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Organization of RNA Transcripts from a Vaccinia Virus Early Gene Cluster

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The detailed organization of the RNAs transcribed from an early gene cluster encoded by vaccinia virus has been determined from the information derived from several complementary techniques. These include hybrid selection coupled with cell-free translation to locate DNA sequences complementary to mRNAs encoding specific polypeptides; RNA filter hybridization to size and locate on the DNA mature RNAs as well as higher-molecular-weight RNAs; S1 nuclease mapping to precisely locate the 5′ and 3′ ends of the RNAs; S1 nuclease mapping to precisely locate the 5′ and 3′ ends of the RNAs; and fractionation of hybrid-selected mRNAs in an agarose gel containing methyl mercury hydroxide followed by the cell-free translation of these mRNAs to definitively ascertain the size of the mRNA encoding each polypeptide. The early gene cluster is located between 21 and 26 kilobases from the left end of the vaccinia virus genome and is encoded by a 5.0-kilobase *Eco*RI fragment which spans the *Hin*dIII-N, -M, and -K fragments. Transcribed towards the left terminus are four mature mRNAs, 1,450, 950, 780, and 400 nucleotides in size, encoding polypeptides of 55, 30, 20, and 10 kilodaltons, respectively. These mRNAs are colinear with the DNA template and are closely spaced such that the 5′ terminus of one mRNA is within 50 base pairs of the 3′ terminus of the adjacent RNA. In addition to the mature size mRNAs, there are higher-molecular-weight RNAs, 5,000, 3,300, 2,350, 2,300, 1,800, 1,700, and 1,350 nucleotides in size. The 5′ and 3′ termini of the high-molecular-weight RNAs are coterminal with the 5′ and 3′ termini of the mature size mRNA. The implications of this arrangement and the biogenesis of these early mRNAs are discussed.

Vaccinia virus is a member of the Poxviridae, the largest and most complex of the DNA virus groups. The poxviruses are distinguished from the other DNA viruses by the fact that their transcription and replication occur in the cytoplasm of the infected cell. This autonomy from the host cell nucleus requires that vaccinia virus encode the entire complement of enzymes required for both viral RNA transcription and DNA replication. In fact, purified virions are capable of synthesizing translatable viral mRNAs (8, 30), thus demonstrating that the functions essential for RNA synthesis and processing are also packaged within the virus particle.

It is therefore appropriate that the virus particle contain a large double-stranded DNA genome of approximately 200 kilobases (kb) which has the potential for encoding 150 polypeptides with an average molecular weight of 50,000 (24). As in the other DNA virus groups, papovavirus, adenovirus, and herpesvirus, vaccinia virus gene expression is divided into two temporally distinct phases separated by viral DNA replication. Those viral genes synthesized before viral DNA replication are referred to as early genes, and those genes whose transcription requires the onset of DNA replication are referred to as late genes (12, 25, 33). Coincident with the onset of viral gene expression is the rapid inhibition of cellular protein synthesis and its replacement by the exclusive synthesis of viral polypeptides (23, 35).

Recently, two groups have demonstrated the potential for the use of vaccinia virus as a vector for the expression of disease-related antigens and the use of these recombinant viruses as live vaccines. The DNA sequences encoding either the hepatitis B surface antigen or the influenza cell surface antigen were ligated to a vaccinia virus-specific promoter element, and the chimeric transcriptional units were introduced into viable vaccinia viruses. The recombinant viruses were capable of expressing either antigen at levels which elicited a protective immunological response in animals (28, 38).

As a prelude to understanding the biogenesis of early mRNAs and the regulation of their synthesis during the infectious cycle, we have determined the organization of the early transcripts from a 5.0-kb fragment of vaccinia virus DNA encoding early mRNAs. In conjunction with other studies on vaccinia virus transcription (5, 10, 11, 14, 20, 44, 45), it will eventually be possible to understand the pattern of transcription of the entire vaccinia virus genome. Moreover, the information derived from the organization of these early mRNAs will facilitate the construction of chimeric transcriptional units capable of mediating the expression of foreign genes by vaccinia virus.

Encoded on the same DNA strand of the 5.0-kb fragment is a cluster of four closely spaced early mRNAs that encode polypeptides which are 55,000 (55K), 30K, 20K, and 10K daltons in size. These early mRNAs are colinear with their DNA template and coterminal with additional higher-molecular-weight RNAs. The implications of these higher-molecular-weight RNAs in regard to the biogenesis of the mature mRNAs are discussed.

MATERIALS AND METHODS

Cell culture and virus. Vaccinia virus (W.R. strain) was a gift from Joseph Kates and was plaque purified twice in our laboratory. All experiments were carried out with mouse L-cells propagated as monolayers in Dulbecco modified Eagle medium supplemented with 5% calf serum. L-cells were infected with vaccinia virus at a multiplicity of infection equal to 30 in Dulbecco modified Eagle medium supplemented with 5% calf serum.

Isolation of RNA from infected cells. Total cytoplasmic

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RNA from vaccinia virus-infected L-cells was prepared as described previously (34). Early vaccinia virus RNA was isolated from L-cells infected in the presence of 100 μg of cycloheximide per ml. The cells were pretreated with cycloheximide for 30 min before the infection, and the RNA was isolated at 2 h postinfection. Polyadenylated [poly(A^+)] RNA was prepared by the method of Aviv and Leder (3) by enrichment with oligodeoxythymidylate-cellulose (Collaborative Research, Inc.) chromatography.

Translation and polypeptide analysis. RNA was translated in the message-dependent reticulocyte system (New England Nuclear) as described before (31). Polypeptides were fractionated by electrophoresis in sodium dodecyl sulfate (SDS)–17.5% polyacrylamide gels (18). Fluorography was carried out by the method of Bonner and Laskey (7), and the dried gels were exposed to Kodak XAR-5 film at −80°C.

Hybrid selection of RNA by DNA immobilized on DBM paper. DNA was immobilized on diazobenzyloxymethyl (DBM) paper either by transfer from an agarose gel or by direct spotting, according to the method of Alwine et al. (1). Filters were hybridized with 25 to 100 μg of total cytoplasmic RNA in a 100-μl reaction volume containing 50% deionized formamide (Matheson Coleman Bell)–0.6 M NaCl–4 mM Na_2 EDTA–80 mM Tris-hydrochloride (pH 7.8). After hybridization at 37°C for 4 to 16 h, the filters were washed 10 times with 50% formamide–30 mM NaCl–2 mM Na_2 EDTA–4 mM Tris-hydrochloride (pH 7.8) at 37°C (1). Each wash was vortexed and removed by aspiration. The RNA was eluted from the DNA by incubating the filters in 200 μl of elution buffer (90% formamide, 100 mM Tris-hydrochloride [pH 7.8], 15 μg of calf thymus RNA [Boehringer Mannheim Biochemicals]) for 5 min at 65°C. The elution buffer was removed and diluted into 240 μl of 0.2 M sodium acetate, pH 5.5. The RNA was precipitated with 2.5 volumes of ethanol at −20°C. In preparation for translation, the precipitated RNA was pelleted at 12,000 × *g* for 10 min at 4°C, washed twice with 70% ethanol, and dried under vacuum.

Fractionation of RNA in methyl mercury hydroxide-containing agarose gel. Horizontal agarose gels (15 cm long and 4 mm thick) containing 12.5 mM methyl mercury hydroxide were run as described by Bailey and Davidson (4). The RNA was denatured by 12.5 mM methyl mercury hydroxide in borate buffer, pH 8.2, and subsequently fractionated by electrophoresis at a constant voltage of 50 V for 18 h at room temperature. For transfer to DBM paper, the procedure of Alwine et al. (2) was followed. The gel was soaked in three changes of 0.5 M ammonium acetate, pH 4.0, for 30 min. The RNA was transferred out of the gel to DBM paper by the method of Southern (39). RNA filter hybridizations were carried out according to the method of Alwine et al. (2), using 10^6 cpm of DNA labeled with ^{32}P by the nick translation method of Rigby et al. (36).

Alternatively, for translation of mRNAs after fractionation in methyl mercury hydroxide-containing agarose gels, the procedure of Miller et al. (22) was followed. The gel was cast with low-melting-point agarose (Bethesda Research Laboratories). After electrophoresis, the gel was soaked for 60 min in 100 mM dithiothreitol. Individual lanes were excised and sliced into 1-mm portions with a Mickel gel slicer. Slices were pooled in contiguous sets of three slices and frozen in 1.5-ml Eppendorf tubes at −70°C before processing. The RNA was pelleted, washed twice with 70% ethanol, dried under vacuum, and translated in 12.5 μl of reticulocyte lysate.

Preparation and labeling of DNA. Vaccinia virus genomic DNA was isolated from purified virions by the method of Gross-Bellard et al. (15). Digestion of DNA was carried out with restriction enzymes purchased from and used according to the procedures outlined by New England Biolabs. DNA fragments were fractionated by electrophoresis in 1% (wt/vol) agarose in 36 mM Tris-hydrochloride (pH 7.6)–36 mM NaH_2PO_4–1 mM Na_2 EDTA or in 6% polyacrylamide gels according to the method of Alwine et al. (1). DNA fragments were purified from agarose gels by the method of Vogelstein and Gillespie (42). For 5′ end labeling, DNA restriction fragments were treated with bacterial alkaline phosphatase in 50 mM Tris-hydrochloride, pH 8.0, at 60°C for 15 min. The DNA was extracted three times with phenol and ethanol precipitated. Fragments were labeled by using [γ-^{32}P]ATP (New England Nuclear) and polynucleotide kinase as specified by New England Biolabs. For 3′ labeling, DNA restriction fragments with 5′ protruding termini were reacted with reverse transcriptase, [α-^{32}P]dTTP, and [α-^{32}P]dATP in a buffer containing 10 mM dGTP–10 mM dCTP–50 mM Tris-hydrochloride (pH 8.3)–10 mM KCl–10 mM $MgCl_2$ for 1 h at 42°C. Alternatively, the Klenow fragment of *Escherichia coli* DNA polymerase was used in the fill-in reaction.

Plasmid DNA was uniformly labeled in vivo with $^{32}PO_4$. A 30-ml culture in phosphate-depleted medium was grown to an optical density at 550 nm equal to 0.5, the culture was made to 25 μg of chloramphenicol per ml, and 0.5 mCi of $^{32}PO_4$ (New England Nuclear) was added. After shaking overnight at 37°C, the cells were harvested and plasmid DNA was prepared according to the alkaline plasmid procedure (17).

S1 and exoVII nuclease analyses. S1 nuclease mapping was done as described by Sharp et al. (37). Labeled DNA (10 to 50 ng) was mixed with 100 ng of poly(A^+) RNA in a buffer containing 80% deionized formamide–0.4 M NaCl–40 mM 1,4-piperazinediethanesulfonic acid (pH 6.4)–1 mM Na_2 EDTA in a 20-μl reaction. The solution was denatured at 65°C for 10 min and then transferred to a 42°C water bath and allowed to hybridize for 3 h. After hybridization, the reactions were diluted with 200 μl of ice-cold S1 buffer containing 280 mM NaCl–30 mM sodium acetate (pH 4.4)–4.5 mM zinc acetate–5% glycerol–25 μg of herring sperm double-stranded DNA (Boehringer Mannheim) per ml–200 U of S1 nuclease (Miles Laboratories) per ml. After 30 min of digestion at 37°C, 3 volumes of ethanol were added and the DNA was precipitated at −20°C. Pelleted DNA was washed twice with 70% ethanol, dried under vacuum, and then fractionated on 1.5% alkaline agarose gels (21). The dried gel was exposed to Kodak XAR-5 film at −80°C with the aid of an intensifying screen.

The determination of exoVII-resistant DNA-RNA hybrids was done according to Sharp et al. (37). DNA-RNA hybridization was done as described above for the S1 nuclease analysis. After hybridization, the reactions were diluted with 200 μl of exoVII buffer containing 30 mM KCl–1 mM Na_2 EDTA–10 mM Tris-hydrochloride (pH 7.4)–1.24 U of exoVII (Bethesda Research Laboratories) per ml and digested at 37°C for 1 h. After digestion, the solutions were made 0.2 M NaCl and the DNA was precipitated with 3 volumes of ethanol at −20°C. The DNA precipitates were processed as described for the S1 nuclease analysis.

RESULTS

Towards the goal of understanding the regulation of vaccinia virus gene expression and viral mRNA biogenesis, our laboratory has constructed two libraries of vaccinia virus recombinant molecules. The *Eco*RI fragments of the viral

genome were inserted into the *Eco*RI site of λ gt wes and propagated as recombinant phage. Our preliminary characterization of several of these recombinant phage demonstrated that the genes encoding both early and late viral genes were often located on the same or adjacent *Eco*RI fragments. However, one phage with a 5.0-kb *Eco*RI insert was shown by hybrid selection analysis to encode exclusively early genes. The early mRNAs hybrid selected by the 5.0-kb vaccinia virus fragment programmed the cell-free synthesis of [^{35}S]methionine-labeled polypeptides, which were 55K, 30K, 20K, and 10K in size. The mRNAs encoding each of these polypeptides were detected at the start of the infectious cycle and were present in the RNA population 12 h after infection.

To study the regulation of individual viral genes and their mRNAs, we constructed a second recombinant library. This library was a cDNA library representative of poly(A$^+$) RNAs isolated at both early and late times of infection. cDNA was prepared by the reverse transcription of an oligodeoxythymidylate-primed poly(A$^+$) RNA template, and second-strand synthesis was accomplished with the large fragment of *E. coli* DNA polymerase. Subsequent to S1 nuclease cleavage, the double-stranded DNA molecules were tailed by terminal transferase and ligated to appropriately tailed *Pst*I-digested pBr322.

The cDNA library was screened by using the colony hybridization method of Grunstein and Hogness (16) and by mRNA hybrid selection, which resulted in some unexpected observations. In particular, several cDNA molecules hybrid selected early mRNAs encoding two or three different polypeptides. One cDNA, pcE5, which is 1,100 nucleotides in length, hybrid selects the mRNAs for the 55K and 30K early polypeptides encoded by the 5.0-kb *Eco*RI fragment (Fig. 1A). That a single cDNA hybrid selects the mRNAs encoding two polypeptides which are presumably encoded by two viral genes limits the use of this cDNA as a probe to study the regulation of an individual viral gene. Moreover, given the definition of a cDNA as a representation of those sequences transcribed into mRNA, this observation raises several important questions concerning the organization of the transcripts for these polypeptides. A number of explanations could account for this observation, namely: transcripts which overlap in either their coding or noncoding sequences; transcripts which overlap on opposite DNA strands; transcripts containing reiterated sequences; or a transcript capable of directing the synthesis of more than one polypeptide.

With these considerations in mind, we undertook an investigation into the detailed organization of the mRNAs transcribed from the 5.0-kb *Eco*RI fragment.

Determination of the DNA strand encoding each polypeptide. We have ruled out the possibility of overlapping transcripts encoded by opposite strands of DNA by determining which DNA strand encodes each polypeptide. DNA strands were separated by denaturing the recombinant phage DNA in the presence of polyuridylate-polyguanylate, followed by electrophoresis in an agarose gel. The separated strands were visualized by ethidium bromide staining, transferred to DBM paper, and utilized to hybrid select early mRNA. The [^{35}S]methionine-labeled polypeptides encoded by the mRNAs hybrid selected by each strand were visualized by fluorography after separation by SDS-polyacrylamide electrophoresis (Fig. 1B). The fast DNA strand hybrid selects the mRNAs encoding the 55K, 30K, 20K, and 10K polypeptides. These data rule out an organization involving overlapping transcripts from opposite DNA strands. To distinguish between the remaining possible organizations of these mRNAs, it was necessary to determine a restriction endonuclease map of the 5.0-kb fragment and the organization of early mRNAs relative to this map.

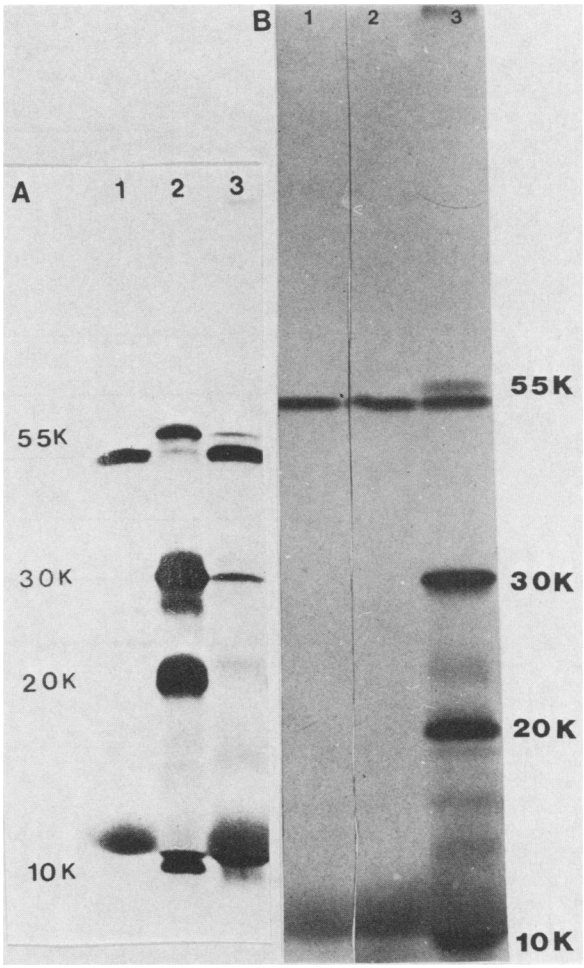
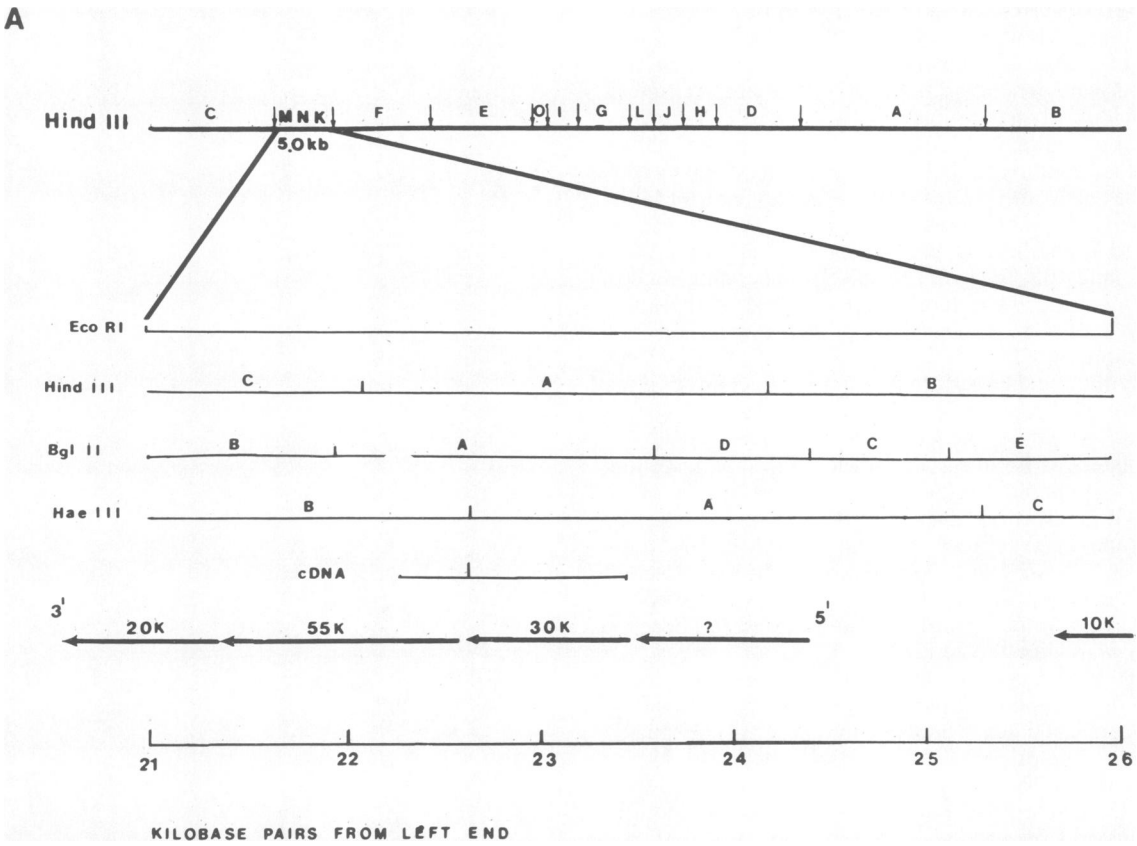


FIG. 1. Hybrid selection by the cDNA complementary to the 5.0-kb fragment and hybrid selection by the separated strands of the 5.0-kb fragment. (A) Fluorography of [^{35}S]methionine-labeled polypeptides synthesized in an mRNA-dependent reticulocyte lysate and fractionated by electrophoresis in an SDS–17.5% polyacrylamide gel. Plasmid DNA of pcE5 was immobilized on DBM paper and used to hybrid select from 75 μg of vaccinia virus early total RNA followed by translation in the reticulocyte cell-free system. Lane 1, No RNA added; lane 2, mRNA hybrid selected by the cDNA, pcE5. (B) Fluorograph of [^{35}S]methionine-labeled polypeptides synthesized in a cell-free system by mRNA hybrid selected to separated DNA strands of the 5.0-kb vaccinia virus insert. DNA strands were separated by denaturation in the presence of polyuridylate-polyguanylate and subsequent electrophoresis in a Tris-acetate agarose gel (13). The fast and slow DNA strands were visualized by ethidium bromide staining and transferred to DBM paper. The immobilized DNA was used to hybrid select early vaccinia virus mRNAs followed by translation in the reticulocyte cell-free system. The polypeptides were fractionated in an SDS–17.5% polyacrylamide gel, and the dried gel was fluorographed for 24 h. Lane 1, No mRNA added; lane 2, mRNAs hybrid selected by the slow DNA strand; lane 3, mRNAs hybridized to the fast DNA strand.

Restriction endonuclease map of the 5.0-kb fragment. The position of this 5.0-kb fragment on the restriction endonuclease map of the vaccinia virus genome was ascertained by labeling recombinant phage DNA with ^{32}P by the nick translation method (36) and then hybridizing this probe to a



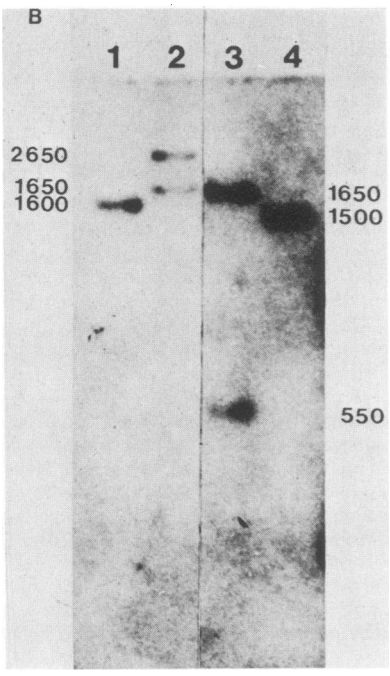


FIG. 2. Diagram of restriction endonuclease sites and location of the cDNA. (A) Diagram of the cleavage sites generated by *Hin*dIII restriction endonuclease within vaccinia virus DNA (19) and location of the 5.0-kb *Eco*RI fragment; expanded view of the 5.0-kb fragment and location of the cleavage sites generated by *Hin*dIII, *Hae*III, and *Bgl*II endonucleases; and location of cDNA relative to this map of restriction endonuclease sites on the 5.0-kb fragment. A diagram of the final map depicting the locations of the mature mRNAs for the 55K, 30K, 20K, and 10K polypeptides relative to the restriction map is shown. (B) Determination of the position of cDNA pcE5 on the map of restriction endonuclease cleavage sites on the 5.0-kb *Eco*RI fragment by Southern blot hybridization and visualization by autoradiography. The purified 5.0-kb *Eco*RI fragment was digested with several different endonucleases, and the fragments were separated by electrophoresis in a Tris-acetate–6% polyacrylamide–agarose gel. The DNA was transferred to DBM paper and hybridized with ^{32}P-labeled pcE5. DNA was digested with (lane 1) *Bgl*II, (lane 2) *Hae*III, (lane 3) *Hin*dIII and *Hae*III, and (lane 4) *Hin*dIII and *Bgl*II.

Southern blot of various restriction endonuclease digests of the vaccinia virus genome. The location of this 5.0-kb fragment relative to the map of the *Hin*dIII sites on the vaccinia virus genome (19) is shown (Fig. 2A). The *Hin*dIII-N fragment is entirely contained within the 5.0-kb *Eco*RI fragment, and portions of the M and K fragments are located, respectively, to the left and right of the N fragment.

To establish a preliminary restriction endonuclease map, the 5.0-kb vaccinia virus *Eco*RI fragment was purified and ligated into the *Eco*RI site of pBr322 and propagated thereafter as a plasmid. With a series of single and double digestions of purified plasmid DNA, followed by sizing of DNA fragments on agarose gels, we were able to determine the restriction endonuclease map of the *Hae*III, *Hin*dIII, and *Bgl*II enzymes (Fig. 2A).

As an initial step towards understanding the organization of the transcripts of the 5.0-kb fragment, the position of the cDNA relative to the restriction map was ascertained. DNA of pcE5 was labeled with ^{32}P by the nick translation method and hybridized to a blot of various restriction endonuclease digests of the 5.0-kb fragment (Fig. 2B). The cDNA hybrid-

izes to the *Hae*III-B and -A fragments and solely to the *Hin*dIII-A and BglII-A fragments. The presence of a single *Hae*III site in the 1,100-base pair (bp) cDNA which generates two fragments, 300 and 700 bp, dictates the position of the cDNA (Fig. 2A).

Hybrid selection of early mRNAs encoded by subfragments of the 5.0-kb fragment. To establish the preliminary organization of the translatable mRNAs in relation to the cDNA's position, the 5.0-kb fragment was digested with the restriction endonuclease *Hin*dIII or *Hae*III. The resultant DNA fragments were fractionated by agarose gel electrophoresis, visualized by ethidium bromide staining, and transferred to DBM paper. The immobilized DNA fragments were used to hybrid select early RNA, and the encoded polypeptides were defined by in vitro translation (Fig. 3).

The leftmost *Hin*dIII-C fragment hybrid selects the mRNAs encoding the 20K and 55K polypeptides. The mRNA encoding the 55K polypeptide is also hybrid selected by the adjacent *Hin*dIII-A fragment, indicating that it spans this *Hin*dIII site. In addition, the *Hin*dIII-A fragment hybrid selects the mRNA encoding the 30K polypeptide. The rightmost *Hin*dIII-B fragment hybrid selects the mRNA encoding the 10K polypeptide, which is often obscured by the large amount of globin protein present in the reticulocyte lysate. The mRNA encoding the 10K polypeptide is also hybrid selected by the *Hae*III-C fragment, which locates its transcript between the rightmost *Hae*III site and the *Eco*RI site.

Similarly to the *Hin*dIII-C fragment, the leftmost *Hae*III-B fragment hybrid selects the mRNAs encoding the 20K and 55K polypeptides. However, the adjacent *Hae*III-A fragment hybrid selects solely the mRNA encoding the 30K polypeptide and not the mRNA encoding the 55K polypeptide. This indicates that the mRNA encoding the 55K polypeptide spans the *Hin*dIII site but terminates before the *Hae*III site. Surprisingly, these hybrid selection data indicate that no mature mRNAs span the *Hae*III site contained within the cDNA. This suggests that the cDNA is not a direct copy of the mature mRNAs encoding the 55K or 30K polypeptides.

The mapping information derived from the hybrid selection analysis and the subsequent analyses is compiled in schematic form (Fig. 4).

Sizing of RNA transcripts by RNA filter hybridization. To determine the size of the mRNAs encoding the 55K, 30K, 20K, and 10K polypeptides as well as their positions along the 5.0-kb *Eco*RI fragment, we have used the RNA filter hybridization technique. RNA was fractionated by electrophoresis in an agarose gel containing methyl mercury hydroxide, transferred to DBM paper, and visualized by hybridization with the ^{32}P-labeled *Hae*III-A, -B, and -C DNA fragments (Fig. 5).

The leftmost *Hae*III-B fragment, which hybrid selects the mRNAs encoding the 20K and 55K polypeptides, hybridizes to six distinct poly(A^+) RNAs, 5,000, 3,300, 2,300, 1,800 nucleotides in size, and two unique transcripts, 1,450 and 780 nucleotides in length (Fig. 5A). The adjacent *Hae*III-A fragment, which hybrid selects exclusively the mRNA encoding the 30K polypeptide, also hybridizes to the RNA transcripts 5,000, 3,300, 2,300, and 1,800 nucleotides in length and to two unique transcripts, 1,000 and 950 nucleotides in size (Fig. 5B). The cDNA fragment which spans the *Hae*III site separating the *Hae*III-B and *Hae*III-A fragments hybridizes to the transcripts 5,000, 3,000, 2,300, and 1,800 nucleotides in size and to the 1,450- and 950-nucleotide RNAs (Fig. 5D). Since the cDNA hybrid selects the mRNAs encoding the 55K and 30K polypeptides, this would suggest that the 55K polypeptide is encoded by the 1,450-nucleotide RNA and the 30K polypeptide is encoded by the 950-nucleotide RNA. Furthermore, the 20K polypeptide is probably encoded by the 780-nucleotide RNA which is the other transcript that is unique to the hybridization with the *Hae*III-B fragment.

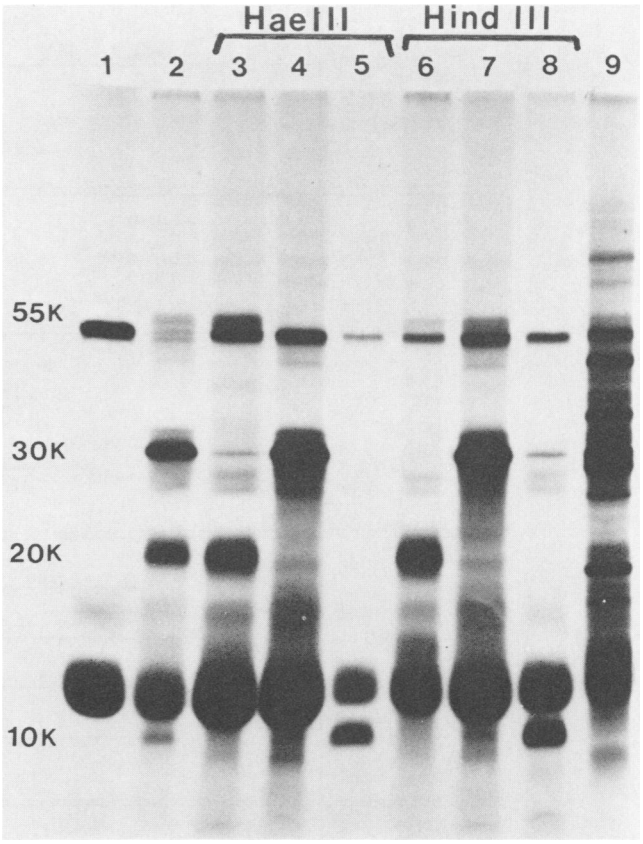


FIG. 3. Hybrid selection of early vaccinia virus mRNAs by DNA fragments generated by restriction endonuclease digestion of the 5.0-kb *Eco*RI fragment. Fluorograph of [^{35}S]methionine-labeled polypeptides synthesized in am mRNA-dependent reticulocyte lysate and fractionated by electrophoresis in an SDS–17.5% polyacrylamide gel. The dried gel was fluorographed for 20 h. Plasmid DNA containing the 5.0-kb fragment was digested with endonucleases and fractionated in a 1% agarose gel. The DNA fragments were visualized by ethidium bromide staining and transferred to DBM paper. Immobilized DNA was used to hybrid select early vaccinia virus mRNAs followed by translation in the reticulocyte cell-free system. Equal volumes of each translation reaction were fractionated in the SDS-polyacrylamide gel except for lanes 2 and 9, where one-fifth the volume of the other reactions was loaded. Translation products directed by mRNA hybridized to: (lane 1) no mRNA added; (lane 2) 5.0-kb *Eco*RI fragment; (lane 3) *Hae*III-B fragment; (lane 4) *Hae*III-A fragment; (lane 5) *Hae*III-C fragment; (lane 6) *Hin*dIII-C fragment; (lane 7) *Hin*dIII-A fragment; (lane 8) *Hin*dIII-B fragment. (Lane 9) Translation products whose synthesis was directed by total early RNA.

The rightmost *Hae*III-C fragment, which hybrid selects the mRNA encoding the 10K polypeptide, hybridizes to four distinct RNAs, 2,350, 1,700, 1,350, and 400 nucleotides in size (Fig. 5C). Based on this information it is not possible to ascertain which RNA encodes the 10K polypeptide.

In summary, when the information obtained from the hybrid selection analysis is correlated with the size of the

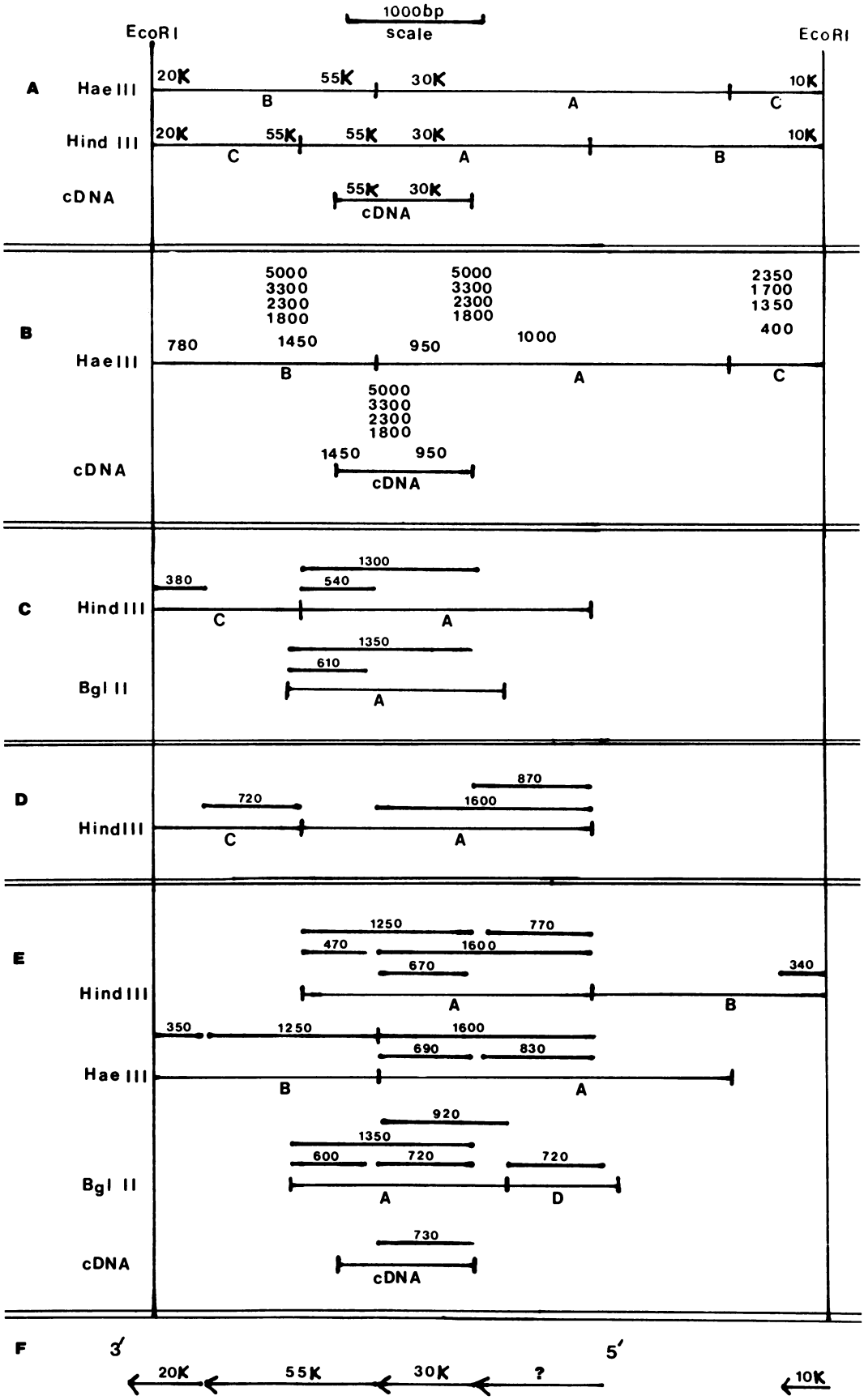


FIG. 4. Compilation in schematic form of the mapping data of the 5.0-kb *Eco*RI fragment. The information derived from all of the different mapping techniques has been compiled and drawn to scale relative to the location of restriction endonuclease sites along the 5.0-kb *Eco*RI fragment. Sizes of polypeptides are indicated in kilodaltons (K), and sizes of RNAs are indicated in nucleotides. Compilation of the data is from: (A) hybrid selection; (B) RNA filter hybridization; (C) S1 nuclease mapping with 5′-end-labeled DNA fragments; (D) S1 nuclease mapping with 3′-end-labeled DNA fragments; (E) S1 nuclease mapping with uniformly labeled DNA fragments. (F) Diagram of the final map of the mature size mRNAs and the size of the polypeptide encoded by each mRNA.

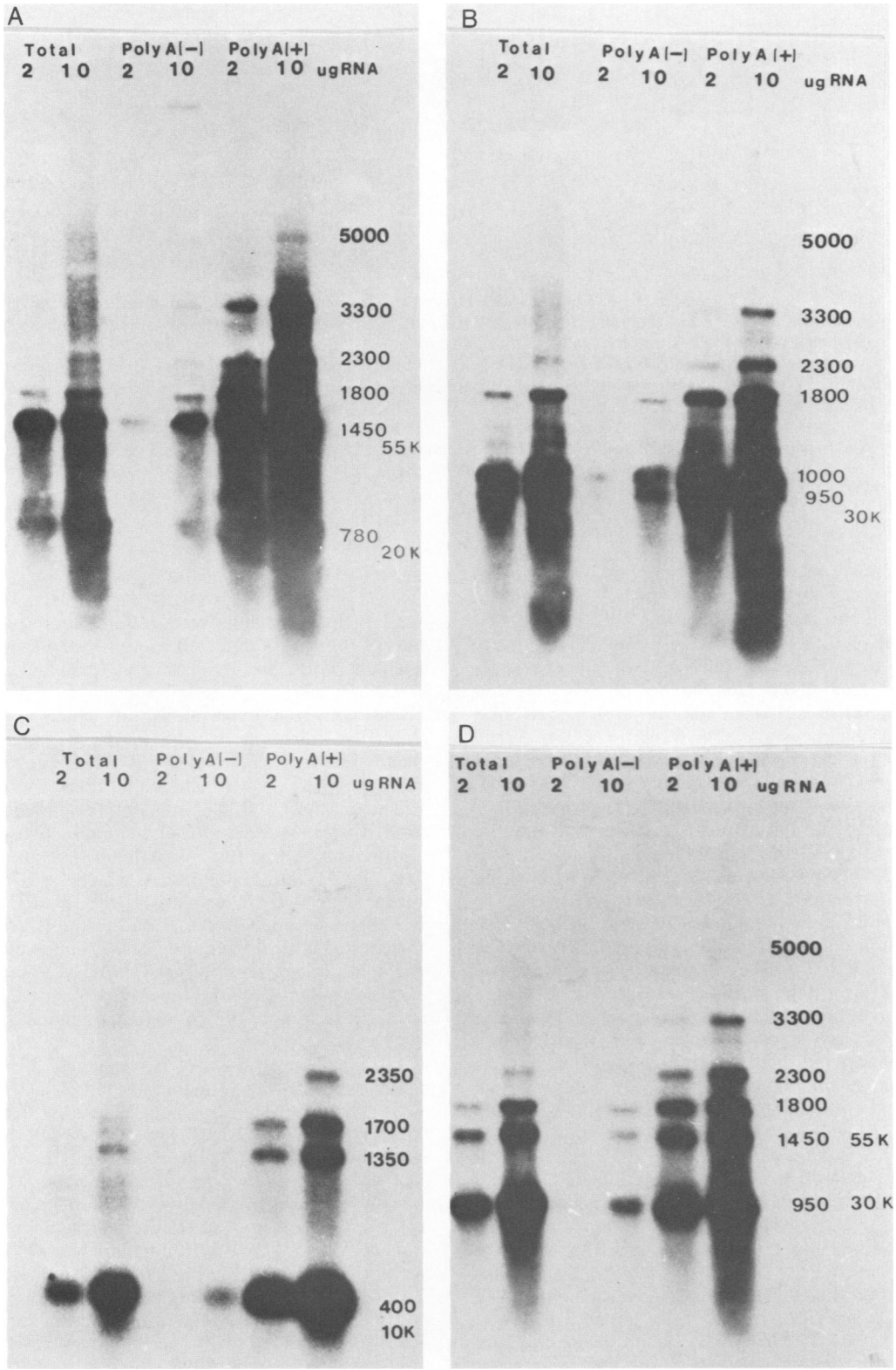


FIG. 5. Size distribution of RNA transcripts from the 5.0-kb *Eco*RI fragment as determined by RNA filter hybridization. Autoradiographs of RNA filters after hybridization with ^{32}P-labeled fragments of the 5.0-kb fragment. Plasmid DNA containing the 5.0-kb insert was digested with *Hae*III and fractionated on a 1% agarose gel. The purified fragments were labeled in vitro with ^{32}P by the nick translation method and hybridized to an RNA filter of (i) 2 μg of total early RNA; (ii) 10 μg of total early RNA; (iii) 2 μg of poly(A$^-$) early RNA; (iv) 10 μg of poly(A$^-$) early RNA; (v) 2 μg of poly(A$^+$) early RNA; (vi) 10 μg of poly(A$^+$) early RNA. The filter was autoradiographed for 24 to 48 h. Hybridization was with the ^{32}P-labeled (A) *Hae*III-B fragment; (B) *Hae*III-A fragment; (C) *Hae*III-C fragment; (D) cDNA pcE5.

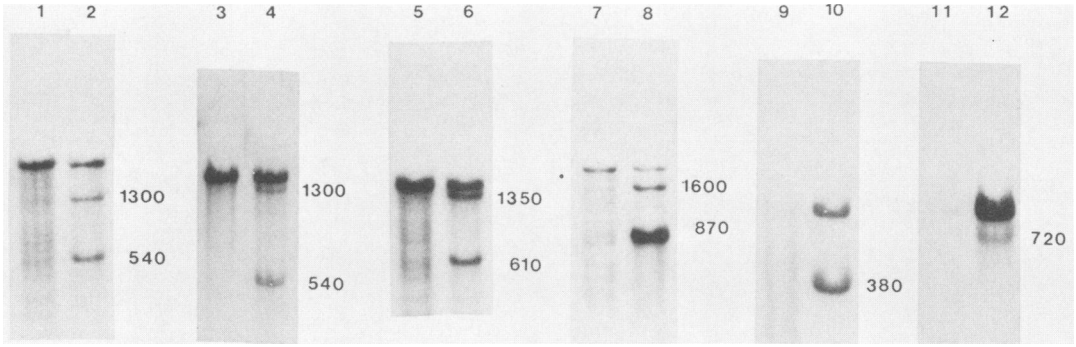


FIG. 6. Mapping of the 5′ and 3′ termini of the RNAs transcribed from the 5.0-kb fragment, using a modification of the S1 nuclease assay. Autoradiograph of an alkaline agarose gel of the S1 nuclease-resistant products of early vaccinia virus RNA hybridized to either ^{32}P-, 5′-end-labeled or ^{32}P, 3′-end-labeled purified restriction fragments of the 5.0-kb fragment. The samples are as follows: (lane 1) *Hin*dIII-A fragment 5′ end labeled, no RNA added; (lane 2) *Hin*dIII-A fragment 5′ end labeled, hybridized to early RNA; (lane 3) *Hin*dIII-A fragment 5′ end labeled and then digested with *Bgl*II, no RNA added; (lane 4) *Hin*dIII-A fragment 5′ end labeled and digested with *Bgl*II, hybridized to early RNA; (lane 5) *Bgl*II-A fragment 5′ end labeled, no RNA added; (lane 6) *Bgl*II-A fragment 5′ end labeled, hybridized to early RNA; (lane 7) *Hin*dIII-A fragment 3′ end labeled, no RNA added; (lane 8) *Hin*dIII-A fragment 3′ end labeled, hybridized to early RNA; (lane 9) *Hin*dIII-C fragment 5′ end labeled at the *Eco*RI site, no RNA added; (lane 10) *Hin*dIII-C fragment 5′ end labeled at the *Eco*RI site, hybridized to early RNA; (lane 11) *Hin*dIII-C fragment 3′ end labeled at the *Hin*dIII site, no RNA added; (lane 12) *Hin*dIII-C fragment 3′ end labeled at the *Hin*dIII site, hybridized to early RNA.

RNAs and their position on the DNA template, we can begin to ascribe a specific polypeptide to its mRNA (Fig. 4A and B). Although the mRNA encoding the 10K polypeptide cannot be clearly identified, the 55K, 30K, and 20K polypeptides appear to be encoded by mRNAs which are, respectively, 1,450, 950, and 780 nucleotides in size.

The surprising result is that, in addition to these mRNAs, there are poly(A$^+$) higher-molecular-weight RNAs of 5,000, 3,300, 2,300, and 1,800 nucleotides in size which are transcribed from the DNA sequences encoding the 55K, 30K, and 20K polypeptides. The position of the high-molecular-weight RNAs relative to the mature size mRNAs cannot be determined from this Northern analysis.

Mapping of the 5′ and 3′ termini of the RNAs by S1 nuclease analysis. The information derived from the hybrid selection and RNA filter hybridization indicates that the 1,450-nucleotide mRNA which encodes the 55K polypeptide spans the left *Hin*dIII site of the *Hin*dIII-A fragment. Therefore, the 5′ and 3′ termini of this mRNA would be expected to be located fixed distances to either the left or right of this site depending on the direction of transcription. We have used a variation of the Berk and Sharp S1 nuclease mapping procedure, as modified by Weaver and Weissmann (43), to determine the direction of transcription and to locate the 5′ and 3′ termini of the RNA which spans this *Hin*dIII site.

The *Hin*dIII-A fragment was labeled at its 5′ termini with $^{32}PO_4$, using polynucleotide kinase and [γ-^{32}P]ATP. This 5′-labeled substrate was hybridized to early vaccinia virus RNA and digested with S1 nuclease (Fig. 6, lanes 1 and 2). The two nuclease-resistant fragments indicate that there are two RNAs whose 5′ ends are 1,300 and 540 nucleotides inward from the terminus of the *Hin*dIII-A fragment. If the rightmost 5′ PO_4 label of *Hin*dIII-A is removed by *Bgl*II digestion, the same S1 nuclease-resistant fragments are generated (Fig. 6, lanes 3 and 4). This establishes the direction of transcription as well as the fact that the two RNAs whose 5′ ends are 1,300 and 540 nucleotides inward from the *Hin*dIII-A terminus are overlapping.

This was further confirmed by using the *Bgl*II-A fragment. The 5′ ends of two RNAs, 1,350 and 610 nucleotides, were detected with the 5′-end-labeled *Bgl*II-A fragment (Fig. 6, lanes 5 and 6). When positioned on the map, the 5′ ends of these RNAs extend inward from the left *Bgl*II site of the *Bgl*II-A fragment and correspond to the positions of the 5′ ends of the RNAs detected by the 5′-end-labeled *Hin*dIII-A fragment which are, respectively, 1,300 and 540 nucleotides in size (Fig. 4C).

The converse experiment, in which a 3′-end-labeled *Hin*dIII-A fragment is used in the S1 nuclease analysis, locates the 3′ terminus of the RNA (Fig. 6, lanes 7 and 8). From these data, we conclude that there are two RNAs with 3′ ends, 1,600 and 870 nucleotides inward of the right *Hin*dIII site of the *Hin*dIII-A fragment (Fig. 4D).

Moreover, since the *Hin*dIII-A fragment is 2,150 bp in size, the 5′ end of the RNA which is 1,300 nucleotides inward from the left terminus of the *Hin*dIII-A fragment is probably <50 bp from the 3′ end of the RNA, which is 870 nucleotides inward from the right terminus of the *Hin*dIII-A fragment. Likewise, the RNAs whose 5′ and 3′ ends are 1,600 and 540 nucleotides inward, respectively, from the left and right termini of the *Hin*dIII-A fragment are also <50 bp apart.

A similar arrangement of RNAs whose 5′ and 3′ ends are closely spaced was found in the adjacent *Hin*dIII-C fragment. The *Hin*dIII-C fragment has an *Eco*RI site at its left boundary and a *Hin*dIII site at its right boundary. The *Hin*dIII-C fragment, 5′ end labeled at its *Eco*RI site, protected from S1 nuclease digestion an RNA whose 5′ end is located 380 bases inward from the *Eco*RI site (Fig. 6, lanes 9 and 10). Conversely, the *Hin*dIII-C fragment, 3′ end labeled at its *Hin*dIII site, hybridized to an RNA whose 3′ end is 720 nucleotides to the left of the *Hin*dIII site (Fig. 6, lanes 11 and 12). Since the *Hin*dIII-C fragment is 1,100 bp in length, the 5′ and 3′ ends of these RNAs are located within 50 bp of each other, as is the case for the RNAs encoded by the *Hin*dIII-A fragment (Fig. 4C and D).

The mRNA encoding the 55K polypeptide, which spans the *Hin*dIII site between the *Hin*dIII-C and -A fragments, was 1,450 nucleotides in size when measured by RNA filter hybridization. The 5′ and 3′ ends of the RNAs which best approximate this size and location are the 540-nucleotide 5′ end encoded by the *Hin*dIII-A fragment plus the 720-nucleo-

tide 3′ end located in the *Hin*dIII-C fragment. The difference in size between these RNAs (1,450 versus 1,260 nucleotides) is probably a reflection of the 150 to 200 adenylate residues which comprise the poly(A) tail of the mRNA as sized by RNA filter hybridization.

The alternate RNA which spans this site consists of the 720-nucleotide 3′ end in the *Hin*dIII-C fragment and the 1,300-nucleotide 5′ end located in the *Hin*dIII-A fragment (Fig. 4C and D). This 2,020-nucleotide RNA most likely does not encode the 55K polypeptide because of the large difference in size and the fact that a significant portion of the 5′ end of the RNA is encoded by the *Hae*III-A fragment, which does not hybrid select the mRNA encoding the 55K polypeptide. With the same allowance for the size differences due to the poly(A) tail, this 2,020-nucleotide RNA may correspond to the 2,300-nucleotide, high-molecular-weight RNA seen by RNA filter hybridization. This RNA hybridizes to both the *Hae*III-B and -A fragments.

The 20K polypeptide is encoded by an mRNA which is hybrid selected by both the *Hin*dIII-C and *Hae*III-B fragments. In addition, the mRNA encoding the 20K polypeptide is hybrid selected by the *Eco*RI fragment located immediately to the left of the 5.0-kb *Eco*RI fragment (A. Mahr and B. E. Roberts, unpublished data). This would suggest that the mRNA encoding the 20K polypeptide spans the left *Eco*RI site of the 5.0-kb fragment. Therefore, the 5′ end of this mRNA is located in the *Hin*dIII-C fragment and is the 5′ terminus which has been measured to be 380 nucleotides inward from the *Eco*RI site (Fig. 4C).

The mRNA which encodes the 30K polypeptide was shown by hybrid selection to be located on both the *Hae*III-A and the *Hin*dIII-A fragments. This would locate this mRNA between the left *Hae*III site of the *Hae*III-A fragment and the right *Hin*dIII-site of the *Hin*dIII-A fragment. In addition, the cDNA, which also hybrid selects the mRNA encoding the 30K polypeptide, hybridizes on the RNA filter to an mRNA 950 nucleotides in size, as does the *Hae*III-A fragment. The RNAs which have been detected by S1 nuclease analysis, using either 5′- or 3′-labeled fragments, do not correspond to the mRNA encoding the 30K polypeptide in either size or position on the DNA template. For example, both of the RNAs encoded by the *Hin*dIII-A fragment whose 5′ ends are 1,300 and 540 nucleotides inward from the left *Hin*dIII site are complementary to the *Hae*III-B fragment which does not hybrid select or hybridize on the RNA filter to the presumptive 950-nucleotide mRNA encoding the 30K polypeptide. Furthermore, the RNAs whose 3′ end is 870 nucleotides inward from the right *Hin*dIII site of the *Hin*dIII-A fragment is approximately the correct size, but the majority of the RNA is to the right of the boundary of the cDNA and the terminus of the 870-nucleotide RNA probably does not overlap with the cDNA. The RNA whose 3′ end is 1,600 nucleotides inward from the right *Hin*dIII site of the *Hin*dIII-A fragment has a position on the DNA template which is consistent with it encoding the 30K polypeptide; however, its size and the additional allowance for the poly(A) tail make it an unlikely candidate for the 950-nucleotide mRNA encoding the 30K polypeptide.

The inability to ascribe a specific S1 nuclease-resistant fragment to the mRNA encoding the 30K polypeptide may be due to a limitation of the technique. The modified S1 nuclease mapping procedure can locate only those mRNAs which span the 5′- or 3′-labeled restriction sites. Those mRNAs which are entirely contained within the DNA fragment will not be detected.

S1 nuclease mapping of mRNAs transcribed from the 5.0-kb *Eco*RI fragment with uniformly labeled DNA. In contrast to the DNA fragment which is either 5′ or 3′ end labeled, a uniformly labeled fragment used in the S1 nuclease analysis can detect all mRNAs transcribed from a DNA fragment regardless of their position on the DNA template. In addition, this analysis will also determine whether the sequence present in these mRNAs are colinear with the DNA template or whether they are comprised of nonadjacent sequences.

The detection of intervening sequences in the DNA is made possible by the different specificities of the S1 nuclease and exoVII enzymes. exoVII is an exonuclease specific for single-stranded DNA, but which has a strict requirement for a free 5′ or 3′ terminus, whereas S1 nuclease is a single-strand-specific endonuclease which will cleave any DNA that is not base paired. The presence of an intervening sequence in the DNA of a DNA-RNA hybrid would result in an internal loop of single-stranded DNA lacking an accessible 5′ or 3′ end and therefore resistant to exoVII digestion yet sensitive to S1 cleavage.

The 5.0-kb *Eco*RI fragment uniformly labeled with $^{32}PO_4$ was used in the S1 nuclease and exoVI analyses (Fig. 7A). The major S1 nuclease-resistant fragments are 1,600, 1,350, 770, and an intense band at 900 nucleotides and several faint higher-molecular-weight bands which probably correspond to the high-molecular-weight RNAs seen by RNA filter hybridization. A comparison of the S1 nuclease-resistant fragments with the exoVII exonuclease-resistant fragments reveals no major differences and is indicative of RNAs that are colinear within the 5.0-kb *Eco*RI fragment.

For a further refinement of the positions of these transcripts, we have determined the location of the S1-resistant fragments along the 5.0-kb DNA fragment and correlated these data directly to those obtained by hybrid selection, RNA filter hybridization, and S1 nuclease analysis with 5′- and 3′-end-labeled DNA. Uniformly labeled DNA was digested with the *Hae*III restriction endonuclease, and the purified fragments were used in the S1 nuclease analysis (Fig. 7B). The leftmost *Hae*III-B fragment hybrid selects the mRNAs encoding the 20K and 55K polypeptides, which are 780 and 1,450 nucleotides in size as determined by RNA filter hybridization. From S1 nuclease analysis, the *Hae*III-B fragment has two S1 nuclease-resistant bands, 350 and 1,250 nucleotides in size (Fig. 7, lanes 1 and 2). S1 nuclease analysis with the 5′- and 3′-end-labeled *Hin*dIII-C fragment showed that there are two RNAs present in the *Hae*III-B region. The 5′ end of one RNA is 380 nucleotides inward from the *Eco*RI site and corresponds to the 350-nucleotide RNA protected by the uniformly labeled *Hae*III-B fragment. This RNA encodes the 20K polypeptide. The other RNA complementary to the *Hae*III-B fragment, as demonstrated by the end-labeled *Hin*dIII-C and -A fragments, has a 3′ end which was 720 nucleotides to the left of the *Hin*dIII site and a 5′ end which was 540 nucleotides to the right of the same *Hin*dIII site. The contiguous length of this RNA (1,260 nucleotides) is equal to the 1,250-nucleotide RNA protected from S1 nuclease digestion by the uniformly labeled *Hae*III-B fragment. The mRNA encoding the 55K polypeptide is exclusively hybrid selected by the *Hae*III-B fragment, and therefore the 1,250-nucleotide, S1-resistant fragment is a representation of the entire transcript encoding the 55K polypeptide. The size and location of this RNA are consistent with it encoding the 55K polypeptide. The 3′ end of this mRNA is adjacent and probably within 50 bp of the 5′ end of the mRNA encoding the 20K polypeptide.

The preceding S1 nuclease analysis using the end-labeled *Hin*dIII-A fragment failed to detect an mRNA of the appro-

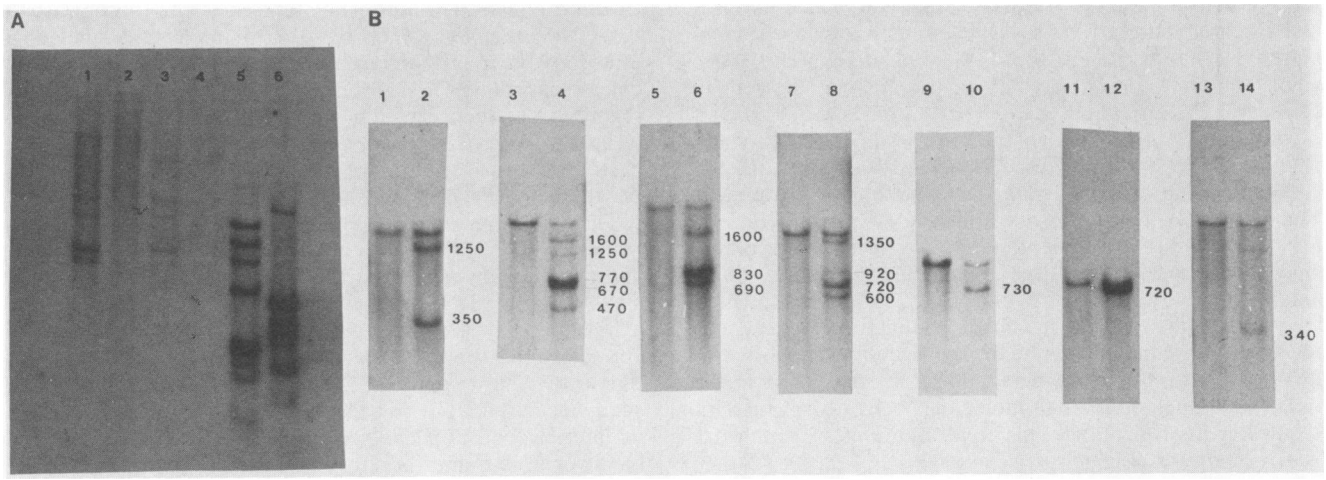


FIG. 7. Autoradiographs of S1- and exoVII-digested early DNA-RNA hybrids from the 5.0-kb *Eco*RI fragment. (A) Alkaline agarose gel of the S1 and exoVII digestion products of early vaccinia virus RNA hybridized to the ^{32}P uniformly labeled 5.0-kb fragment. The samples are as follows: (lane 1) S1-resistant DNA after hybridization to early vaccinia virus RNA; (lane 2) S1-resistant DNA with no added RNA; (lane 3) exoVII-resistant DNA after hybridization to early vaccinia virus RNA; (lane 4) exoVII-resistant DNA with no added RNA; (lane 5) ^{32}P-labeled φX174 DNA cleaved with *Hae*III with sizes of 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 77 nucleotides; (lane 6) 5′ ^{32}P-labeled pBr322 DNA cleaved with *Hin*fI with sizes of 1,631, 516, 506, 396, 344, 298, 221, and 154 nucleotides. (B) Alkaline agarose gel of the S1-resistant products of early vaccinia virus RNA hybridized to ^{32}P uniformly labeled fragments of the 5.0-kb *Eco*RI fragment. Uniformly labeled plasmid DNA was digested with endonucleases, and the purified fragments were used in S1 nuclease analysis. The restriction fragments used in S1 nuclease analysis are as follows: (lane 1) *Hae*III-B fragment, no RNA added; (lane 2) *Hae*III-B fragment hybridized to early RNA; (lane 3) *Hin*dIII-A fragment, no RNA added; (lane 4) *Hin*dIII-A fragment hybridized to early RNA; (lane 5) *Hae*III-A fragment, no RNA added; (lane 6) *Hae*III-A fragment hybridized to early RNA; (lane 7) *Bgl*II-A fragment, no RNA added; (lane 8) *Bgl*II-A fragment hybridized to early RNA; (lane 9) cDNA fragment, no RNA added; (lane 10) cDNA fragment hybridized to early RNA; (lane 11) *Bgl*II-D fragment, no RNA added; (lane 12) *Bgl*II-D fragment hybridized to early RNA; (lane 13) *Hin*dIII-B fragment, no RNA added; (lane 14) *Hin*dIII-B fragment hybridized to early RNA.

priate size and location to encode the 30K polypeptide. The uniformly labeled *Hin*dIII-A fragment protects five RNAs from S1 nuclease digestion, 1,600, 1,250, 770, 670, and 470 nucleotides in size (Fig. 7, lanes 3 and 4). Four of the five RNAs must correspond in size and position to the two 5′ ends (1,300 and 540 nucleotides) and the two 3′ ends (1,600 and 870 nucleotides) located previously with the end-labeled *Hin*dIII-A fragment.

These data suggest that the 5′ ends which are 1,300 and 540 nucleotides inward from the left *Hin*dIII site correspond, respectively, to the 1,250- and 470-nucleotide RNAs detected by the uniformly labeled *Hin*dIII-A fragment, whereas the 3′ ends which are 1,600 and 870 nucleotides inward from the right *Hin*dIII site correspond, respectively, to the 1,600- and 770-nucleotide RNAs detected by the uniformly labeled *Hin*dIII-A fragment. The additional RNA, 670 nucleotides in size, which is of an appropriate size to encode the 30K polypeptide, must be located somewhere within the *Hin*dIII-A fragment (Fig. 3E). This 670-nucleotide RNA is of the correct size such that it could be located in the central region of the *Hin*dIII-A fragment between the RNA whose 5′ end is 540 nucleotides inward from the left *Hin*dIII site and the RNA whose 3′ end is 870 nucleotides inward from the right *Hin*dIII site. This type of arrangement would be similar to the arrangement of the 5′ and 3′ ends of the mRNAs encoding, respectively, the 20K and 55K polypeptides. Moreover, this location is a region of DNA which is common to the *Hin*dIII-A, *Hae*III-A, and cDNA fragments. All three of these fragments hybrid select the mRNA encoding the 30K polypeptide, and the cDNA as well as the *Hae*III-A fragment hybridize to a common 950-nucleotide RNA as ascertained by RNA filter hybridization.

Additional S1 nuclease data with the uniformly labeled *Hae*III-A, *Bgl*II-A, *Bgl*II-D, and cDNA fragments confirm the map of the transcripts already elucidated and delineate the coordinates for the 670-nucleotide RNA complementary to the uniformly labeled *Hin*dIII-A fragments. The uniformly labeled *Hae*III-A fragment protects three RNAs from S1 nuclease digestion which are 1,600, 830, and 690 nucleotides in size (Fig. 7, lanes 5 and 6). The region of DNA common to the *Hae*III-A and *Hin*dIII-A fragments is located between the left *Hae*III site and the right *Hin*dIII site. When the 1,600-, 830-, and 690-nucleotide RNAs are arranged in this region, the 1,600- and 830-nucleotide RNAs correspond, respectively, to the 3′ ends located 1,600 and 870 nucleotides inward from the right *Hin*dIII site (Fig. 4E). The 690-nucleotide RNA could correspond to several RNAs: the 670-nucleotide RNA complementary to the uniformly labeled *Hin*dIII-A fragment, a portion of the RNA whose 5′ end is 1,250 nucleotides inward from the left *Hin*dIII site, or both of these RNAs. In any case, this locates the 670-nucleotide RNA complementary to the uniformly labeled *Hin*dIII-A fragment between the left *Hae*III site and the right *Hin*dIII site.

The uniformly labeled *Bgl*II-A fragment has three major S1 nuclease-resistant fragments, 1,350, 720, and 600 nucleotides, and a faint band at 920 nucleotides (Fig. 7, lanes 7 and 8). If the RNAs are aligned with the RNAs complementary to the *Hin*dIII-A fragment, the 1,350- and 600-nucleotide RNAs correspond, respectively, to the 5′ ends of the RNAs located 1,300 and 540 nucleotides inward from the left *Hin*dIII site (Fig. 4E). The 920-nucleotide RNA corresponds to a portion of the RNA whose 3′ end extends 1,600 nucleotides inward from the right *Hin*dIII site of the

*Hin*dIII-A fragment. Again, the 720-nucleotide RNA could correspond to several RNAs: the 670-nucleotide RNA complementary to the uniformly labeled *Hin*dIII-A fragment, a portion of the RNA whose 5′ end is 1,250 nucleotides inward from the left *Hin*dIII site, or both RNAs.

If the S1 nuclease data from the *Hin*dIII-A, *Hae*III-A, and *Bgl*II-A fragments are all correlated, the 670-nucleotide RNA complementary to the uniformly labeled *Hin*dIII-A fragment must be located within the left *Hae*III site and the right *Bgl*II site of the *Bgl*II-A fragment.

Further resolution of the position of the 670-nucleotide RNA was achieved with the uniformly labeled cDNA fragment. The major RNA protected from S1 nuclease digestion by the cDNA fragment was 730 nucleotides in size (Fig. 7, lanes 9 and 10). If the 670-nucleotide transcript complementary to the uniformly labeled *Hin*dIII-A fragment was close to the *Bgl*II site and was not located between the 5′ end of the RNA which is 540 nucleotides inward from the left *Hin*dIII site and the 3′ end of the RNA which is 870 nucleotides inward from the right *Hin*dIII site, the RNA protected by the uniformly labeled cDNA fragment would be expected to be much less than 670 nucleotides.

A compilation of all of the S1 nuclease mapping data of the *Hin*dIII-A fragment (Fig. 4) demonstrates that the RNA whose 5′ end is 1,300 nucleotides inward from the left *Hin*dIII site overlaps the entire sequence of the 5′ end of the mRNA encoding the 55K polypeptide which is 540 nucleotides inward from the left *Hin*dIII site. Moreover, the same 1,300-nucleotide RNA overlaps the sequences of the 670-nucleotide transcript whose 3′ end is adjacent to the 5′ end of the mRNA encoding the 55K polypeptide. Within the limits of this S1 nuclease analysis, the 5′ terminus of the 1,300-nucleotide RNA is coterminal with the 5′ end of the 670-nucleotide RNA. Conversely, an RNA whose 3′ end is 1,600 nucleotides inward from the right *Hin*dIII site overlaps the entire sequences of the RNA whose 3′ end is 870 nucleotides inward from the same *Hin*dIII site. In addition, the same 1,600-nucleotide RNA overlaps the sequences of a 670-nucleotide RNA whose 5′ end is adjacent to the 3′ end of the 870-nucleotide RNA. Within the limits of this S1 nuclease analysis, the 3′ end of the 670-nucleotide transcript is coterminal with the 3′ end of the 1,600-nucleotide RNA.

The 670-nucleotide RNA, located in the center of the *Hin*dIII-A fragment, has the appropriate size and location to encode the 30K polypeptide. The location of the 670-nucleotide RNA coincides with the mRNA encoding the 30K polypeptide, which is hybrid selected by the *Hae*III-A, *Hin*dIII-A, and cDNA fragments. With the addition of 200 adenylate residues to account for the poly(A) tail of an mRNA, the 670-nucleotide transcript approximates the size of the 950-nucleotide RNA which hybridizes to the *Hae*III-A and cDNA fragments on the RNA filter. Therefore, we conclude that the 670-nucleotide transcript, complementary to the uniformly labeled *Hin*dIII-A fragment, is the mRNA which encodes the 30K polypeptide.

Adjacent to the transcript encoding the 30K polypeptide is an RNA complementary to the uniformly labeled *Hae*III-A fragment which is 830 nucleotides in size. The location of this transcript suggests that it represents the 1,000-nucleotide RNA which hybridizes on the RNA filter to the *Hae*III-A fragment and not the cDNA fragment. Furthermore, the addition of 200 adenylate residues to the 830-nucleotide transcript would make it the approximate size of the 1,000-nucleotide RNA.

The uniformly labeled *Bgl*II-D fragment protects an RNA from S1 nuclease digestion which is 720 nucleotides in size (Fig. 7, lanes 11 and 12). This would suggest that the 5′ end of the 1,000-nucleotide RNA is 720 nucleotides from the left *Bgl*II site of the *Bgl*II-D fragment. This 1,000-nucleotide RNA does not program the cell-free synthesis of a polypeptide with either [^{35}S]methionine or [^{3}H]leucine as a label (data not shown).

When compared with the levels of the other RNAs in S1 nuclease analysis, the 1,000-nucleotide RNA appears to be the most abundant. The transcripts corresponding to this RNA are the 870-nucleotide RNA complementary to the 3′-end-labeled *Hin*dIII-A fragment (Fig. 6, lanes 7 and 8), the 770-nucleotide RNA complementary to the uniformly labeled *Hin*dIII-A fragment (Fig. 7, lanes 3 and 4), and the 830-nucleotide RNA complementary to the uniformly labeled *Hae*III-A fragment (Fig. 7, lanes 5 and 6).

Finally, the mRNA encoding the 10K polypeptide which was shown to be hybrid selected by both the *Hae*III-C and *Hin*dIII-B fragments was detected by using the uniformly labeled *Hin*dIII-B fragment. This fragment protected an RNA from S1 nuclease digestion which was 340 nucleotides in size. This would suggest that the RNAs of 2,350, 1,700, 1,350, and 400 nucleotides in size, which hybridize to the *Hae*III-C fragment on the RNA filter, all have a common 3′ end. However, these data alone cannot ascertain which of these RNAs encodes the 10K polypeptide.

Sizing of hybrid-selected mRNAs by fractionation in an agarose gel containing methyl mercury hydroxide and translation in a cell-free system. The combined mapping data have sized and located three of the four translatable mRNAs transcribed from the 5.0-kb *Eco*RI fragment. The 55K polypeptide is encoded by an mRNA which is 1,450 nucleotides as sized by RNA filter hybridization and 1,250 nucleotides when sized by the S1 nuclease procedure. Likewise, the 30K polypeptide is encoded by an mRNA whose size is 950 nucleotides on the RNA filter and 670 nucleotides in the S1 nuclease analysis. The mRNA encoding the 20K polypeptide was 780 nucleotides when sized by RNA filter hybridization, and a portion of the mRNA, 350 nucleotides, has been located by S1 nuclease analysis. As for the mRNA encoding the 10K polypeptide, the information derived from both the S1 nuclease and the RNA filter hybridization analyses cannot determine which of the 2,350-, 1,700-, 1,350-, and 400-nucleotide RNAs encodes the 10K polypeptide.

Moreover, in addition to the mature size RNAs encoding the 55K, 30K, and 20K polypeptides, there are high-molecular-weight RNAs of 5,000, 3,300, 2,300, and 1,800 nucleotides in size which are transcribed from the 5.0-kb fragment. At least two of these high-molecular-weight RNAs appear to be coterminal with the 5′ and 3′ ends of the mature size mRNA encoding the 30K polypeptide. A question remains regarding whether or not these RNAs can program the synthesis of polypeptides in the cell-free system.

To definitively size the mRNAs encoding each of these polypeptides, we have size fractionated the RNAs complementary to the 5.0-kb *Eco*RI fragment and determined which RNAs program the synthesis of each polypeptide in a cell-free system. In addition, this analysis ascertains that each polypeptide represents a complete round of translation and that each polypeptide is specified by a separate mRNA.

The size of the mRNA encoding each polypeptide was determined in the following manner. RNA complementary to the 5.0-kb region was prepared by hybrid selection, denatured with methyl mercury hydroxide, and fractionated by electrophoresis in an agarose gel containing methyl mercury hydroxide. Subsequently, the gel was sliced into 3-mm fractions, and the polypeptides encoded by the RNA eluted

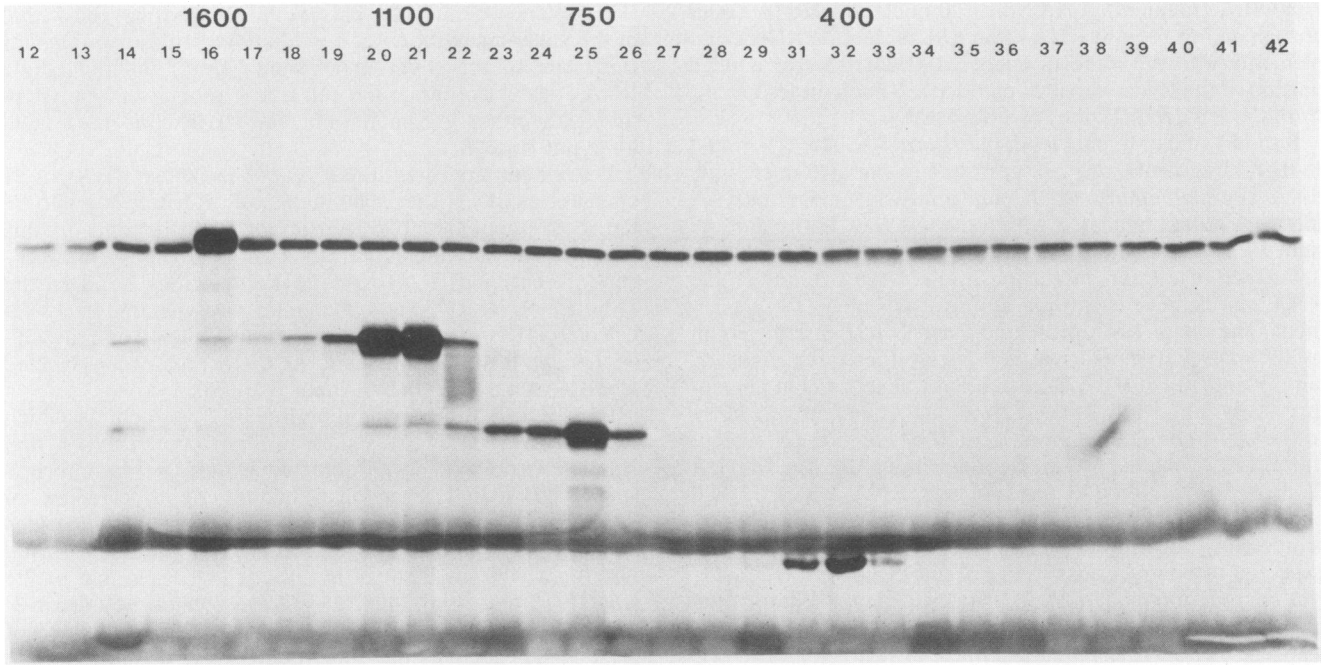


FIG. 8. Size distribution of translatable mRNAs complementary to the 5.0-kb *Eco*RI fragment. Fluorograph of an SDS–17.5% polyacrylamide gel of the [^{35}S]methionine-labeled cell-free products directed by mRNAs hybrid selected by the 5.0-kb *Eco*RI fragment and fractionated in a methyl mercury hydroxide-agarose gel. Plasmid DNA (50 μg) was immobilized on DBM paper and used to hybrid select mRNAs from 100 μg of total early vaccina virus RNA in a hybridization reaction of 100 μl. The eluted RNA was fractionated in a 1.3% low-melting-point agarose gel containing 12.5 mM methyl mercury hydroxide. The gel was sliced and the RNA was isolated from contiguous 3-mm portions of the gel. The isolated RNA from each fraction was then used to direct the synthesis of [^{35}S]methionine-labeled polypeptides in a 12.5-μl reticulocyte cell-free system. Equal volumes (5 μl) of each translation reaction were fractionated on an SDS–17.5% polyacrylamide gel, and the dried gel was fluorographed for 14 h. Only fractions 12 to 42 are shown because fractions 1 to 11 did not program the synthesis of any [^{35}S]methionine-labeled polypeptides. The sizes of the mRNAs in nucleotides were computed from the migration of DNA restriction fragments of known molecular weight, namely, the *Hae*III digest of simian virus 40 DNA and the *Bgl*I digest of pBr322.

from each fraction were defined by in vitro translation. The fluorograph of the [^{35}S]methionine-labeled cell-free products after fractionation by SDS-polyacrylamide gel electrophoresis is shown in Fig. 8.

The majority of the 55K, 30K, 20K, and 10K polypeptides are encoded by distinct and separate mRNAs which are, respectively, 1,600, 1,050, 750, and 400 nucleotides in size. It should be noted, however, that there are also low levels of the 30K and 20K polypeptides encoded by larger mRNAs. These mRNAs range in size from 2,100 to 980 nucleotides, which encode the 30K polypeptide, and 980 to 560 nucleotides, which encode the 20K polypeptide. These mRNAs could be greater in size because of additional sequences at either the 5′ or 3′ region of the mRNA. From this analysis, the synthesis of the trace quantities of the 30K and 20K polypeptides from larger mRNAs could be due to two types of mRNAs: separate, distinct species that encode the individual polypeptide, or a single mRNA that could program the synthesis of both polypeptides.

The sizes of the translatable mRNAs for the 55K, 30K, and 20K polypeptides are in close agreement with the sizes as determined by RNA filter hybridization and S1 nuclease analyses. Although size fractionation of mRNAs in an agarose gel containing methyl mercury hydroxide and subsequent translation in a cell-free system comprise a definitive assay for the size of a translatable mRNA, the assay is perhaps the least accurate of the RNA sizing techniques due to the perturbation of the agarose gel during the slicing and pooling of the contiguous gel fractions. This is probably reflected in the larger size of the mRNA encoding the 55K polypeptide, 1,600 versus 1,450 nucleotides.

Nevertheless, this procedure has given reasonable sizes for the mRNAs encoding the 55K, 30K, and 20K polypeptides, consistent with the previous mapping data. Moreover, we can conclude that the 10K polypeptide is encoded by the 400-nucleotide RNA complementary to the *Hae*III-C fragment and that the 2,350-, 1,700-, and 1,350-nucleotide RNAs cannot program the synthesis of the 10K polypeptide in a cell-free system.

DISCUSSION

Essential to unraveling the mechanisms controlling the temporal regulation of vaccinia virus genes as well as the biogenesis of their mRNAs is an understanding of the organization of viral RNA transcripts. Towards this goal we have located the mRNAs transcribed from a 5.0-kb *Eco*RI fragment which encompasses the *Hin*dIII-N, -M, and -K fragments, previously shown to be located between 21 and 26 kb from the left terminus of the vaccinia virus genome (19).

Transcribed in the leftward direction from the same DNA strand of this 5.0-kb fragment are four early mRNAs whose synthesis commences within 30 min after the addition of virus to L-cells (J. R. Morgan and B. E. Roberts, unpublished data). The left 21 kb of the vaccinia virus genome has been extensively mapped by Moss and co-workers (10, 11, 44, 45), and of the 11 early mRNAs they have located, 9 are transcribed in the same leftward direction. Moreover, the

mRNAs encoding six early polypeptides have been located in the *Hin*dIII-F fragment which is adjacent to the *Hin*dIII-K fragment. All six of these early mRNAs are also transcribed towards the left terminus (14). Taken together, 19 of the 21 early mRNAs encoded by 39 kb of the left terminus are transcribed in the leftward direction.

The overall pattern of the transcription of early genes of the entire genome remains to be established. However, the information currently available suggests some general rules for the organization of early genes and their transcription. The genes encoding early polypeptides are distributed throughout the vaccinia virus genome. Early genes are usually grouped together as clusters. Within a given cluster, the RNA transcripts of the genes are closely spaced and transcribed from the same DNA strand. In addition, that 19 of 21 early mRNAs encoded by the left 39 kb of the genome are transcribed in the leftward direction suggests that the mRNAs of adjacent clusters are usually transcribed in the same direction.

This type of arrangement is conceivably a mechanism for the coordinate control and rapid expression of early mRNAs. Early upon infection, vaccinia virus is known to induce the synthesis of several enzymatic activities which are probably encoded by the viral genome (24). Enzymes such as the capping enzyme complex, poly(A) polymerase, and DNA-dependent RNA polymerase are multimeric enzymes composed of heterologous subunits. Since the subunits of these enzymes are assembled in precise stoichiometric amounts, the synthesis of these polypeptides would be expected to be regulated. One manner in which the coordinate regulation of the synthesis of various subunits could be mediated is by the arrangement of related early genes into gene clusters. The control of transcription within a gene cluster and between gene clusters could facilitate the coordinate synthesis of the appropriate amounts of individual subunits.

The organization of early genes into clusters transcribed from the same DNA strand could also facilitate their rapid expression. We have detected, by hybrid selection, the mRNAs encoding early polypeptides as early as 0.5 h after the addition of virus. Since each virion has an estimated 150 to 200 RNA polymerase molecules and over 75 early genes must be transcribed at the start of the infectious cycle (24), the predominant coding of clusters of early mRNAs by one DNA strand may facilitate their rapid synthesis.

The refinement of the organization of the transcripts originating from the 5.0-kb fragment was a compilation of the information derived from several complementary techniques: (i) hybrid selection to locate DNA sequences complementary to mRNAs encoding specific polypeptides; (ii) RNA filter hybridization to size and locate, on the DNA, mature RNAs as well as higher-molecular-weight RNAs; (iii) S1 nuclease mapping to determine whether or not these transcripts are colinear with their DNA templates and to precisely locate their 5′ and 3′ termini; (iv) fractionation of hyrid-selected mRNAs in an agarose gel containing methyl mercury hydroxide followed by the cell-free translation of these mRNAs to ascertain the size of each mRNA encoding each polypeptide.

This combined information demonstrates that, within this 5.0-kb *Eco*RI DNA fragment, there are four early mRNAs encoding polypeptides of molecular weights 55K, 30K, 20K, and 10K that are (i) transcribed from the same DNA strand, (ii) colinear with the DNA template, (iii) arranged such that the 5′ terminus of one mRNA is within 50 bp of the 3′ terminus of the adjacent mRNA, and (iv) contained within and coterminal with the sequences of the higher-molecular-weight RNAs.

In addition, we have detected one poly(A^+) RNA for which there is no corresponding polypeptide. The 1,000-nucleotide RNA adjacent to the mRNA encoding the 30K polypeptide does not program the synthesis of a polypeptide in the reticulocyte lysate with either [^{35}S]methionine or [^{3}H]leucine as label. The function of this RNA is unknown. One possible explanation is that this gene may have been mutated to a pseudogene whose mRNA is not fully translated because of the premature termination of translation. In support of this possibility is the fact that a deletion mutant of vaccinia virus which lacks 9.0 kb of DNA encoding eight early genes is still viable (26, 27). This would suggest that the viral genome contains nonessential genes which are not required for growth in tissue culture and that mutations could accumulate in these genes without affecting the viability of the virus.

The map of the RNAs transcribed from the 5.0-kb *Eco*RI fragment explains the enigma of a cDNA which hybrid selects two of these early mRNAs. The cDNA is not an exclusive copy of the sequences of a single mature mRNA but instead represents a portion of a higher-molecular-weight RNA. In fact, this cDNA contains the sequences of the 5′ start of the mRNA encoding the 55K polypeptide as well as sequences from the 3′ terminus of the adjacent mRNA encoding the 30K polypeptide. As a result, the cDNA pcE5 can hybrid select the mRNAs encoding both of these polypeptides.

The presence of high-molecular-weight RNAs transcribed from this region has been detected by S1 nuclease analysis and most convincingly by RNA filter hybridization. The largest RNA of 5,000 nucleotides is big enough to encompass the sequences of the mRNAs encoding the 55K, 30K, and 20K polypeptides. In addition, intermediate sized RNAs of 3,300, 2,300, and 1,800 nucleotides are obvious, and conceivably the cDNA could specify a portion of the sequences of any of these RNAs. The presence of high-molecular-weight RNAs which are overlapping readthrough transcripts has been detected in other regions of the viral genome (5, 11, 14, 20, 44, 45).

The location of the position of the 5′ and 3′ termini of the RNAs within the *Hin*dIII-A fragment as determined by S1 nuclease indicates that the termini of two high-molecular-weight RNAs coincide with the 5′ and 3′ termini of the mature mRNA encoding the 30K polypeptide. From the knowledge of this arrangement, two predictions can be made regarding the biogenesis of these early mRNAs as well as the nature of the high-molecular-weight RNAs.

Transcription for the entire cluster of genes could begin at a single promoter located between 24.5 and 25.5 kb from the left terminus, whereby the RNA polymerase would transcribe a large primary transcript that would be subsequently cleaved to yield mature size mRNAs. That being the case, the high-molecular-weight RNAs could be intermediates in a cleavage pathway for vaccinia virus early mRNA biogenesis, which has been previously postulated (29, 40). The enzyme 5′-phosphate polyribonucleotide kinase, which could facilitate the formation of 5′-terminal cap structures at RNA cleavage sites, has been isolated from vaccinia virions (40). This enzyme catalyzes the conversion of 5′-monophosphate-terminated RNA chains to di- and triphosphate-terminated RNA molecules, using the (γ) phosphate group of ATP. After a hypothetical RNA cleavage event, the resultant internal RNA could have either a 5′-OH or a 5′-monophosphate terminus depending on the specificity of the site-

specific RNase. Neither of these termini is a substrate for the vaccinia virus capping enzyme. The 5'-phosphate polyribonucleotide kinase could convert the monophosphate terminus to a di- or triphosphate terminus, both of which are suitable substrates for the capping enzyme complex.

The second possibility is that each gene contains the sequences responsible for the promotion and termination of transcription by the viral DNA-dependent RNA polymerase. In this event, the high-molecular-weight RNAs might not be involved in a cleavage pathway of mRNA biogenesis but instead represent products of improper termination by RNA polymerase and readthrough to a downstream termination signal. Therefore, the DNA sequences responsible for the promotion and termination of transcription would be expected to be confined to the 50-bp region between the 5' and 3' termini of the mature mRNAs. At another early gene cluster located in the inverted terminal repeat, UV inactivation studies and the labeling of RNA termini with [β-^{32}P]GTP suggested that the transcription of these genes was independently initiated (10, 41). However, in contrast to the mRNAs encoded by the 5.0-kb *Eco*RI fragment, the 5' and 3' termini of the mRNAs encoded by the inverted terminal repeat are separated by more than 2.0 kb of DNA.

In addition to the DNA sequences which promote transcription in this early gene cluster, the presence of DNA sequences which terminate transcription can be inferred from the locations of the 3' termini of the RNAs. There are two types of 3' ends located in this early gene cluster. The first category is exemplified by the 3' ends of the mRNAs encoding the 55K and 30K polypeptides. These 3' ends are located on mRNAs which are situated in the center of the early gene cluster and are in close proximity ($\leq$50 bp) to the 5' ends of the adjacent mRNAs. In addition, there are high-molecular-weight RNAs which are coterminal with these 3' ends as well as larger RNAs which span the inter-mRNA region occupied by these 3' termini.

The second category of 3' ends is exemplified by the mRNA encoding the 10K polypeptide. The 3' end of this mRNA is located 1,000 bp from the 5' end of the nearest RNA. Moreover, the mRNA encoding the 10K polypeptide is not centrally located but is situated at the 3' end of possibly another early gene cluster located in the adjacent *Hin*dIII-F fragment. The 3' end of the mature mRNA is coterminal with the 3' ends of three larger RNAs, 2,350, 1,700, and 1,350 nucleotides in size. Unlike the first category, there is no evidence that these larger RNAs extend past the 3' end of the mRNA encoding the 10K polypeptide.

The distinctions between the 3' ends may reflect differences in the functions of the DNA sequences at the ends of each gene. Two processes are potentially mediated by the 3'-proximal DNA sequences: termination of transcription or RNA cleavage or both. The location of the RNA transcripts suggests that the DNA sequences proximal to the 3' end of the mRNA encoding the 10K polypeptide mediate solely the termination of RNA transcription. At this single site, the transcription of four distinct early RNAs are terminated and there is no evidence for a transcript which reads through this site.

On the other hand, the DNA sequences proximal to the 3' end of the mRNA encoding the 30K polypeptide could be involved in either the termination of transcription or RNA cleavage. If these DNA sequences serve to terminate transcription, the termination signal is not as strong as the one located near the 3' end of the mRNA encoding the 10K polypeptide. This point is supported by the fact that the RNA polymerase is able to transcribe high-molecular-weight RNAs through this region. Alternatively, these sequences could specify a site for cleavage by a site-specific RNase. The high-molecular-weight RNAs contain the sequences of this intergene region, and these sequences could serve as a recognition site for processing. It remains to be established whether these intergene DNA sequences mediate the termination of transcription or direct the action of a site-specific RNase.

In summary, we have determined the organization of the RNAs transcribed from an early gene cluster encoded by vaccinia virus. The genes encoding four early polypeptides are tandemly arranged and are transcribed from the same DNA strand. The 5' and 3' termini of the mature mRNAs are closely spaced and are coterminal with the 5' and 3' termini of high-molecular-weight RNAs. The information derived from the map of these transcripts will help to determine whether the high-molecular-weight RNAs are precursors to the mature mRNAs. Moreover, the DNA elements responsible for the promotion and termination of transcription can be inferred from the locations of the 5' and 3' termini. These elements can be incorporated into chimeric transcription units to further our understanding of the regulation of transcription and to mediate the high-level expression of heterologous genes by vaccinia virus.

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