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Mapping of a Gene Coding for a Major Late Structural Polypeptide on the Vaccinia Virus Genome

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Cell-free translation of total RNA isolated from vaccinia virus-infected cells late in infection results in a complex mixture of polypeptides. A monospecific antibody directed against one of the major structural proteins of the virus particle immunoprecipitated a single polypeptide with a molecular weight of 11,000 (11K) from this mixture. Immunoprecipitation was therefore used to identify the structural polypeptide among the in vitro translation products of RNA purified by hybridization selection to restriction fragments of the vaccinia virus genome. This allowed us to map the mRNA coding for the 11K polypeptide to the extreme left-hand end of the *Hin*dIII E fragment. Detailed transcriptional mapping of this region of the genome by nuclease S1 analysis revealed the presence of a late RNA transcribed from the rightward-reading strand. Its 5′ end mapped at ca. 130 base pairs to the left of the *Hin*dIII site at the junction between the *Hin*dIII F and E fragments. The map position of this RNA coincided precisely with the map position of the late message coding for the 11K polypeptide.

Vaccinia virus is a complex animal virus with a large double-stranded DNA genome of ca. 180 kilobase pairs (reviewed in reference 26). Expression of this large amount of genetic information occurs in a temporally tightly regulated fashion (reviewed in reference 13). A first class of genes (early genes) is transcribed shortly after infection by the viral RNA polymerase. After viral DNA replication, a second class (late genes) is expressed. The molecular basis for the switch from early to late gene expression is not known.

To learn more about the fine structure and regulation of vaccinia virus genes, detailed transcription maps of selected regions of the genome encoding predominantly early RNAs have been established (1; reviewed in reference 26). Based on these maps, four early genes have been DNA sequenced, and their putative promoter regions upstream of the initiation site of transcription were found to lack the consensus sequences typical of most eucaryotic promoters (21, 22, 24).

Very little is known about vaccinia virus late genes. Extensive symmetrical transcription late in infection and the enormous length heterogeneity of late RNA transcripts (reviewed in reference 26) render late genes less accessible to analysis. However, a detailed fine structure analysis of late genes is an important prerequisite for understanding gene regulation in vaccinia virus.

In this communication we describe the mapping of a vaccinia virus late gene encoding a polypeptide with a molecular weight of 11,000 (11K). This structural polypeptide represents a significant fraction of the total protein mass of the virion. By combining hybridization selection of RNA, in vitro translation, and immunoprecipitation with nuclease S1 mapping, we were able to map precisely the gene on the vaccinia virus DNA.

MATERIALS AND METHODS

Viruses and cells. The vaccinia virus strains WR (obtained from Bernard Moss, National Institutes of Health, Bethesda, Md.) and IHD (obtained from Keith Dumbell, St. Mary's Hospital Medical School, London) were used in this study. HeLa cells were grown in suspension in Eagle minimal essential medium (Spinner modification) supplemented with 10% horse serum. Infection of cells and purification of virus have been described (10, 12). Rabbit kidney cells (RK-13) were grown as monolayer cultures in Eagle minimal essential medium supplemented with 5% fetal calf serum. Cells were infected with 10 PFU of vaccinia virus per cell and then maintained in medium containing 2% fetal calf serum.

Labeling of cells and preparation of cell lysates for immunoprecipitation. For in vivo labeling of polypeptides, the growth medium of infected cell monolayers was removed and replaced with medium containing 1/10 of the regular methionine concentration and 10 μCi/ml of [^{35}S]methionine (specific activity, 800 Ci/mmol). After being labeled for 1 h, the cells were washed with phosphate-buffered saline and lysed by the addition of 0.1 ml per 10^6 cells of 50 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl–1 mM phenylmethylsulfonyl fluoride–0.1 mM 2-mercaptoethanol–1% Triton X-100–0.5% sodium deoxycholate. The lysate was transferred to a centrifuge tube, and insoluble material was removed by centrifugation at 10,000 × *g* for 15 min at 4°C. Samples of the cleared lysate were used for immunoprecipitation (see below) or analyzed directly by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after the addition, per 30 μl of lysate, of 10 μl of a solution containing 0.3125 M Tris-hydrochloride (pH 6.8), 10% 2-mercaptoethanol, 50% glycerol, 0.015% bromophenol blue, and 10 μl of 20% SDS and then heating the sample in a boiling water bath for 3 min.

RNA extraction. Total cytoplasmic RNA was purified from infected HeLa cells by sedimentation through a CsCl cushion (4, 7). Early RNA was isolated at 4 h after infection from cells maintained in medium containing either 100 μg of cycloheximide per ml (cycloheximide RNA) or 40 μl of cytosine arabinoside (CAR) per ml (CAR RNA) (4). Late RNA was extracted at 6 h after infection from cells that were not treated with either inhibitor (4).

Total RNA from infected RK-13 monolayers was isolated by a procedure communicated by Hans Koblet (University of Berne, Berne, Switzerland). The procedure is described for one culture flask with a 175-cm^2 growth area. Infected cells were washed once with ice-cold phosphate-buffered

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saline and then lysed by the addition of 9 ml of 0.02 M sodium acetate–8 M guanidinium hydrochloride–0.1 M 2-mercaptoethanol (pH 5). The viscous solution was transferred to a Dounce homogenizer and homogenized with 10 strokes of a tight-fitting pestle. RNA was then precipitated selectively by the addition of 4.5 ml of absolute ethanol, and the solution was allowed to stand at −20°C for at least 2 h. After centrifugation, the pellet was washed twice with absolute ethanol and then dissolved in 4.5 ml of 50 mM sodium acetate (pH 5)–10 mM EDTA–1% SDS–200 μg of proteinase K per ml. The solution was incubated at 37°C for 1 h. The RNA was then extracted several times with phenol, followed by several extractions with chloroform, and then precipitated with ethanol.

Hybridization selection of RNA. Approximately 20 μg of recombinant plasmid DNA carrying cloned vaccinia DNA restriction fragments was cleaved with *Hin*dIII, denatured in alkali, and immobilized on nitrocellulose filters as described (5). Alternatively, DNA restriction fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose sheets by blotting (19). Total cytoplasmic RNA (200 μg) was hybridized to 10 μg of immobilized DNA essentially as described (5), except that all hybridizations were performed at 42°C for 6 to 18 h. After hybridization, filters were washed three times at room temperature with 5-ml portions of 10 mM Tris-hydrochloride (pH 7.6)–0.1% SDS and three times with 10 mM Tris-hydrochloride (pH 7.6). Filters were then washed twice for 15 min each with 80% formamide–0.4 M NaCl–0.04 M sodium-PIPES [sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.001 M EDTA at 42°C. RNA was eluted from the filters with 90% formamide–0.04 M sodium-PIPES (pH 6.4)–0.001 M EDTA by heating at 65°C for 10 min and then was alcohol precipitated with 2.25 μg of calf liver tRNA (Boehringer, Mannheim, Federal Republic of Germany) as carrier after sodium acetate was added to a final concentration of 0.2 M. The precipitate was washed twice with 75% ethanol and dried.

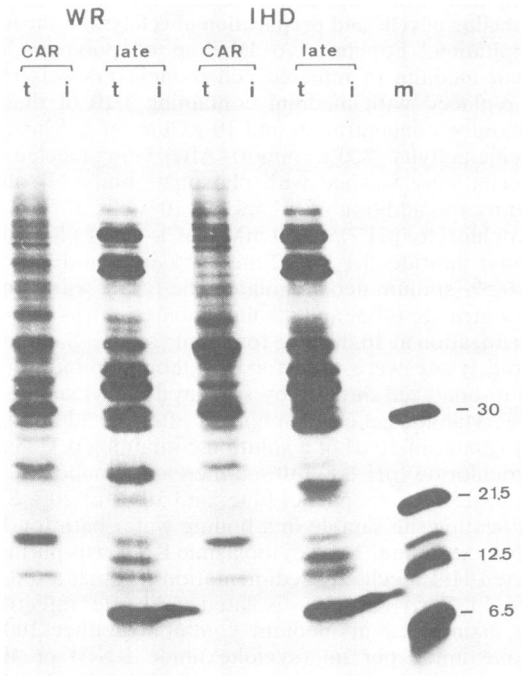

FIG. 1. Immunoprecipitation of the 11K polypeptide from vaccinia virus- (strain WR or IHD) infected ells. Cells were maintained in medium containing CAR or in medium without the inhibitor (late) and labeled for 1 h with [^{35}S]methionine at 3 and 4 h, respectively, after infection. Total cell lysates were analyzed either directly (lanes t) or after immunoprecipitation (lanes i) with the anti-11K antibody. A fluorograph of a 20% polyacrylamide gel is shown. Lane m, molecular weights ($\times 10^3$) of polypeptides used as size markers.

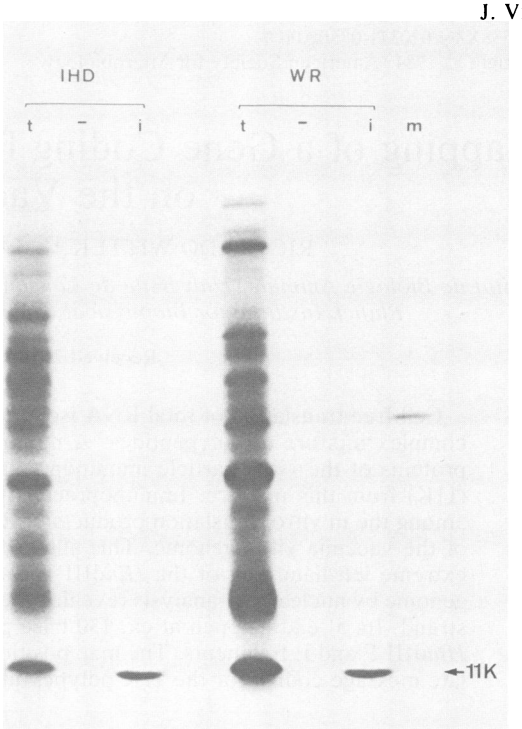

FIG. 2. Immunoprecipitation of in vitro translation products. Late RNA was isolated from vaccinia virus- (strain IHD or WR) infected cells and translated in vitro. Samples of the incubation mixture were analyzed either directly (lanes t) or after immunoprecipitation (lanes i) or mock-immunoprecipitation (lanes –). A fluorograph of a 20% polyacrylamide gel is shown. Lane m, molecular weight markers.

In vitro translation. A micrococcal nuclease-treated rabbit reticulocyte lysate was prepared and used as described in detail by Jackson and Hunt (9). In vitro translation reactions contained [^{35}S]methionine (specific activity, 800 Ci/mmol) at a final concentration of 1 μCi/μl and total cytoplasmic RNA (final concentration, 100 μg/ml). RNA selected by hybridization was translated after the dried RNA pellet was dissolved directly in 20 μl of the in vitro translation mixture.

Immunoprecipitation. A monospecific antibody directed against a major structural polypeptide was used in this study. Preparation and characterization of this antibody has been described in detail (8). Cleared cell lysates (see above) were used directly for immunoprecipitation. For immunoprecipitation of polypeptides made in vitro, the translation reactions were first diluted with 10 to 20 volumes of buffer A (50 mM Tris-hydrochloride [pH 7.5], 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM 2-mercaptoethanol, 1% Triton X-100). Two to three microliters of antiserum (or water for controls) was then added, and the mixture was rotated end over end at 4°C for ca. 15 h. Forty microliters of a 50% protein A-Sepharose (Pharmacia, Uppsala, Sweden) slurry in buffer A was then added, and mixing was continued for 2 h at room temperature. The protein A-Sepharose was harvested by centrifugation and washed three times with 1-ml portions of buffer A, three times with buffer B (same as buffer A but containing 300 mM NaCl), and finally three times with 20 mM Tris-hydrochloride (pH 6.8)–80 mM NaCl.

After the last wash, the protein A-Sepharose was suspended in sample buffer (0.0625 M Tris-hydrochloride [pH 6.8], 4% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.003% bromophenol blue) and heated in a boiling water bath for 3 min. The supernatant was recovered after centrifugation.

Immunoprecipitated material was analyzed by electrophoresis on 20% SDS-polyacrylamide gels. After electrophoresis, gels were treated with either En^{3}Hance or Enlightning (New England Nuclear Corp., Boston, Mass.) according to the instructions of the manufacturer, dried, and exposed to prefogged Kodak X-Omat R film at −70°C.

Nuclease S1 analysis. Nuclease S1 analysis (3) was carried out as described previously (25). Various DNA restriction fragments were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase to map the 5′ ends of transcripts (23). After hybridization and nuclease S1 digestion, resistant fragments were analyzed by electrophoresis on either neutral agarose gels or sequencing polyacrylamide gels (16).

Molecular cloning. An almost complete library of cloned *Hin*dIII restriction fragments was obtained from Bernard Moss (National Institutes of Health). Restriction fragments derived from this recombinant DNA were subcloned in pBR322 and propagated in *Escherichia coli* K-12 HB101 cells by standard procedures. A *Cla*I restriction fragment of particular interest (see below) was cloned from genomic vaccinia DNA. Bacteria containing the desired plasmid were identified by colony hybridization (20) with a DNA fragment ^{32}P-labeled by nick translation (15) as a probe.

RESULTS

Immunoprecipitation of polypeptides synthesized in vivo. A monospecific antibody directed against a vaccinia virus structural 11K polypeptide has recently been described in detail (8). Since many structural polypeptides are made late in infection, this antibody appeared to be particularly useful for the mapping of a vaccinia virus late gene by the strategy outlined below. To confirm that the 11K polypeptide is indeed expressed as a late viral function, cells were infected with two strains of vaccinia virus, and the polypeptides were labeled with [^{35}S]methionine for 1 h at 5 h after infection. Parallel infected cultures were maintained in medium containing CAR, an inhibitor of DNA synthesis. Under these conditions, only early polypeptides are made. Cells treated with the inhibitor were labeled for 1 h starting at 3 h after infection. After being labeled, the cells were lysed, and the polypeptides were analyzed either directly (Fig. 1, lanes t) or after immunoprecipitation with the anti-11K antibody (lanes i). Cells labeled in the presence or absence of CAR yielded the typical patterns (14) of early and late polypeptides, respectively. No band was detected after immunoprecipitation of the early polypeptides. In contrast, immunoprecipitation of polypeptides labeled in the late phase of infection resulted in an intense band at the expected position, and this band was seen with both virus strains used. A polypeptide with a very similar electrophoretic mobility was present as a major band in the nonimmunoprecipitated samples. This polypeptide is a good candidate for the 11K structural polypeptide since the amount of immunoprecipitated material loaded onto the gel was equivalent to only approximately four times the amount of total cell extract present in the nonimmunoprecipitated samples (lanes t). The 11K polypeptide is thus made in large amounts late in infection. The slight difference in mobility was probably due to the detergents which were used to solubilize the cells, which were present in the total polypeptide samples but which were presumably removed during extensive washing of the immunoprecipitate.

Immunoprecipitation of in vitro translation products. The strategy for mapping the gene coding for the 11K polypeptide involved immunoprecipitation of the in vitro translation product. To determine whether the anti-11K antibody indeed detects such a target, total RNA was isolated at 7 h after infection from cells infected with either strain WR or IHD of vaccinia virus. This RNA was translated in vitro, and the products were analyzed directly (Fig. 2, lanes t), after immunoprecipitation (lanes i), or after mock-immunoprecipitation without the addition of antibody (lanes –). As expected, a complex polypeptide pattern was obtained upon in vitro translation of total late RNA, and no bands were detected after mock-immunoprecipitation of parallel samples. After immunoprecipitation, a polypeptide of the expected size was observed. The amount of immunoprecipitated material analyzed on the gel was equivalent to twice the amount of total in vitro translation products present in the corresponding lanes. As judged from the intensities of the bands, the 11K polypeptide appears to be made in large amounts in reticulocyte lysates programmed with total late RNA.

In vitro translation of RNA selected on *Hin*dIII restriction

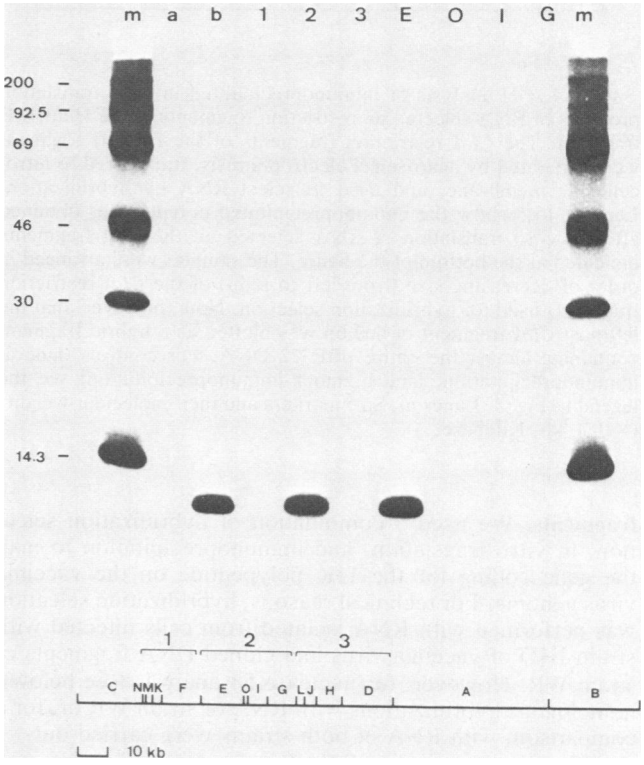

FIG. 3. Immunoprecipitation of in vitro translation products of RNA selected on *Hin*dIII restriction fragments. Polypeptides synthesized in vitro by late RNA purified by hybridization selection to pools of cloned *Hin*dIII fragments (lanes 1, 2, and 3) and to individual *Hin*dIII fragments (lanes E, O, I, and G) were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. Total unselected RNA was translated in vitro, and the products were either immunoprecipitated (lane b) or mock-immunoprecipitated (lane a) as controls. The molecular weights ($\times 10^3$) of polypeptides used as size markers (lanes m) are indicated. The *Hin*dIII restriction map of vaccinia (strain WR) DNA (6, 11) is shown at the bottom of the figure. kb, Kilobases.

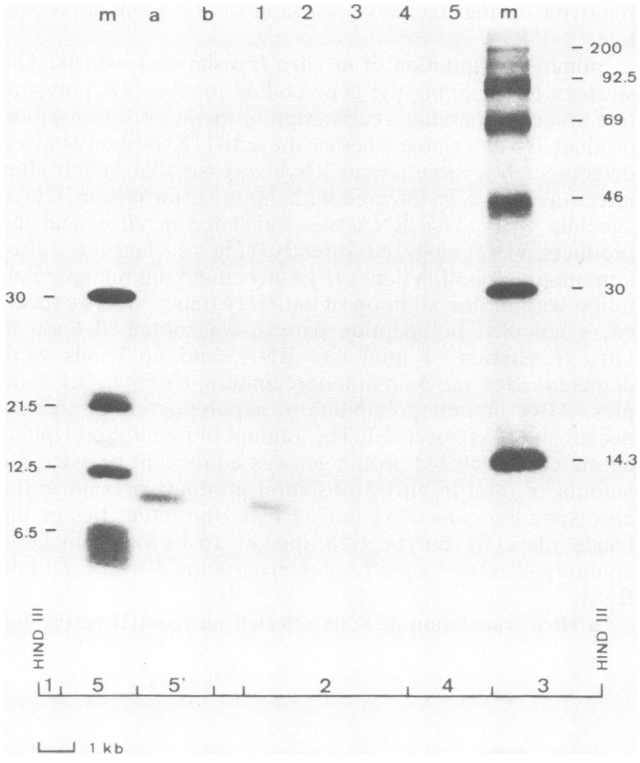

FIG. 4. Gel analysis of immunoprecipitated in vitro translation products of RNA selected on restriction fragments of the *Hin*dIII E fragment. The *Cla*I restriction fragments of the *Hin*dIII fragment were separated by agarose gel electrophoresis, transferred to nitrocellulose membrane, and used to select RNA by hybridization. Lanes 1 to 5 show the immunoprecipitated polypeptides obtained after in vitro translation of RNA selected on the *Cla*I fragments indicated at the bottom of the figure. The samples were arranged in order of decreasing size (from left to right) of the *Cla*I restriction fragments used for hybridization selection. Note, however, that the leftmost *Cla*I fragment of 600 bp was blotted as a hybrid fragment containing almost the entire pBR322 DNA. For controls (lane a, immunoprecipitation; lane b, mock-immunoprecipitation) see the legend to Fig. 3. Lanes m, Size markers and their molecular weights ($\times 10^3$). kb, Kilobase.

fragments. We used a combination of hybridization selection, in vitro translation, and immunoprecipitation to map the gene coding for the 11K polypeptide on the vaccinia virus genome. For technical reasons, hybridization selection was performed with RNA isolated from cells infected with strain IHD of vaccinia virus and cloned DNA fragments of strain WR. However, for nuclease S1 analysis (see below), homologous hybridizations with RNA of strain WR or, for a comparison, with RNA of both strains were carried out.

In the first experiment, RNA from vaccinia virus-infected cells isolated at 7 h after infection (late RNA) was purified by hybridization selection to cloned *Hin*dIII DNA restriction fragments. To keep the number of samples to be processed small, three nitrocellulose filters, with the DNA of four recombinant plasmids immobilized on each, were used in the first screening. Selected RNA was translated in vitro, and the products were immunoprecipitated with the monospecific antibody directed against the 11K polypeptide and analyzed by polyacrylamide gel electrophoresis (Fig. 3). As a control, total unselected RNA from infected cells was translated in vitro, and the products were either immunoprecipitated with the antibody or mock-immunoprecipitated without the addition of antibody but otherwise with the same manipulations as for immunoprecipitation. As expected, a polypeptide with a molecular weight of ca. 11,000 was precipitated from an in vitro translation mixture of total RNA (lane b), but no band was detected in the absence of the antibody (lane a).

When RNA selected by hybridization to the pooled *Hin*dIII restriction fragments N, M, K, and F (Fig. 3, lane 1) and L, J, H, and D (lane 3) was translated in vitro, no polypeptide was immunoprecipitated with the anti-11K antibody. In contrast, RNA selected on a mixture of the *Hin*dIII fragments E, O, I, and G (lane 2) directed the synthesis of the 11K polypeptide. RNA was therefore selected on each of the *Hin*dIII fragments E, O, I, and G individually and translated in vitro, and the polypeptides were immunoprecipitated. Only the *Hin*dIII E fragment selected the message for the 11K polypeptide (lane E).

In vitro translation of RNA selected on *Cla*I restriction fragments. For finer mapping of the gene coding for the 11K polypeptide, the *Cla*I restriction sites were mapped within the *Hin*dIII E fragment (Fig. 4). The recombinant plasmid containing the *Hin*dIII E fragment was cleaved with *Cla*I, and the fragments were separated by gel electrophoresis and blotted onto nitrocellulose membrane. Strips containing individual restriction fragments were cut from the membrane, and these fragments were used to isolate RNA by hybridization selection. Total unselected RNA was translated, and the products were either immunoprecipitated (Fig. 4, lane a) or

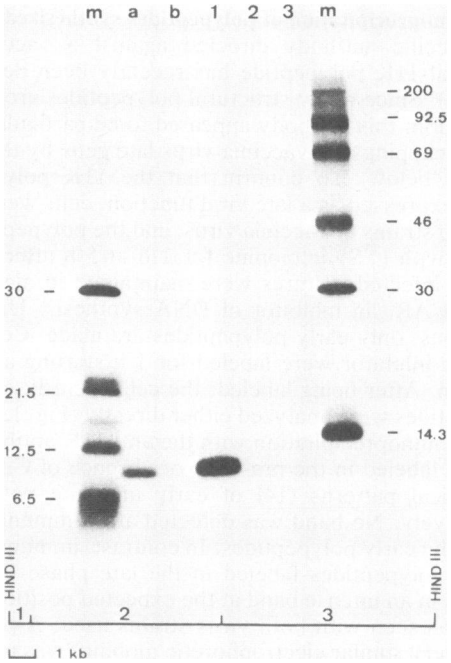

FIG. 5. Immunoprecipitation of in vitro translation products of RNA selected on *Bam*HI restriction fragments of the *Hin*dIII E fragment. The three *Bam*HI fragments of the *Hin*dIII E fragment were used to purify late RNA by hybridization selection. The RNA was translated in vitro, and the products were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. Lanes 1, 2, and 3 show the products obtained by RNA selected on the corresponding fragments (bottom of the figure). For controls (lane a, immunoprecipitation; lane b, mock-immunoprecipitation) see the legend to Fig. 3. Lanes m, size markers and their molecular weights ($\times 10^3$). kb, Kilobase.

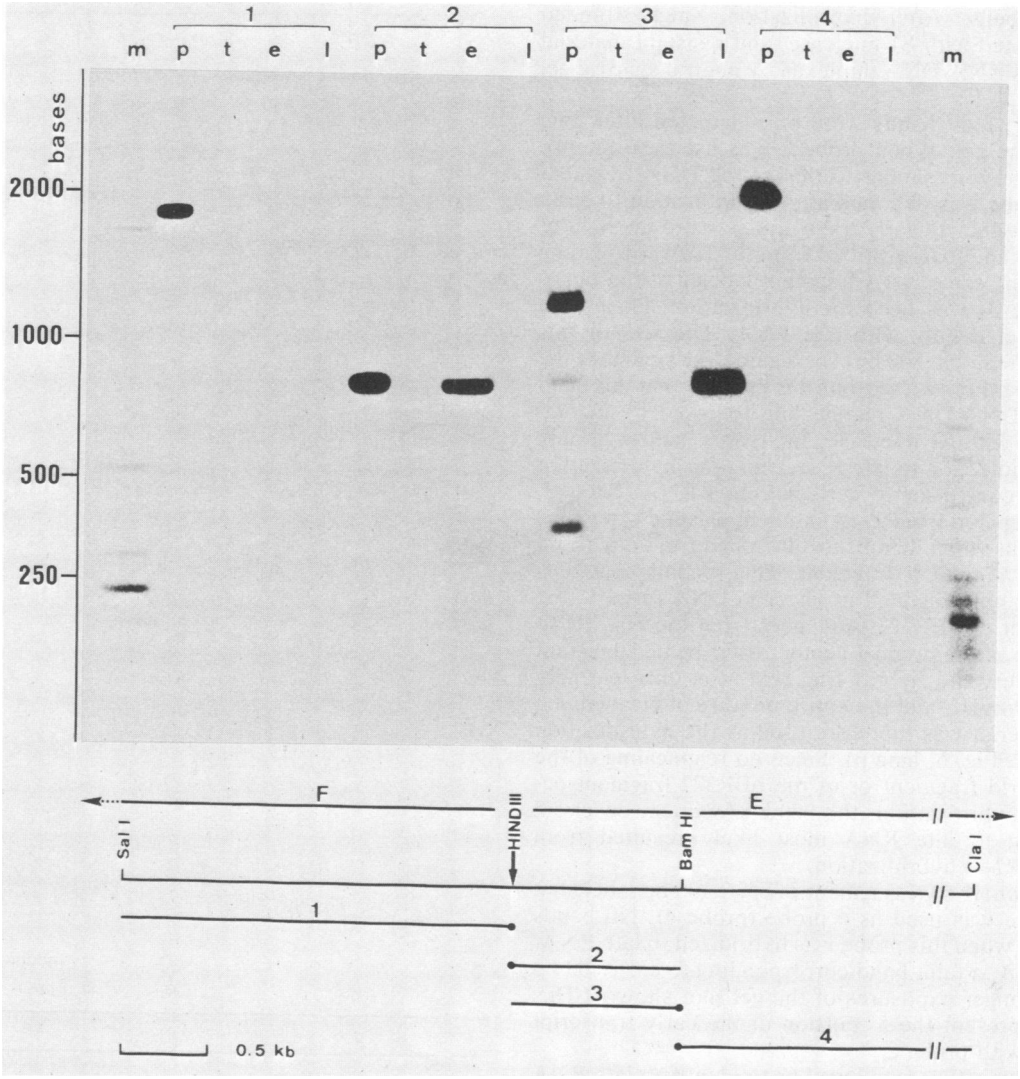

FIG. 6. Fluorograph of electrophoretically separated nuclease S1-resistant DNA-RNA hybrids. DNA fragments derived from the *Hin*dIII E and F fragments (see bottom part of the figure) were labeled at the 5′ end (solid circle). Each probe (1 to 4) was either analyzed directly (lanes p) or after hybridization to tRNA (lanes t) and to early (lanes e) and late (lanes l) RNA from vaccinia virus- (strain WR) infected cells. After hybridization, samples were treated with S1 nuclease, and resistant material was resolved by electrophoresis on a neutral 2% agarose gel. m, Molecular weight markers. kb, Kilobase.

mock-immunoprecipitated (lane b) as a control. The in vitro translation products of RNA selected on the *Cla*I restriction fragments were immunoprecipitated and analyzed on the same gel (lanes 1 to 5). Significant amounts of the 11K polypeptide were only detected when polypeptides made by RNA selected on the leftmost *Cla*I fragment were immunoprecipitated (lane 1).

In vitro translation of RNA selected on cloned *Bam*HI restriction fragments. To confirm this result with cloned DNA fragments, three *Bam*HI restriction fragments, one of ca. 800 base pairs (bp) and two with a very similar size of ca. 7,100 bp, were isolated from the *Hin*dIII E fragment and subcloned in pBR322. Restriction site mapping showed that the 800-bp fragment maps to the left-hand end of the *Hin*dIII E fragment. The recombinant DNA was immobilized on nitrocellulose membranes and used to select RNA by hybridization. Selected RNA was translated in vitro, and the resulting polypeptides were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis (Fig. 5, lanes 1 to 3) together with the immunoprecipitation products of in vitro translated, unselected RNA (lane a) or mock-immunoprecipitated material (lane b) serving as controls.

As expected, only the ca. 800-bp leftmost *Hin*dIII-*Bam*HI fragment selected the mRNA coding for the 11K polypeptide (Fig. 5, lane 1). Thus, by combining hybridization selection, in vitro translation, and immunoprecipitation, it was possible to map the gene for the 11K polypeptide to the extreme left hand end of the *Hin*dIII E fragment.

Mapping of transcripts by nuclease S1 analysis. To map the gene more precisely and also to determine the direction of transcription of the mRNA, nuclease S1 analysis of RNA-DNA duplexes was performed. Various DNA fragments labeled at the 5′ end were used as hybridization probes (Fig. 6). Each end-labeled probe was either analyzed directly (lanes p) or after hybridization with tRNA (lanes t), early RNA (lanes e), and late RNA (lanes l) isolated from vaccinia

virus-infected cells. After hybridization, single-stranded DNA was digested with S1 nuclease, and resistant material was resolved as RNA-DNA duplexes by neutral agarose gel electrophoresis.

With probe 1, faint bands were only detected after long exposures of the gel. When probe 2 was used, an intense band with a size very similar to that of the DNA fragment used as the probe was obtained after hybridization to early RNA, indicating that an early transcript starts to the right of the *Bam*HI site and is transcribed from the leftward-reading strand. When the same DNA fragment labeled at the opposite end (probe 3) was used for hybridization, an intense band was detected only with late RNA. The size of this protected fragment (ca. 800 bp) was again very similar to the size of the probe. For two reasons it is unlikely that this band resulted from DNA-DNA reannealing followed by incomplete digestion with S1 nuclease. First, this band was only seen after hybridization to late RNA, not after hybridization to tRNA as a control or to early vaccinia RNA. Second, probe 3 was prepared from a recombinant plasmid carrying a *Bam*HI fragment which had been subcloned from a plasmid containing the *Hin*dIII E fragment. This recombinant thus contains the 800-bp fragment of vaccinia DNA fused to the 375-bp fragment of pBR322 sequences from the *Hin*dIII to the *Bam*HI site of the original vector. After being labeled at the *Bam*HI sites, the hybrid fragment was only partially cleaved with *Hin*dIII, and the entire mixture of cleaved and uncleaved material was therefore used as the hybridization probe (probe 3; Fig. 6, lane p). Since no reannealing of the uncleaved hybrid fragment or of the pBR322 fragment occurred during hybridization, the 800-bp fragment observed after annealing to late RNA most likely resulted from specific DNA-RNA hybridization.

Finally, a *Bam*HI-*Cla*I fragment of ca. 2,100 bp labeled at the *Bam*HI site was used as a probe (probe 4). No bands were observed when this probe was hybridized to late RNA. With early RNA, a faint band corresponding to ca. 1,000 bp was seen on longer exposures of the gel (not shown). This band could represent the 5′ portion of the early transcript also detected with probe 2.

The S1 analysis (Fig. 6) allowed us to identify a late RNA that maps to the left-hand end of the *Hin*dIII E fragment. The map position of this RNA thus agrees very well with the map position obtained by hybridization selection and in vitro translation of the late message coding for the 11K polypeptide. Furthermore, the initiation site of the RNA is located very close to the *Hin*dIII site at the junction between the *Hin*dIII F and *Hin*dIII E fragments, and transcription occurs from the rightward-reading strand. However, with the DNA fragments used as probes for S1 analysis, it was not possible to more precisely map the 5′ end of the transcript. Therefore, a *Cla*I fragment spanning the *Hin*dIII site was cloned from genomic vaccinia DNA in pBR322. The clone was identified by colony hybridization by using as a probe the vaccinia DNA fragment from the *Hin*dIII site to the first *Cla*I site at the left-hand end of the *Hin*dIII E fragment.

Two probes derived from this fragment were used for further nuclease S1 analysis to map more precisely the 5′ end of the late RNA (Fig. 7). The first probe (probe 1) consisted of the entire *Cla*I fragment labeled at the 5′ ends. Second, a *Taq*I fragment of ca. 800 bp from the left *Cla*I site to the first *Taq*I site at 165 bp to the right of the *Hin*dIII site was isolated from the recombinant DNA and also labeled at the 5′ ends (probe 2). Samples of each probe were analyzed either after cleavage with *Hin*dIII (lanes g) or uncleaved (lanes f). The small *Hin*dIII-*Taq*I fragment of 165 bases

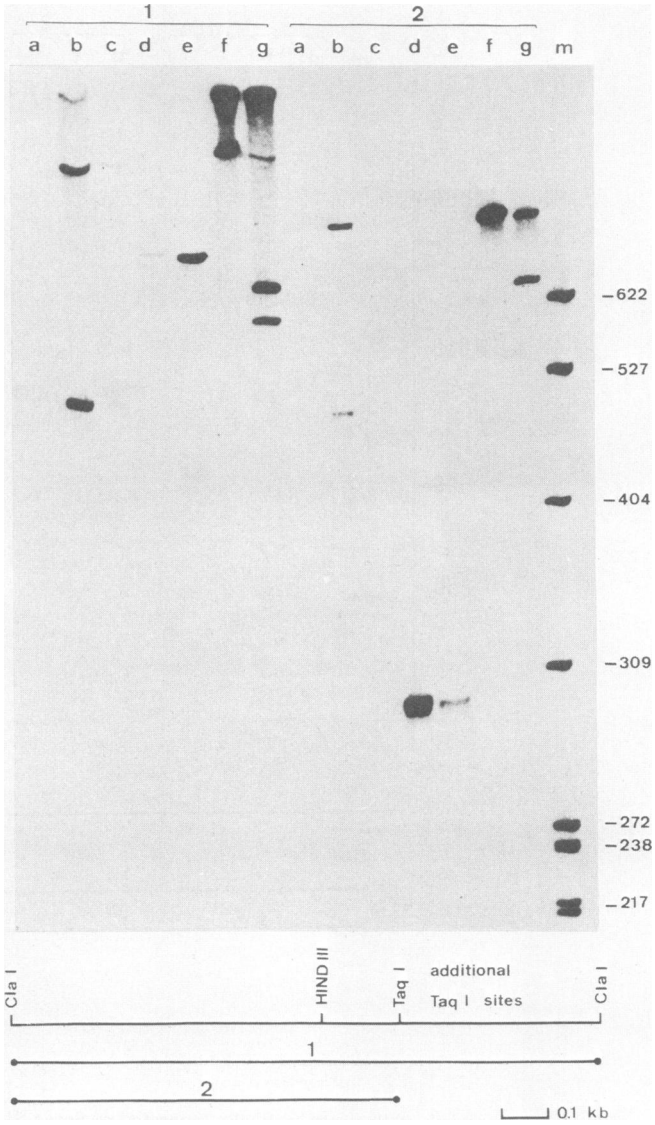

FIG. 7. Fluorograph of electrophoretically separated nuclease S1-resistant DNA fragments. Two fragments (labeled 1 and 2 on the figure) derived from a cloned *Cla*I fragment spanning the *Hin*dIII site at the junction between the *Hin*dIII E and F fragment were labeled at the 5′ ends and used as probes for nuclease S1 analysis. Each probe was analyzed either uncleaved (lanes f) or after cleavage with *Hin*dIII (lanes g). Hybridizations were performed to tRNA (lanes a) as controls and to cycloheximide RNA (lanes b), CAR RNA (lanes c), and late RNA (lanes d) isolated from cells infected with strain WR. Each probe was also hybridized to late RNA isolated from cells infected with strain IHD of vaccinia virus (lanes e). After hybridization, samples were treated with S1 nuclease, and resistant DNA fragments were resolved by electrophoresis on a 4% polyacrylamide sequencing gel. Lane m, sizes (in bases) of DNA fragments used as markers. kb, Kilobase.

obtained upon cleavage of probe 2 with *Hin*dIII is not shown. Hybridizations were performed with the probes that were not digested with *Hin*dIII. Each probe was hybridized to tRNA as a control (lanes a), to early RNA isolated from cells infected with strain WR of vaccinia virus and treated with either cycloheximide (lanes b) or CAR (lanes c), and to late RNA (lanes d). For a comparison, each probe was also hybridized to late RNA isolated from cells infected with

strain IHD (lanes e). With cycloheximide RNA hybridized to probe 1, an S1-protected fragment of ca. 500 bases was detected (lane b). A much fainter band was also seen with CAR RNA (lane c) after longer exposures of the gel. Similar bands were obtained after hybridization of early RNA to probe 2, indicating that an early RNA is transcribed from the leftward-reading strand since the two probes have a common left-hand terminus. When probe 1 was hybridized to either WR or IHD late RNA, S1-protected fragments of ca. 720 bases were obtained. (The intensities of the bands cannot be directly compared since hybridizations with the late RNAs were performed on different occasions with different preparations of the probe.) If the observed fragment resulted from hybridization of the late RNA transcribed from the rightward-reading strand, the 5′ end would map at 130 bases to the left of the *Hin*dIII site, and a much shorter fragment should be obtained with probe 2. Indeed, a fragment of 300 bases was detected after hybridization of late RNA from both virus strains to probe 2 (lanes d and e). With this probe, the 5′ end of the late RNA mapped at 135 bases to the left of the *Hin*dIII site, which is in excellent agreement with the map position obtained with probe 1.

The map position of the late mRNA coding for the 11K polypeptide is shown in Fig. 8.

DISCUSSION

In contrast to the steadily growing information on the fine structure of vaccinia virus early genes, very little is known about late genes. This is mainly because the first regions of the genome that were chosen for detailed transcriptional mapping encoded very few late transcripts and probably no major ones (reviewed in reference 26). In addition, two unusual properties of late RNAs (reviewed in reference 26) render late genes less accessible to analysis by conventional procedures such as RNA blotting and nuclease S1 analysis with uniformly labeled DNA as probes. First, late RNAs are extremely heterogeneous in length, presumably as a result of incorrect termination of transcription late in infection. Second, a large fraction of late RNA is capable of forming double-stranded RNA upon self-annealing. Thus, in all mapping procedures involving DNA-RNA hybridizations with RNA in solution, competing RNA-RNA annealing will take place. As a consequence, if late RNAs are purified by hybridization selection and translated in vitro, the intensity of a particular band may not reflect the abundance of the corresponding message since significant amounts may be lost as RNA-RNA duplexes during selection.

Structural polypeptides are good candidates for late polypeptides. However, such genes cannot be mapped simply on the basis of comigration of in vitro translation products with authentic structural polypeptides since many of these are known to undergo posttranslational modifications (13), most of which will not be performed by the in vitro translation system.

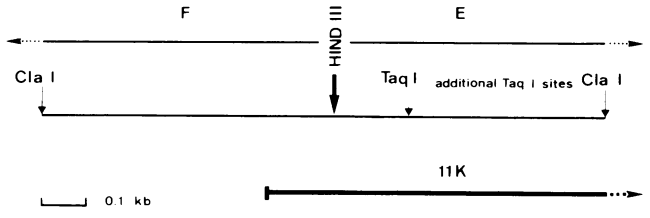

FIG. 8. Map position and direction of transcription of the late mRNA coding for the 11K structural polypeptide. kb, Kilobase.

Immunoprecipitation with antibodies directed against major structural polypeptides in combination with hybridization selection and in vitro translation offers a possibility of mapping representative late genes despite the problems mentioned above.

In this study we used an antibody directed against a structural polypeptide with a molecular weight of 11,000 to map a late gene on the vaccinia virus DNA. This biologically interesting polypeptide is exposed on the surface of mature virus particles and interacts with actin-containing cytoskeletal elements of the infected cell (8). The antibody appeared to be particularly useful for our mapping study mainly for two reasons. First, the antibody has been characterized in great detail and shown by two-dimensional immunoblotting to recognize a single target and thus to be monospecific (8). Second, the 11K polypeptide contributes ca. 10% to the total protein mass present in purified virions (13, 17). We therefore assumed that the mRNA would be present in large amounts in infected cells and thus facilitate mapping of the gene. Indeed, from quantitative aspects of in vitro translation and immunoprecipitation experiments, the 11K polypeptide appears to be a major product made in response to total unselected late RNA.

As expected for a structural protein, significant amounts of the 11K polypeptide were immunoprecipitated from cells labeled in the late phase of infection only and not from cells treated with CAR, in which late viral functions are not expressed.

By combining hybridization selection, in vitro translation, and immunoprecipitation, we were able to map the gene coding for the 11K structural polypeptide to the extreme left-hand end of the *Hin*dIII E fragment. A late polypeptide of similar size has previously been detected after in vitro translation of RNA selected on the *Hin*dIII E fragment (2). Finer mapping of the gene was accomplished by nuclease S1 analysis with 5′ end labeled DNA fragments as probes. The message was found to be transcribed from left to right and to initiate within the adjacent *Hin*dIII F fragment at ca. 130 bp to the left of the *Hin*dIII site. Because of the size heterogeneity of late transcripts, we made no attempt to map the 3′ end of the mRNA. However, from the size of the polypeptide the length of the coding sequence should not exceed ca. 300 nucleotides. Interestingly, an early RNA is transcribed in the opposite direction from the same region of the genome.

Since the gene maps within a region of the genome that is highly conserved between poxviruses (11, 18), it is not surprising that the 11K polypeptide is immunoprecipitated from cells infected with both vaccinia virus strains used in this study.

We are currently sequencing the late gene and its 5′ flanking region to compare its putative promoter sequence with corresponding sequences of early genes (21, 22, 24).

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