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Vaccinia Virus Thymidine Kinase and Neighboring Genes: mRNAs and Polypeptides of Wild-Type Virus and Putative Nonsense Mutants

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Enzymatically active thymidine kinase (TK) was made in reticulocyte lysates programmed with early vaccinia mRNA that hybridized to plasmid recombinants containing either of two adjacent small DNA subsegments of the viral *Hin*dIII-J fragment. The map position of an early polypeptide, with a molecular weight of 19,000 (19K), coincided precisely with that of the TK. The absence of the 19K polypeptide in cell-free translation products of hybridization-selected mRNAs from several TK-negative mutants provided an independent identification of the TK polypeptide. The small size of the TK polypeptide of vaccinia virus distinguishes it from that of procaryotes, eucaryotes, and herpesvirus. Five early mRNAs of 3,840, 2,390, 1,790, 1,070, and 590 nucleotides were mapped within the *Hin*dIII-J fragment by RNA blotting and nuclease S1 digestion of RNA-DNA hybrids. The RNAs of 590 and 2,380 nucleotides were found to have 5′ coterminal ends and represent major and minor forms, respectively, of the TK message. The 3′ end of the minor TK mRNA appeared to be coterminal with the 3′ end of the 1,790-nucleotide transcript which encodes a 41K polypeptide. The 1,070-nucleotide RNA was identified as the message for a 21K polypeptide. All of these RNAs, including the two forms of the TK message, were made by the putative TK-negative nonsense mutants.

Thymidine kinase (TK; EC 2.7.1.75), an enzyme of the pyrimidine salvage pathway that catalyzes phosphorylation of thymidine to thymidine 5′-monophosphate, is present in procaryotes and eucaryotes. The dispensability of TK under usual laboratory conditions and the ability to readily select TK-positive (TK^+) or TK^- variants have made the enzyme particularly useful for studying gene expression (27). In addition, the herpesvirus TK has served as a selectable marker for genetic engineering (21, 33). Vaccinia virus also encodes TK; however, further characterizations of the enzyme, mRNA, and gene are required to exploit suggested uses of this system (32).

An early increase in TK activity within the cytoplasm of TK^- cells infected with vaccinia virus and the isolation of TK^- vaccinia mutants led Dubbs and Kit (10) to propose that the enzyme is virus coded. More recent experiments by Hruby and Ball (14) indicated an accumulation in vaccinia virus-infected cells of mRNA that can be translated in vitro to form enzymatically active TK. Marker rescue experiments from our laboratory (32) demonstrated that the TK gene maps within the 5,000-base-pair (bp) *Hin*dIII-J fragment of the 180,000-bp DNA genome. Furthermore, enzymatically active TK was made in reticulocyte lysate translation systems programmed with mRNAs selected by hybridization to the *Hin*dIII-J fragment (32; Hruby and Ball, personal communication). Inspection of a translational map of the vaccinia genome (2) shows that the *Hin*dIII-J fragment encodes several polypeptides that are possible candidates for TK. The objectives of the present investigation were to further map the TK gene and identify its mRNA and polypeptide products.

MATERIALS AND METHODS

Cells. Human 143 cells, a TK^- variant of cell line R970-5 (23) from K. Huebner (Wistar Institute, Philadelphia, Pa.), were maintained in Eagle medium supplemented with 8% fetal bovine serum and 25 μg of 5-bromodeoxyuridine per ml.

Virus. Wild-type vaccinia virus (strain WR) was originally obtained from the American Type Culture Collection, and independent TK^- mutants were selected in the presence of 5-bromodeoxyuridine (32).

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Recombinant plasmids and phage. A pBR322 plasmid (4) containing the vaccinia virus *Hin*dIII-J fragment was described previously (2, 32). Recombinants containing subfragments of *Hin*dIII-J were constructed by using pBR322 (3) and derivatives of phage M13 (19, 20).

Purification of RNA. Cytoplasmic RNA was obtained by Dounce homogenization of cells infected at a multiplicity of 15 PFU of vaccinia virus and purified by CsCl centrifugation as described previously (7). Routinely, early RNA was isolated 4 h after infection in the presence of 100 μg of cycloheximide per ml. When specified, 40 μg of cytosine arabinoside per ml was used instead. Late RNA was isolated at 6 h after infection in the absence of drugs.

Hybridization selection. Approximately 20 μg of recombinant DNA was boiled in 0.5 ml of 0.2 N NaOH for 8 min and then diluted with 14.5 ml of ice-cold 6.6× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate) containing 10 μl of concentrated HCl. The solution was passed at about 1 ml/min through a 2.5-cm-diameter nitrocellulose filter that had been prepared by boiling in 0.1% sodium dodecyl sulfate for 15 min. The filter was then washed with 10 ml of 6.6× SSC and baked at 80°C for 2 h. Approximately one-third of the filter was cut into pieces approximately 3 by 3 mm and incubated with 350 μg of cytoplasmic RNA in 0.3 ml of 80% formamide (deionized)–0.4 M NaCl–0.04 M Na PIPES [sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–1 mM EDTA–0.1% sodium dodecyl sulfate at 42°C for 18 h (8). Filters were washed briefly at room temperature with three 5-ml portions of 10 mM Tris-hydrochloride (pH 7.6)–0.1% sodium dodecyl sulfate followed by three 5-ml washes with 10 mM Tris-hydrochloride (pH 7.6). Filters were then incubated two times in succession at 42°C for 15 min in 1 ml of hybridization buffer lacking sodium dodecyl sulfate and then rinsed at room temperature with 0.08 M NaCl–0.04 M Na PIPES (pH 6.4)–1 mM EDTA–80% formamide. Specifically bound RNA was eluted at 65°C for 10 min with 0.3 ml of 0.04 M Na PIPES (pH 6.4)–1 mM EDTA–90% formamide. The liquid was removed, and 0.075 ml of 1 M sodium acetate (pH 5.5) containing 2.25 μg of calf liver tRNA was added followed by 3 volumes of 95% ethanol. After storage at −20°C overnight, the precipitate was collected by centrifugation and washed twice with 75% ethanol and dried briefly in vacuo.

Size fractionation of translatable mRNA. Approximately 4 mg of cytoplasmic RNA, purified from cells infected with vaccinia virus in the presence of cycloheximide, was hybridized to 80 μg of immobilized recombinant DNA containing the *Hin*dIII-J fragment. The selected RNA was ethanol precipitated, treated with 10 mM methylmercuric hydroxide at 60°C in a total volume of 50 μl, and then subjected to electrophoresis in a 1.25% agarose gel (3 mm by 16 cm) containing 5 mM methylmercuric hydroxide (1, 8). Denatured phage λ DNA restriction fragments were run in parallel as size markers. Electrophoresis was carried out at 20 V until the bromophenol blue marker was 3 cm from the bottom of the gel. After detoxification in 0.5 M ammonium acetate, the gel was sliced, and the RNA was eluted as previously described (8, 16).

Cell-free translation. Cell-free translations were carried out for 50 min at 30°C with [^{35}S]methionine as described previously (7), except that 0.01 M thymidine was added to stabilize the TK (14). When TK assays were performed, 1 mM unlabeled methionine was added instead of radioactively labeled amino acid, and translation was continued for up to 4 h.

RNA blots. Purified cytoplasmic RNA was treated with 10 mM methylmercuric hydroxide and subjected to agarose gel electrophoresis in 5 mM methylmercuric hydroxide as indicated above. After detoxification of the gel, the RNA was transferred to nitrocellulose as described by Thomas (28), except that 6.6× SSC was used. The nitrocellulose sheet was baked at 80°C for 2 h, cut into 5-mm-wide strips, and then treated with prehybridization buffer (28) at 42°C for at least 24 h. Recombinant DNA was labeled in vitro with ^{32}P by nick translation (25) and then incubated with the nitrocellulose sheet in hybridization buffer (28) at 42°C for 48 h. The sheets were washed (28), and X-ray film fluorographs were prepared with an intensifying screen.

Nuclease S1 analysis. Hybrids between vaccinia virus RNA and purified ^{32}P-labeled vaccinia virus DNA fragments were formed in 30 μl of 80% formamide–0.4 M NaCl–0.04 M PIPES (pH 6.4)–0.001 M EDTA at 42°C for 16 h, treated with nuclease S1, and analyzed by electrophoresis in 2% neutral or alkaline agarose gels (34).

Materials. Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. Nitrocellulose filters (0.45 μm) were from Schleicher & Schuell Co., [^{35}S]methionine was from New England Nuclear Corp., and [γ-^{32}P]ATP and α-^{32}P-nucleotide triphosphates were from Amersham-Searle.

RESULTS

Localization of the TK gene within the *Hin*dIII J fragment. The TK gene has been mapped by marker rescue and cell-free translation of hybrid-selected mRNA to the 5,000-bp *Hin*dIII-J fragment located near the center of the vaccinia virus genome. To facilitate further studies, restriction maps of the J fragment were derived by agarose gel electrophoresis of single- and double-enzyme digests of a recombinant plasmid containing this genomic segment (2). With this information, a series of pBR322 subclones, pJ1 to pJ4, was constructed (Fig. 1). Early mRNA, purified from the cytoplasm of cells infected with vaccinia virus in the presence of cycloheximide, an inhibitor of protein synthesis, was hybridized to the recombinant plasmids immobilized on nitrocellulose filters. Specifically selected mRNAs were translated in a micrococcal nuclease-treated reticulocyte cell-free system (22), and the synthesis of TK was measured by a functional enzyme assay (14). Elevated TK activity was found in lysates programmed with mRNA that hybridized to the left side of the *Hin*dIII-J fragment (Table 1), suggesting that the gene is located between the left *Hin*dIII site and the *Ava*I site and crosses the unique *Eco*RI site.

To determine the direction of transcription

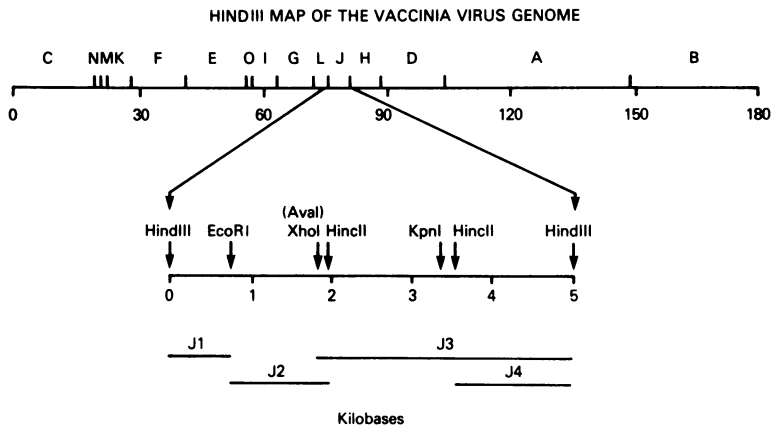

FIG. 1. Restriction endonuclease maps and construction of plasmid recombinants. Restriction endonuclease maps were prepared by agarose gel electrophoresis of single- and double-enzyme digests of a recombinant pBR322 plasmid containing the *Hin*dIII-J fragment. Recombinant plasmids containing segments of the *Hin*dIII-J fragment were prepared by standard methods. The DNA segments contained within these recombinants, designated pJ1 to pJ4, are indicated.

and confirm hybridization of TK mRNA to the left *Hin*dIII-*Eco*RI fragment, the latter segment of *Hin*dIII-J was ligated to *Hin*dIII- and *Eco*RI-cleaved replicative forms of mp8 and mp9 derivatives of phage M13 (19, 20). Since the *Hin*dIII and *Eco*RI sites of mp8 and mp9 are in opposite orientations, opposite strands of the vaccinia virus DNA segment were present in the single-stranded DNAs of mp8 and mp9, respectively. The TK message was selected specifically by hybridization to the phage M13mp8 recombinant containing the rightward-reading strand of vaccinia virus DNA immobilized on nitrocellulose filters as judged by enzyme assays after translation of the eluted RNA.

Mapping of polypeptides within the *Hin*dIII-J fragment. Three major polypeptides of 17K, 21K, and 41K are synthesized in reticulocyte lysates programmed with early mRNA selected by hybridization to the *Hin*dIII-J fragment (2). To determine whether one or more of these are TK subunits, mRNA was selected by hybridization to the cloned subfragments of *Hin*dIII-J and translated in a reticulocyte lysate containing [^{35}S]methionine. A fluorograph of translation products resolved by polyacrylamide gel electrophoresis is shown in Fig. 2. A 19K polypeptide (previously estimated as 17K [2]) was synthesized with mRNA that hybridized to recombinants pJ1 and pJ2, making it a candidate for the TK subunit. The relative amounts of the 19K polypeptide made with RNA that hybridized to pJ1 and pJ2 varied somewhat, probably reflecting differences in the efficiency of DNA immobilization, hybridization, and recovery after ethanol precipitations. In some experiments, prolonged fluorographic exposure revealed a very faint 19K band with RNA that hybridized to pJ3 (Fig. 2). To further correlate the 19K polypeptide with the TK subunit, RNA that hybridized to the rightward-reading strand of the *Hin*dIII-*Eco*RI fragment cloned in phage M13mp8 was translated. The only product detected was the 19K polypeptide.

The mRNA for the 41K polypeptide hybridized to recombinants pJ2 and pJ3, whereas mRNA for the 21K polypeptide hybridized to pJ3 exclusively (Fig. 2). Thus, these two polypeptides map to the right of TK. No major early polypeptides were detected by translation of mRNA that hybridized to the extreme right fragment contained in pJ4 (Fig. 2).

When early mRNA made in the presence of cytosine arabinoside (40 μg/ml), an inhibitor of DNA synthesis, was hybridized to the cloned

TABLE 1. Translation of TK mRNA selected by hybridization to recombinant plasmids

Recombinant plasmid[a]	TK activity[b] (cpm × 10^3)
pJ1	34
pJ2	145
pJ3	7
pJ4	8

[a] Recombinant plasmids are identified in the legend to Fig. 1.

[b] Early RNA that hybridized to the designated recombinant DNA immobilized on nitrocellulose filters was translated in reticulocyte lysates. Samples of the latter were assayed for TK activity, and background values obtained with control lysates were subtracted.

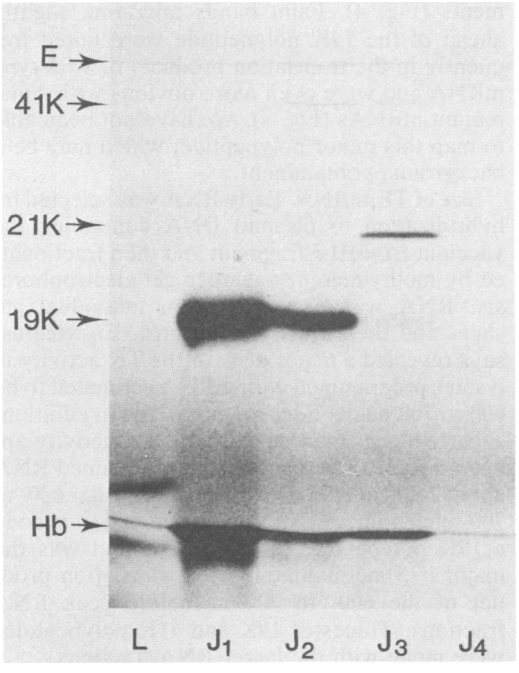

FIG. 2. Cell-free translation products of early RNA selected by hybridization to cloned subfragments of *Hin*dIII-J. Purified cytoplasmic RNA, obtained 4 h after infection of cells in the presence of cycloheximide, was hybridized to recombinants containing the *Hin*dIII-L fragment or *Hin*dIII-J subfragments indicated in Fig. 1. The selected mRNAs were eluted and translated in a reticulocyte cell-free system containing [^{35}S]methionine, and the products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular weights were determined by coelectrophoresis of labeled marker proteins. A fluorograph is shown. E, Endogeneous protein band; Hb, globin band.

subfragments of *Hin*dIII-J, a polypeptide pattern similar to that shown in Fig. 2 was obtained. However, less translatable RNA was isolated under these conditions.

Previous studies (2) indicated that some late mRNAs are encoded within the adjacent *Hin*dIII-L, -J, and -H fragments. The resolution provided by two-dimensional polyacrylamide gel electrophoresis suggested that multiple polypeptides of about 30K are encoded within *Hin*dIII-L and that at least one of these is also made by RNA selected to *Hin*dIII-J. To determine whether this late transcript overlaps the putative TK gene, mRNA was isolated from cells at 6 h after infection and hybridized to the cloned subfragments of *Hin*dIII-J as well as to recombinants containing *Hin*dIII-L and -H. The [^{35}S]methionine cell-free translation products of the selected mRNAs were similar to those found before (2), although the molecular weight assignments are slightly different (Fig. 3). The mRNA for the major late 28K polypeptide of *Hin*dIII-L appeared to hybridize to the *Hin*dIII-*Eco*RI (pJ1) and the *Eco*RI-*Hin*cII (pJ2) subfragments of *Hin*dIII-J, suggesting that it overlaps the TK gene. Additional data (J. Weir and B. Moss, unpublished observations) indicate that the mRNAs for the late polypeptide and TK are transcribed from the same DNA strand. The translation products in Fig. 3 also suggest that some mRNAs encoding early polypeptides including TK are still present at 6 h after infection. We have not accumulated sufficient data to comment on other late polypeptides encoded within *Hin*dIII fragments L, J, and H.

Analysis of putative nonsense mutants. Although the mapping data served to identify the 19K polypeptide as the TK subunit, we wished to obtain independent evidence. For this purpose, we used four independent TK$^-$ mutants that were isolated as individual plaques by 5-bromodeoxyuridine selection (32). No increases in TK activity were detected in cytoplasmic extracts of TK$^-$ 143 cells infected with these mutants, and each mutant could be rescued with the recombinant *Hin*dIII-J fragment originally derived from the wild-type virus. Since the *Hin*dIII-J fragments of the TK$^-$ mutants and wild-type virus comigrated on agarose gel electrophoresis (data not shown), the presence of large deletions could be ruled out.

We considered that if any of the mutants were

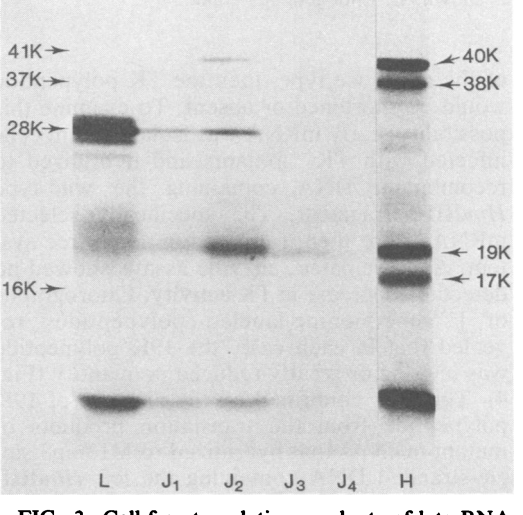

FIG. 3. Cell-free translation products of late RNA selected by hybridization to recombinants containing *Hin*dIII-L, subfragments of *Hin*dIII-J, or *Hin*dIII-H. Purified cytoplasmic RNA was isolated 6 h after infection and selected, translated, and analyzed as in Fig. 2.

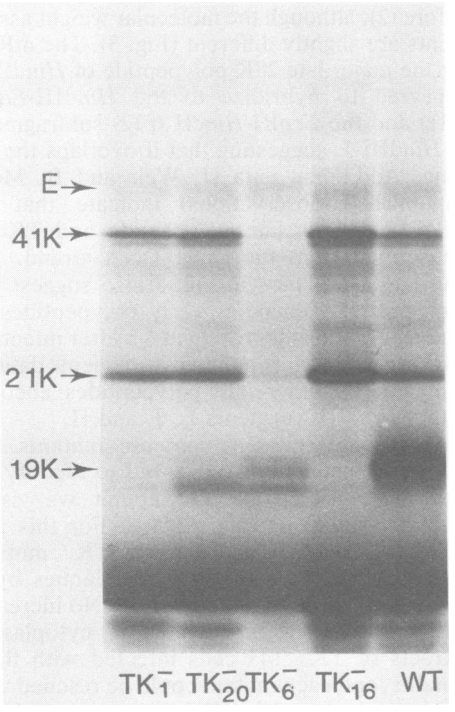

FIG. 4. Cell-free translation products of immediate early mRNAs of wild-type and of TK$^-$ mutants. Purified cytoplasmic RNAs from cells infected with wild-type virus (WT) and four independently isolated TK$^-$ mutants (TK$^-_1$, TK$^-_{20}$, TK$^-_6$, and TK$^-_{16}$) were selected by hybridization to *Hin*dIII-J recombinant DNA and translated in a reticulocyte lysate containing [^{35}S]methionine. Translation products were resolved by polyacrylamide gel electrophoresis. A fluorograph is shown. E, Endogeneous band.

of the nonsense type, then the TK polypeptide would be shortened or absent. To examine this possibility, early mRNA was isolated from cells infected with TK$^-$ mutants and hybridized to recombinant DNA containing the wild-type *Hin*dIII-J fragment. The specifically selected mRNAs were then translated in a cell-free system. As anticipated, enzyme assays showed no detectable increase in TK activity. Fluorographs of [^{35}S]methionine-labeled polypeptides revealed that in each case, the 19K polypeptide was missing or greatly reduced in quantity (Fig. 4). This was confirmed by the absence of 19K polypeptide from the translation products of mutant mRNAs that hybridized to M13mp8 single-stranded DNA containing the left *Hin*dIII-*Eco*RI segment. Significantly, with mRNA from three of the four mutants, the 21K and 41K polypeptides were made in usual amounts (Fig. 4). The fourth mutant, TK$^-$ 6, appeared odd in that several polypeptides were reduced and a faint 19K polypeptide was seen in some experiments (Fig. 4). Faint bands migrating slightly ahead of the 19K polypeptide were noted frequently in the translation products of wild-type mRNA and were even more obvious with some mutant mRNAs (Fig. 4). We have not been able to map this minor polypeptide, which may be a background contaminant.

Size of TK mRNA. Early RNA was selected by hybridization to plasmid DNA containing the vaccinia *Hin*dIII-J fragment and then fractionated by methylmercury-agarose gel electrophoresis. RNA was extracted from individual gel slices and then translated in vitro. Enzyme assays revealed a major peak of the TK activity in lysates programmed with mRNA estimated to be 600 to 700 nucleotides long (Fig. 5). In addition, slightly higher than background TK activity appeared to peak in reactions that contained RNA about 2,200 nucleotides long. The smaller 600- to 700-nucleotide mRNA is large enough to encode a 19K polypeptide; as expected, that was the major [^{35}S]methionine-labeled translation product of the 600- to 700-nucleotide peak RNA fractions. Traces of 19K and 41K polypeptides were made with the larger RNA fractions.

Size and map positions of early mRNAs encoded within *Hin*dIII-J fragment. Purified cytoplasmic RNA from cells infected with the wild type and TK$^-$ mutants of vaccinia virus was fractionated by methylmercuric hydroxide-ag-

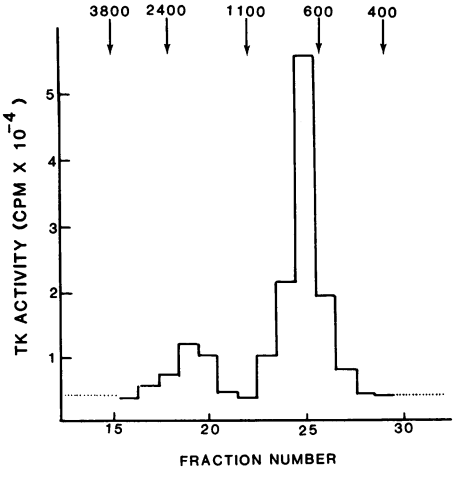

FIG. 5. Synthesis of enzymatically active TK in cell-free extracts programmed with hybridization-selected and size-fractionated mRNA. Purified early cytoplasmic RNA was selected by hybridization to immobilized *Hin*dIII-J recombinant DNA and then fractionated by electrophoresis on methylmercury agarose gel. Eluted RNA was translated in reticulocyte lysates and assayed for TK activity. Values for nucleotide size were obtained by coelectrophoresis of denatured DNA restriction fragments.

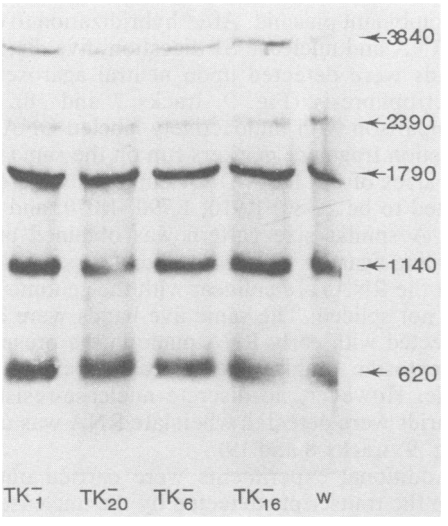

FIG. 6. Hybridization of ^{32}P-labeled *Hin*dIII-J DNA to RNA species resolved by methylmercuric hydroxide agarose gel electrophoresis. Approximately 80 μg of purified cytoplasmic RNA from cells infected with the wild type (w) and four independent TK$^-$ mutants in the presence of cycloheximide was denatured and subjected to electrophoresis in a 1.25% agarose gel containing 5 mM methylmercuric hydroxide. After transfer to a nitrocellulose filter, the RNA was hybridized to ^{32}P-labeled plasmid DNA containing the *Hin*dIII-J fragment of vaccinia virus. A fluorograph is shown. The length of the RNAs in nucleotides was determined by coelectrophoresis of denatured restriction fragment markers.

arose gel electrophoresis and transferred to nitrocellulose sheets. After hybridization to ^{32}P-labeled plasmid DNA containing the *Hin*dIII-J fragment, five RNA species were detected (Fig. 6). In each case, the RNAs were estimated to be 3,840, 2,390, 1,790, 1,140, and 620 nucleotides long. The 620- and 2,390-nucleotide RNAs were candidates for the major and minor TK messages.

For purposes of genetic mapping, strips of nitrocellulose containing electrophoretically separated early RNA from cells infected with wild-type virus were hybridized to ^{32}P-labeled recombinant plasmids pJ1 to pJ4 as well as to recombinants containing the entire *Hin*dIII-L and *Hin*dIII-H fragments. The fluorograph in Fig. 7 demonstrates that (i) the RNA of 620 nucleotides hybridized to pJ1 and pJ2 only; (ii) the RNA of 2,390 nucleotides hybridized to pJ1, pJ2, and pJ3, but not to pJ4; (iii) the RNA of 1,790 nucleotides hybridized to pJ2 and pJ3, but not to pJ1 or pJ4; (iv) the RNA of 1,140 nucleotides hybridized exclusively to pJ4; and (v) the RNA of 3,840 nucleotides hybridized to pJ3, pJ4, and the adjacent *Hin*dIII-H fragment. By correlating these data with the results obtained by cell-free translation of mRNA selected by hybridization to the same recombinant DNA plasmids (Fig. 2) and fractionated by size (Fig. 5), we concluded that the TK or 19K polypeptide is made predominantly by the 620-nucleotide RNA and also by the overlapping 2,390-nucleotide RNA. The 1,790- and 1,140-nucleotide RNAs code for the 41K and 21K polypeptides, respectively. In this study, we have not been able to assign a polypeptide product to the large 3,840-nucleotide RNA that maps at the right end of the *Hin*dIII-J fragment and extends into *Hin*dIII-H.

When late RNA, isolated 6 h after infection, was analyzed by Northern blotting, smears were obtained instead of discrete bands. Similar results had previously been obtained when other segments of the vaccinia virus genome were used as hybridization probes and were attributed to extreme 3′ terminal heterogeneity of late RNAs (9).

Refinement of the transcriptional map by analysis of nuclease S1-resistant RNA-DNA hybrids. The precise RNA sizes and map positions used to prepare Fig. 8 were obtained by analysis of nuclease S1-resistant RNA-DNA duplexes. This procedure is based on the observation that DNA segments hybridized to RNA are protected against nuclease digestion, whereas unhybrid-

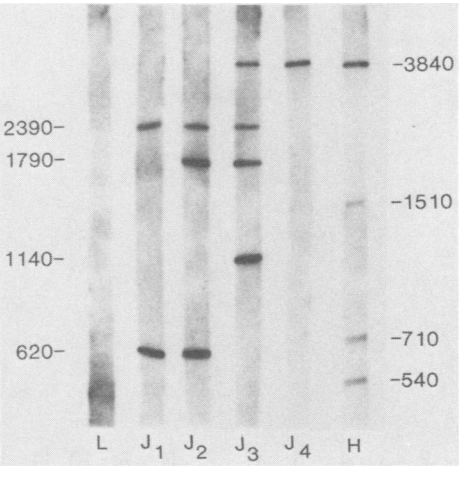

FIG. 7. Fluorograph of electrophoretically separated early RNA species that hybridized to ^{32}P-labeled DNA recombinants. Approximately 480 μg of purified cytoplasmic RNA from cells infected with wild-type virus in the presence of cycloheximide was denatured and subjected to electrophoresis in a 1.25% agarose gel containing 5 mM methylmercuric hydroxide. After transfer to a nitrocellulose membrane, 5-mm strips were hybridized to ^{32}P-labeled recombinants containing *Hin*dIII-L, *Hin*dIII-H, and the subfragments J1 to J4 indicated in Fig. 1. A fluorograph is shown.

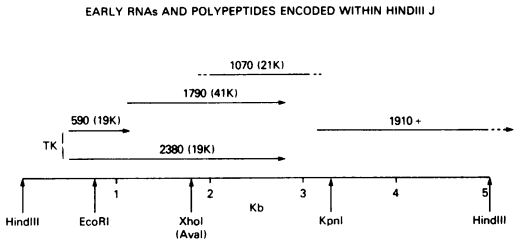

FIG. 8. Transcriptional map of the *Hin*dIII-J fragment. Sizes of RNAs are in nucleotides as determined by nuclease S1 mapping. Polypeptide translational products are indicated in parentheses. The direction and exact map coordinates of the 1,070-nucleotide RNA were not determined.

ized single-stranded DNA tails are trimmed away (3). As a consequence, the size of the transcript can be determined by gel electrophoresis of the radioactively labeled DNA-RNA hybrid.

For initial experiments of this type, the intact *Hin*dIII-J fragment was purified by agarose gel electrophoresis from uniformly ^{32}P-labeled recombinant plasmid. After hybridization to early RNA and nuclease S1 digestion, five discrete bands were detected upon neutral agarose gel electrophoresis (Fig. 9, tracks 7 and 18). By comparison with radioactively labeled DNA restriction fragment markers run on the same gel, the sizes of the RNA-DNA duplexes were estimated to be 2,380, 1,910, 1,790, 1,070, and 590 bp. A similar size pattern was obtained upon alkaline agarose gel electrophoresis, suggesting that the RNAs are colinear with the genome and are not spliced. The same five bands were also detected with early RNA made in the presence of cytosine arabinoside instead of cycloheximide. However, no discrete nuclease-resistant hybrids were detected when late RNA was used (Fig. 9, tracks 8 and 19).

Additional experiments were carried out to map the transcripts detected by the nuclease S1 procedure and to correlate them with the RNAs identified by blotting and cell-free translation. Uniformly ^{32}P-labeled recombinant DNA containing the *Hin*dIII-J fragment was cleaved with *Hin*dIII and a second restriction enzyme, either *Kpn*I, *Xho*I, or *Eco*RI. In each case, the two

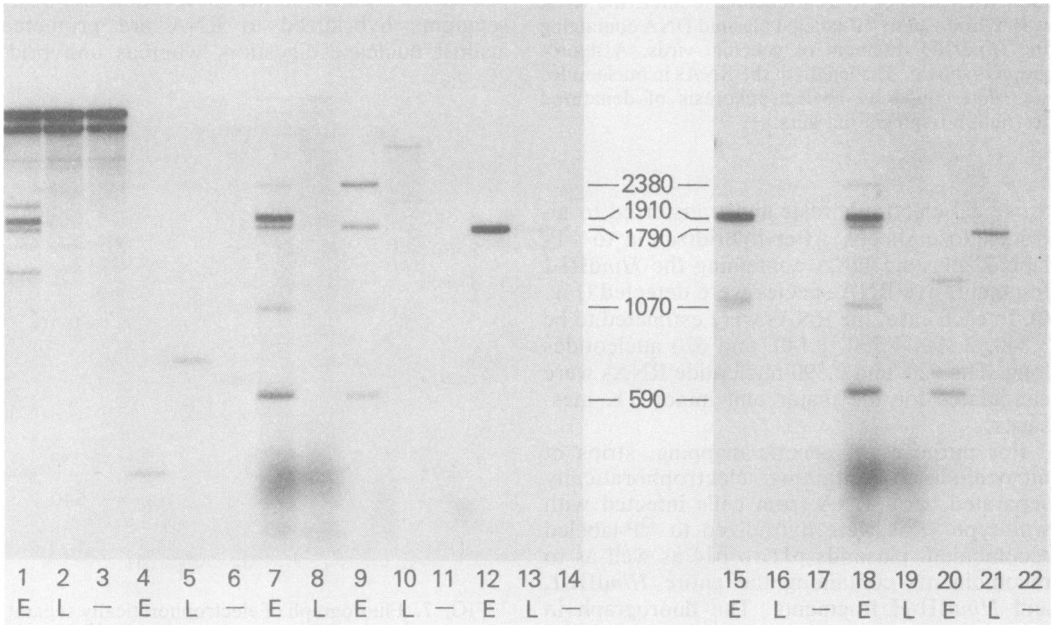

FIG. 9. Fluorograph of electrophoretically separated nuclease S1-resistant duplexes formed by hybridization of RNA to uniformly ^{32}P-labeled DNA fragments. Uniformly ^{32}P-labeled recombinant DNA containing the *Hin*dIII-J fragment was digested with *Hin*dIII and either *Eco*RI, *Kpn*I, or *Xho*I. The fragments were purified by agarose gel electrophoresis and then hybridized to early RNA. After nuclease S1 digestion, the resistant DNA-RNA hybrids were resolved by electrophoresis on neutral 2% agarose gels. Molecular weights were determined by coelectrophoresis of ^{32}P-labeled DNA restriction fragment markers. Tracks: 1 through 3, right *Hin*dIII-*Eco*RI fragment; 4 through 6, left *Hin*dIII-*Eco*RI fragment; 7 and 8, *Hin*dIII-J fragment; 9 through 11, left *Hin*dIII-*Kpn*I fragment; 12 through 14, right *Hin*dIII-*Kpn*I fragment; 15 through 17, right *Hin*dIII-*Xho*I fragment; 18 and 19, *Hin*dIII-J fragment; 20 through 22, left *Hin*dIII-*Xho*I fragment. E, Early RNA; L, late RNA; −, no RNA.

*Hin*dIII-J subfragments were purified by agarose gel electrophoresis. The protected segments of *Hin*dIII-J and *Hin*dIII-J subfragments were identical in size for transcripts encoded entirely within the latter. Changes in size of protected DNA segments occurred when a transcript extended beyond a subfragment. However, from the extent of truncation, the precise position of the transcript relative to the restriction site was determined.

Results obtained with the two *Hin*dIII-*Kpn*I fragments were particularly simple. When the larger (left) *Kpn*I fragment was hybridized to early RNA, bands of 2,380, 1,790, 1,070, and 590 bp formed, indicating that these transcripts map entirely to the left of the *Kpn*I site (Fig. 9, track 9). Since the 1,910-bp band was absent, the corresponding transcript must cross the *Kpn*I site. When the smaller (right) *Kpn*I fragment was used, a single band of 1,764 bp was detected (Fig. 9, track 12). This small reduction in size of the 1,910-bp band maps the left end of the transcript about 145 bp to the left of the *Kpn*I site (Fig. 8). An RNA of about 3,840 nucleotides was identified by Northern blot hybridizations with DNA fragments from the right end of *Hin*dIII-J and the adjacent *Hin*dIII-H fragment (Fig. 7). Therefore, the 1,910-bp band must represent the left half of this transcript, and the right half must extend about 1,900 nucleotides into the *Hin*dIII-H fragment.

Similar experiments were carried out with the *Hin*dIII-*Xho*I fragments. Full-size bands of 1,910 and 1,070 bp were detected with the larger (right) *Xho*I fragment (Fig. 9, track 15), whereas a full size band of 590 bp was found with the smaller (left) fragment (Fig. 9, track 20). Since bands of 2,380 and 1,790 bp were not obtained with either DNA fragment, the corresponding transcripts must cross the *Xho*I site. Indeed, two truncated bands of 1,267 and 678 bp were found with the smaller (left) *Xho*I fragment (Fig. 9, track 20), thus mapping the left ends of the two transcripts. The latter result suggested that both transcripts extend about 1,100 nucleotides to the right of the *Xho*I site. A single 1,100-bp band was resolved just above the 1,070-bp band (Fig. 9, track 15) when the right *Xho*I fragment was used, suggesting that the two RNAs have a common right end (Fig. 8).

Results obtained with the *Hin*dIII-*Eco*RI fragments were of particular importance for mapping the 590-nucleotide TK mRNA and the 2,380-nucleotide RNA, both of which cross this site. The left end of these transcripts was mapped by hybridization to the small (left) *Eco*RI fragment. A single nuclease-resistant band of about 316 nucleotides was detected (Fig. 9, track 4), suggesting that these two RNAs have common left ends. From this result, we would predict that nuclease-protected, truncated bands of about 300 and 2,100 bp would form by using the larger (right) fragment for hybridization. In this case, some DNA-DNA reannealing caused protected bands in the absence of RNA (Fig. 9, track 3). Nevertheless, it was evident that the 1,910-, 1,790-, and 1,070-bp bands were fully protected, whereas the 2,380- and 590-bp bands were absent (Fig. 9, track 1). Moreover, truncated bands of about 316 and 2,142 bp were detected. The origin of faint additional bands of about 1,300 bp is uncertain. Clearer results were obtained by using end-labeled *Eco*RI fragments for hybridization as shown below.

Comparison of the Northern blots (Fig. 7) with the nuclease S1 maps (Fig. 8) revealed excellent correlation with respect to both map position and size of all five early transcripts (Table 2).

As previously mentioned, only smears were observed when the apparently heterogeneous late RNAs were examined by Northern blotting. Similarly, only limited information was obtained by nuclease S1 analysis of late RNAs. Bands of approximately the size expected for complete protection of the left *Eco*RI and *Xho*I and right *Kpn*I fragments were detected (Fig. 9, tracks 5, 21, and 10). Since these bands were not found in the absence of RNA or with early RNA (Fig. 9, tracks 6, 22, and 11), we assume that they formed from annealing of late RNA.

Polarity of transcripts. The direction of transcription was determined by nuclease S1 analysis with purified restriction fragments labeled at their 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase or at the 3′ end with an appropriate α-^{32}P-deoxynucleotide triphosphate and the Klenow fragment of *Escherichia coli* DNA polymerase (31). In one set of experiments, the plasmid recombinant containing the *Hin*dIII-J

TABLE 2. Comparison of RNA sizes determined by blotting and nuclease procedures

Polypeptide	RNA size (nucleotides)		
	Minimum[a]	RNA blot	Nuclease S1
19K	496	620	590
19K	496	2,390	2,380
21K	548	1,140	1,070
41K	1,070	1,790	1,790
110K[b]	2,896	3,840	1,910+[c]

[a] The minimum size of the message was determined from the length of the polypeptide.

[b] Previous results (2) suggested that a 110K polypeptide is made by RNA overlapping *Hin*dIII-J and -H fragments.

[c] This RNA is encoded within the right segment of *Hin*dIII-J and the left segment of *Hin*dIII-H. Only the 1,910-nucleotide portion encoded within *Hin*dIII-J was measured by nuclease analysis.

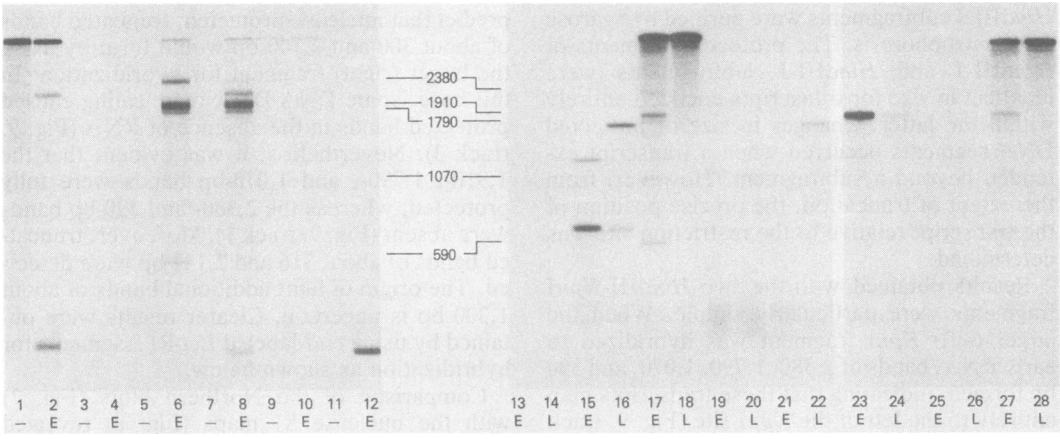

FIG. 10. Fluorograph of electrophoretically separated nuclease S1-resistant duplexes formed by hybridization of RNA to end-labeled DNA fragments and resolved by agarose gel electrophoresis. *Hin*dIII-J recombinant DNA was cleaved with the designated restriction enzyme, labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase and at the 3′ end with the appropriate α-^{32}P-deoxynucleotide triphosphate and DNA polymerase and then cleaved with a second restriction enzyme. The DNA fragments were purified by agarose gel electrophoresis, hybridized to early RNA, and resolved by electrophoresis in neutral 2% agarose gels. Tracks: 1 and 2, 3′ *Eco*RI end-labeled right *Hin*dIII fragment; 3 and 4, 3′ *Eco*RI end-labeled left *Hin*dIII fragment; 5 and 6, uniformly labeled *Hin*dIII-J fragment; 7 and 8, uniformly labeled *Hin*dIII-J fragment cleaved with *Eco*RI; 9 and 10, 5′ *Eco*RI end-labeled right *Hin*dIII fragment; 11 and 12, 5′ *Eco*RI end-labeled left *Hin*dIII fragment; 13 and 14, 5′ *Xho*I end-labeled right *Hin*dIII fragment; 15 and 16, 5′ *Xho*I end-labeled left *Hin*dIII fragment; 17 and 18, uniformly labeled *Hin*dIII-J fragment; 19 and 20, 3′ *Xho*I end-labeled right *Hin*dIII fragment; 21 and 22, 3′-*Xho*I end-labeled left *Hin*dIII fragment; 23 and 24, 5′ *Hin*dIII labeled right *Xho*I fragment; 25 and 26, 5′ *Hin*dIII labeled left *Xho*I fragment; 27 and 28, uniformly labeled *Hin*dIII-J fragment; E, Early RNA; L, late RNA; –, no RNA.

fragment was cleaved with *Eco*RI and divided into two portions for 5′ and 3′ end labeling. Both labeled preparations were then digested with *Hin*dIII, and the resulting fragments, each labeled either at the 3′ or 5′ ends, were purified by agarose gel electrophoresis. When early RNA was hybridized to the 5′ end-labeled left *Eco*RI fragment, a single band of 300 bp was detected (Fig. 10, track 12). Two bands of about 2,140 and 300 bp were protected when the 3′ end labeled right *Eco*RI fragment was used (Fig. 10, track 2). No specific bands were detected when the 3′ end-labeled left fragment (Fig. 10, track 4) or the 5′ end labeled right fragment (Fig. 10, track 10) was used for hybridization. This result proves that both the 590- and 2,380-nucleotide RNAs are transcribed from left to right and is consistent with the cell-free translation experiments that demonstrated hybridization selection of TK mRNA to the rightward-reading DNA strand cloned in phage M13mp8. The nuclease-digested samples were also analyzed on 6% polyacrylamide gels to accurately map the 5′ and 3′ ends of the 590-nucleotide TK mRNA (data not shown). The 5′ end mapped 285 nucleotides to the left of the *Eco*RI site and 305 nucleotides to the right of it. Under these conditions, the 3′ ends appeared to resolve into multiple closely spaced bands suggesting either 3′ terminal heterogeneity or extensive nibbling by nuclease S1.

The polarity of the 1,790-nucleotide RNA was determined by labeling the recombinant *Hin*dIII-J plasmid at the *Xho*I site and then cleaving with *Hin*dIII. After hybridization of the 5′ end-labeled left *Xho*I fragment to early RNA, the bands of about 1,267 and 678 bp were detected (Fig. 10, track 15). With the 3′ end-labeled right *Xho*I fragment, a single band of 1,100 bp was found (Fig. 10, track 19). No protected bands were detected with the other 5′ and 3′ end-labeled *Xho*I DNA fragments (Fig. 10, tracks 13 and 21). Thus, the 1,790-nucleotide RNA and the 590- and 2,142-nucleotide species are transcribed from left to right (Fig. 8). Apparently, late RNAs protect both the 5′ and 3′ end-labeled left *Xho*I fragments (Fig. 10, tracks 16 and 22), suggesting that both DNA strands are transcribed at late times.

To determine the polarity of the transcript mapping at the extreme right end of the *Hin*dIII-J fragment, the recombinant was labeled at the *Hin*dIII sites. After cleavage with *Xho*I, the fragments were purified and hybridized to early RNA. An intense band of 1,764 bp was obtained with the 5′ end-labeled right fragment (Fig. 10, track 23), indicating that this RNA also is transcribed from left to right (Fig. 8).

Because of the unavailability of a unique

restriction site in the gene for the 1,070-nucleotide RNA, neither its polarity or exact 5′ and 3′ map positions have been determined (Fig. 8).

DISCUSSION

The TK gene was previously localized within the 5,000-bp *Hin*dIII-J fragment of vaccinia virus (32). Since several early polypeptides are encoded within this DNA segment (2), finer mapping was required to identify the TK subunit. Using a TK assay developed by Hruby and Ball (14), we found that mRNA coding for TK hybridized to the left segment of the *Hin*dIII-J fragment. Only one polypeptide, of approximately 19K, was synthesized from mRNA that hybridized to this part of the genome. mRNAs for two other polypeptides, with molecular weights of 21K and 41K, hybridized further to the right and therefore cannot be TK subunits. Furthermore, mRNA that hybridized specifically to the rightward-reading strand of that *Hin*dIII-*Eco*RI fragment cloned in phage M13mp8 programmed synthesis of active TK enzyme and the 19K polypeptide exclusively.

Independent evidence that the 19K polypeptide is the TK subunit was obtained by analysis of TK^- vaccinia mutants. Immediate early RNA made in cells infected with four of these mutants and a wild-type control was hybridized to the cloned *Hin*dIII-J fragment of wild-type virus. When the selected mRNAs were translated in the reticulocyte cell-free system, the 19K polypeptide was absent in each case. For three of these mutants, the 21K and 41K polypeptides were made in usual amounts, and no other differences in the fluorographs were noted. Although an understanding of the nature of these variants must await more detailed analysis, we suspect that they are nonsense mutants. Identification of predicted prematurely terminated polypeptides is technically difficult because of the large globin front artifact in electropherograms of reticulocyte lysates. In addition, such aberrant polypeptides might be susceptible to proteolytic degradation in reticulocyte lysates (12). Other types of mutations that theoretically could give the observed result include loss of a translation initiation codon or ability to bind to ribosomes. A large deletion or promoter mutation can be ruled out since the appropriate size TK mRNA is made.

The isolation of so high a percentage of apparent nonsense mutants could reflect their spontaneous origin as well as the stringency of the 5-bromodeoxyuridine selection. Presumably, missense mutants that make low levels of TK would be selected against (26). A high percentage of putative nonsense herpesvirus mutants were also obtained by 5-bromodeoxyuridine selection, and for some of these shortened polypeptides were not identified (26).

The size of the TK message was determined by performing functional enzyme assays after cell-free translation of RNA that had been selected by hybridization to the *Hin*dIII-J fragment and then fractionated by methylmercuric hydroxide agarose gel electrophoresis. The major peak of TK activity was obtained with RNA 600 to 700 nucleotides long. A similar size for the TK message was determined by Hruby and Ball (personal communication). In addition, we found a minor peak of TK activity with RNA about 2,200 nucleotides long. Four early mRNAs of about 590, 1,070, 1,790, and 2,140 nucleotides were mapped by blotting and nuclease S1 analysis entirely within the *Hin*dIII-J fragment. Size, map coordinates, and direction of transcription all served to identify the 590- and 2,140-nucleotide RNAs as the major and minor TK messages. The 5′ ends of these two mRNAs appeared identical as judged by nuclease S1 analysis. The 3′ end of the larger mRNA coincided with that of the next mRNA downstream, suggesting that it arises from incomplete termination (Fig. 8).

From their map coordinates, the 1,070- and 1,790-nucleotide mRNAs were shown to encode 21K and 41K polypeptides, respectively. A fifth early RNA of 3,840 nucleotides is encoded at the extreme right end of the *Hin*dIII-J fragment and within the adjacent *Hin*dIII-H fragment. In the present study, we could not demonstrate a translation product for this RNA. Previously (2), a faint 110K band was detected among the translation products of early RNA selected by hybridization to the *Hin*dIII-J and -H fragments. Both the size of this mRNA and its map position make it a prime candidate for the 110K polypeptide message. However, further work is needed to optimize translation conditions and extend previous results.

Although the *Hin*dIII-L, -J, and -H fragments encode late polypeptides, it is quite difficult to map their mRNAs, apparently because of extreme 3′ terminal heterogeneity. However, the data suggest that late transcripts overlap the TK gene. It is possible that early promoter sites become less accessible to RNA polymerase because of overlapping late mRNAs or that in vivo annealing of early and late transcripts occurs (5, 6). Both transcriptional and translational regulation of vaccinia TK have been suggested (15, 18).

Perhaps most surprising is the small size of the vaccinia virus TK subunit. The TK subunits of procaryotes (24), eucaryotes (11), and herpesviruses (13, 29) all are in the range of 42 to 48K, and a similar size for the vaccinia virus TK subunit was suggested previously (17). The

small size of the vaccinia TK polypeptide observed here is not due to proteolytic degradation or other in vitro translation artifacts since the TK mRNA is too small to code for a much larger polypeptide. Since the native vaccinia virus TK made in vivo (17) or in vitro (14) is about 80 to 85K, the enzyme must be a tetramer. We are in the process of sequencing the TK gene of vaccinia virus and will soon be able to compare it with the known sequence of the herpesvirus TK gene (30).

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