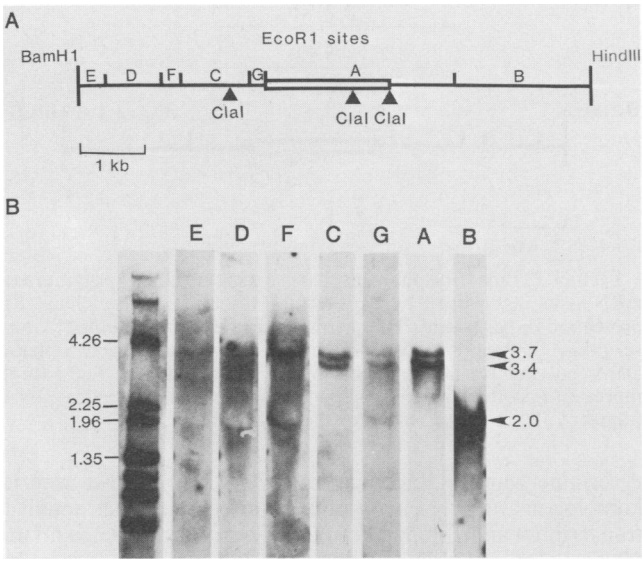

FIG. 1. (A) Restriction endonuclease map of the 7.5-kb *Bam*HI-*Hin*dIII fragment containing the DNA polymerase gene. The open box indicates the smallest DNA fragment active in the rescue of the PAA^r marker, the 2.0-kb *Eco*RI-*Cla*I fragment (9). (B) Autoradiogram of Northern blot of total electrophoretically separated early vaccinia RNA hybridized to *Eco*RI subclones spanning the 7.5-kb *Bam*HI-*Hin*dIII fragment. HeLa cells were suspended at 5×10^6 cells per ml in Eagle medium containing 5% horse serum and 100 μg of cycloheximide per ml. After 10 min at 37°C, 30 PFU of purified vaccinia virus (strain WR) per cell was added. After a 30-min incubation, the infected cells were diluted 10-fold in medium containing cycloheximide. Total cytoplasmic RNA isolated at 4 h was purified by sedimentation through CsCl (5), denatured with glyoxal and dimethyl sulfoxide, electrophoretically separated on 1.1% agarose gels, and transferred to nitrocellulose filters (16). The seven *Eco*RI subclones (labeled A through G in order of decreasing size) were ^{32}P labeled in vitro and hybridized at 42°C to the RNA blots in the presence of 50% formamide–0.75 M NaCl–0.075 M sodium citrate–1× Denhardt solution (13)–50 mM sodium phosphate (pH 6.8)–250 μg of salmon sperm DNA per ml. The unmarked lane shows the migration of ^{32}P-labeled *Hin*dIII fragments of λ DNA in kb.

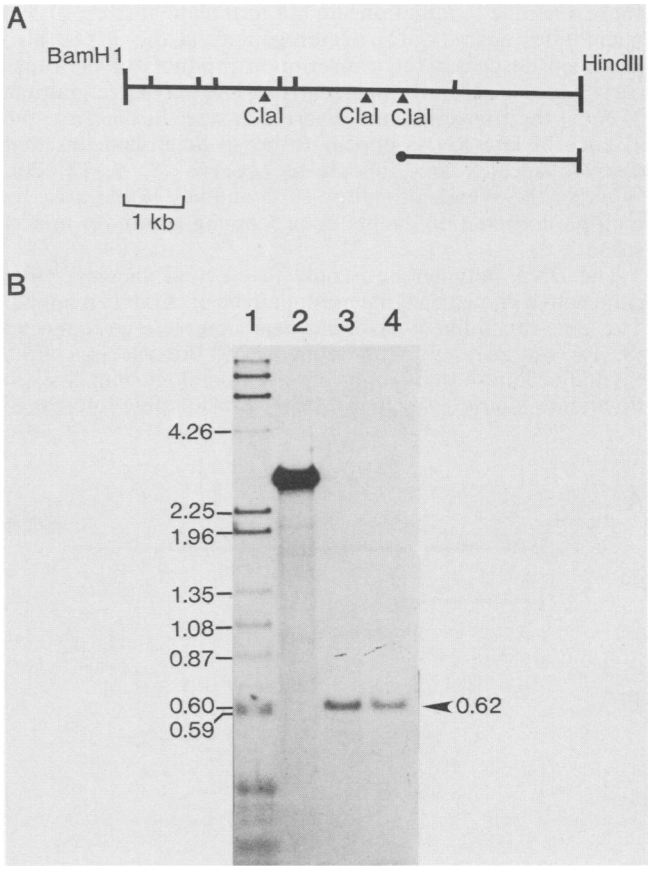

FIG. 2. Mapping of the 5′ end of DNA polymerase mRNA by nuclease S1 protection. (A) Schematic diagram defining the 5′-end-labeled probe. The plasmid containing the 7.5-kb *Bam*HI-*Hin*dIII fragment was cleaved with *Cla*I and 5′ end labeled by using [γ-^{32}P]dATP and T4 polynucleotide kinase. After secondary cleavage with *Hin*dIII, a 3.0-kb *Cla*I-*Hin*dIII fragment, 5′ labeled at the *Cla*I site, was purified. (B) Autoradiogram of electrophoretically separated fragments protected from nuclease S1 digestion after hybridization to the 5′-end-labeled *Cla*I-*Hin*dIII probe. RNA isolated from cycloheximide-treated vaccinia-infected HeLa cells was mixed with the 5′-end-labeled probe in the presence of 80% formamide–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.4 M NaCl and heated to 70°C for 10 min before hybridization for 3 h at 42°C. Single-stranded DNA was digested with nuclease S1 (60 U) at room temperature for 60 min. After several ethanol precipitations, the RNA-DNA hybrids were resolved by electrophoresis on nondenaturing 1.5% agarose gels. ^{32}P-labeled *Hin*dIII fragments of λ DNA and *Hae*III fragments of φX174 DNA in kb (lane 1), intact 3.0-kb *Cla*I-*Hin*dIII 5′-end-labeled probe (lane 2), and nuclease S1-resistant products with 1× (lane 3) and 3.3× (lane 4) concentrations of the probe hybridized to 17 μg of immediate early RNA.

DNA to RNA was increased, the lower band became more abundant (Fig. 3B, lanes 4, 7, and 8). Evidently, the longer RNA competes with or displaces the smaller one when DNA is limiting. A similar phenomenon was described previously (21). In addition to a minor 1,040-nucleotide band, a small amount of fully protected probe was present (Fig. 3B, lanes 7 and 8). This could result from either self-annealing of the probe or the presence of still longer RNA species.

A summary of the transcriptional analysis of the DNA polymerase gene is shown in Fig. 4. To code for a polypeptide of 110,000 daltons, a mRNA of ca. 2,900 nucleotides or more is required. Based on transcriptional mapping of the 5′ and 3′ ends by S1 nuclease analysis, two major mRNA species of 3,450 and 3,900 nucleotides and a minor one of 4,160 nucleotides were derived. These values for the major species are in good agreement with the 3,400- and 3,700-bp mRNAs observed by Northern blot analysis. The S1 data also correctly predict termination of the smaller mRNA within fragment *Eco*RI-C and of the larger mRNA species near the junction of fragments *Eco*RI-F and -D. Although it would be difficult to resolve the putative minor 4,160-nucleotide RNA species by blotting, cell-free translation experiments demonstrated that mRNA encoding a polypeptide with an M_r of 100,000 is hybrid selected by *Eco*RI-D and even fragment *Bam*HI-*Eco*RI-E (data not shown). The latter suggests that there may even be longer, minor RNA species contributing to protection of the full-length probe observed in Fig. 3. While this work was in progress, Traktman and co-workers (17) reported mRNAs of 3,400 and 3,900 nucleotides corresponding to the two major transcripts of 3,400 and 3,700 nucleotides described here.

The heterogeneity observed at the 3′ ends of the vaccinia virus DNA polymerase transcripts is not unique. The thymidine kinase of vaccinia is encoded by two mRNAs which

share a unique 5′ initiation site but terminate at sites ~1,790 nucleotides apart (1, 11). Heterogeneity at the 3′ end also has been described for transcription products of the other early genes (7, 18). Although early RNAs may have multiple 3′ ends, the transcripts are discrete in size. In contrast, the 3′ ends of late RNAs appear to be so heterogeneous that discrete species are difficult to observe (5, 6, 12, 20). Whether the 3′ ends of mature early and late RNAs arise by multiple termination events or processing is not yet understood.

The DNA polymerase is only the second vaccinia virus gene with a known enzyme product to be accurately mapped. The first, thymidine kinase, has been completely sequenced (8, 19). Interestingly, the sequence of the vaccinia virus thymidine kinase shows considerable homology with chicken thymidine kinase (10). It will be of considerable interest to determine whether vaccinia virus DNA polymerase also is homologous to its eucaryotic counterpart. The detailed transcriptional mapping of the DNA polymerase gene should facilitate future studies of this kind.

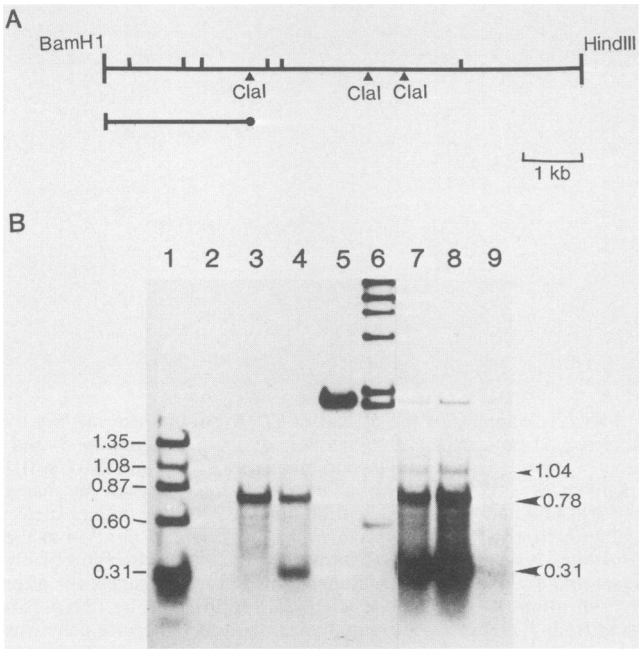

FIG. 3. Mapping of the 3′ ends of DNA polymerase mRNAs by nuclease S1 protection. (A) Illustration of the probe labeled at its 3′ terminus. Plasmid DNA containing the 7.5-kb *Bam*HI-*Hin*dIII fragment was cleaved with *Cla*I. The DNA was 3′ end labeled by using the Klenow fragment of *E. coli* DNA polymerase to fill in the recessed end with [α-^{32}P]dGTP. After cleavage with *Bam*HI, a 2.0-kb fragment from the left end of the 7.5-kb *Bam*HI-*Hin*dIII DNA, 3′ end labeled at the *Cla*I site, was purified. (B) Autoradiogram of electrophoretically separated nuclease-resistant products after hybridization of the 3′-end-labeled *Bam*HI-*Cla*I probe to early vaccinia RNA. RNA isolated from cycloheximide-treated vaccinia-infected HeLa cells was hybridized to the probe as previously described. Single-stranded DNA was digested with nuclease S1 (20 U), and the resistant hybrids were analyzed on nondenaturing 1.5% agarose gels. Shown are ^{32}P-labeled *Hae*III fragments of ϕX174 DNA in kb (lane 1), nuclease S1-resistant material after mock hybridization with 1× concentration of the probe in the absence of RNA (lane 2), nuclease S1-resistant products with 1× concentration of the probe hybridized to 26 μg (lane 3) and 13 μg (lane 4) of immediate early RNA, intact 2.0-kb *Bam*HI-*Cla*I probe (lane 5), ^{32}P-labeled *Hin*dIII fragments of λ DNA (lane 6), nuclease S1-resistant products with 2.5× concentration of the probe hybridized to 13 μg (lane 7) and 26 μg (lane 8) of immediate early RNA, and nuclease S1-resistant material after mock hybridization of the 2.5× concentration of probe in the absence of RNA (lane 9).

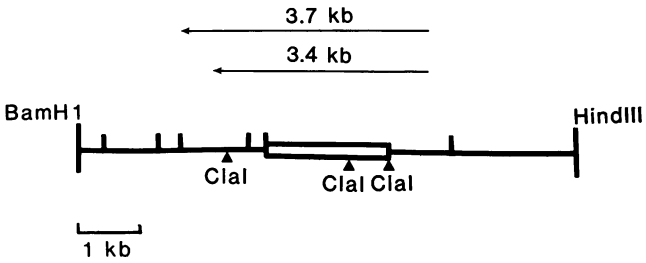

FIG. 4. Transcriptional map of vaccinia DNA polymerase mRNAs as determined by Northern blot analysis and nuclease S1 protection experiments. The arrows indicate the direction of transcription, the length in kb, and the map positions of the two major DNA polymerase RNA species. The short vertical lines (top) represent *Eco*RI sites, and the open box indicates the smallest DNA fragment active in the rescue of the PAA^r marker.

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