JOURNAL OF VIROLOGY, Aug. 1985, p. 450–457
0022-538X/85/080450-08$02.00/0

Vol. 55, No. 2

Transcriptional and Translational Analysis of the Vaccinia Virus Late Gene L65†

SCOTT L. WEINRICH,[1] EDWARD G. NILES,[2] AND DENNIS E. HRUBY[1]*

Center for Gene Research and Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804,[1] and Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214[2]

Received 28 January 1985/Accepted 23 April 1985

Among the products of vaccinia virus genes which are expressed late in infection is a major polypeptide (M_r, 65,000) designated L65. Pulse-chase analyses indicated that L65 is not subject to posttranslational cleavage as is the core polypeptide p4b which migrates to a similar position in sodium dodecyl sulfate-polyacrylamide gels. A polypeptide of 65,000 molecular weight produced in reticulocyte lysates programmed with viral mRNA isolated late in infection was identified as L65 by peptide mapping. L65 mRNA was purified by hybridization selection to restriction fragments of the viral genome and translated in vitro. This allowed the gene encoding L65 to be mapped to the rightmost 4.5 kilobase pairs of the *Hin*dIII D fragment. Transcriptional mapping of this region of the genome detected a late mRNA which was initiated at 450 base pairs to the right of the *Hin*dIII D-A junction, was transcribed in the leftward direction, and was terminated in the nondescript manner typical of vaccinia virus late mRNAs.

The prototypic poxvirus vaccinia (VV) is a large, double-stranded DNA virus that replicates in the cytoplasm of infected cells (10). The 185-kilobase-pair (kbp) genome is expressed in a temporally regulated fashion (35). Shortly after infection, early genes are transcribed by a viral RNA polymerase contained in the virion (23). Concomitant with or after the onset of DNA synthesis, late genes are expressed. Among the early polypeptides are enzymes involved in the synthesis and maturation of RNA and DNA; among the late polypeptides are the major structural components of the virion. The mechanisms involved with the switch between the early and late modes of gene expression are unknown.

As a first step toward deciphering the regulatory mechanisms employed by VV, it is of interest to locate and compare sequences which may be responsible for promoting and terminating early and late VV gene transcription. Several early genes have been precisely mapped, and a few have been sequenced (16, 30, 31, 33). The 5′ flanking sequences of early genes, when fused to heterologous genes recombined back into infectious virus, promote expression with the expected temporal regulation (18). Correct regulation of a foreign gene has also been shown with a late promoter (34). These upstream sequences of VV genes show little resemblance to previously recognized eucaryotic or procaryotic regulatory signals. It has been suggested that VV has evolved unique signal sequences (27).

An unusual feature of late transcripts is their extreme heterogeneity in size; RNA encoding a specific late polypeptide may vary severalfold in length, unlike the early transcripts, which have discrete sizes. Hybrid selection and translation of RNA are an effective means to initially map late genes. A growing number of late genes have been precisely mapped (9, 20, 36–38), and the sequence of one late gene has been reported (34). In each case a specific 5′ end was detected. Presumably, termination signals or mechanisms late in infection are relaxed, resulting in 3′-terminal heterogeneity. To interpret this and other characteristics of VV late-gene expression, more extensive information on genomic location and transcript structure of individual late genes is required.

In this study, the map position of a 65,000-molecular-weight polypeptide (65K polypeptide)-encoding late gene was determined by hybrid-selected translation and S1 nuclease mapping. The 65K polypeptide is a major product late in infection and does not appear to be associated with the virion.

MATERIALS AND METHODS

Cells and virus. BSC-40 monkey kidney cells were maintained in monolayer cultures in Eagle minimal essential medium plus 10% heat-inactivated fetal calf serum. HeLa cells were maintained in suspension cultures in minimal essential medium plus 10% heat-inactivated fetal calf serum.

VV strain WR was grown in suspensions of HeLa cells maintained at a concentration of 5×10^5 cells per ml. Isotopically labeled VV was grown in the presence of 2 μCi of ^{14}C-amino acids (55 mCi/mmol; New England Nuclear Corp., Boston, Mass.) per ml. Cells were infected at a multiplicity of infection of 1. After 48 h of infection, intracellular virus was released by lysing the infected cells, and the virus was purified by sucrose gradient centrifugation as previously described (14). The titer of the purified virus was determined by plaque titration on confluent monolayers of BSC-40 cells. All media and sera were obtained from GIBCO Laboratories, Grand Island, N.Y.

Plasmid construction and isolation. Recombinant plasmids containing VV *Hin*dIII restriction fragments D through O (making up 50% of the VV genome) in the *Hin*dIII site of pBR322 were constructed and generously provided by B. Moss (National Institutes of Health). Plasmids were purified from amplified cultures of *Escherichia coli* HB101 grown in the presence of 20 μg of ampicillin per ml.

Recombinant plasmids containing VV *Hin*dIII-D, *Eco*RI subfragments A through I, and *Bam*HI subfragments A through D were constructed as follows. VV *Hin*dIII-D plasmid (100 μg) was cleaved with *Hin*dIII, the products

* Corresponding author.

† Oregon State University Agricultural Experiment Station Technical Paper no. 7417.

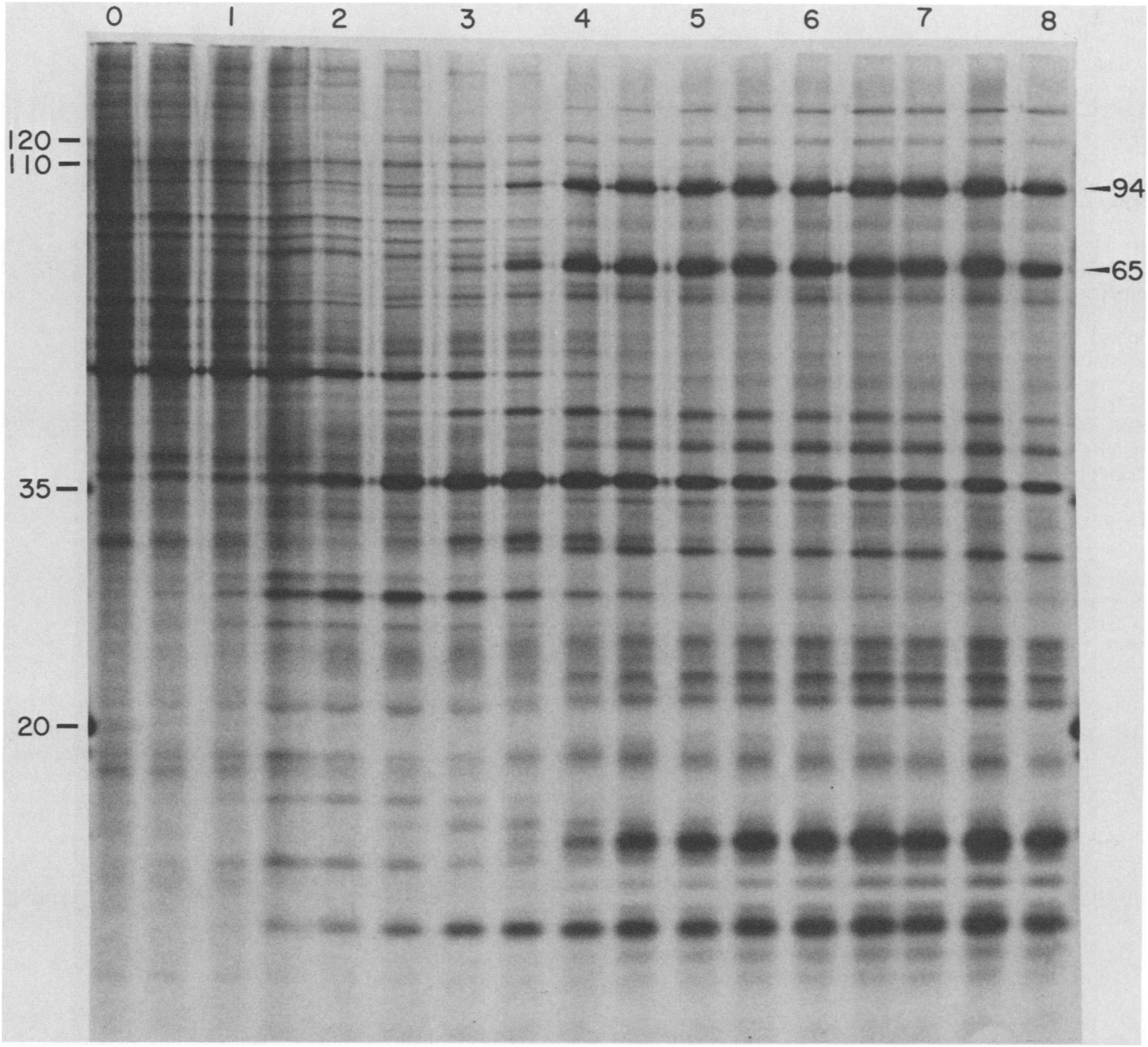

FIG. 1. Gel electrophoresis of the polypeptides synthesized in VV-infected BSC-40 cells. Confluent monolayers were infected (25 PFU per cell), and polypeptides were pulse-labeled by the addition of [^{35}S]methionine (25 μCi/ml) every 30 min throughout the first 8 h of infection. At the end of each labeling period, cells were collected by centrifugation and suspended in sample buffer containing sodium dodecyl sulfate. Lysates were subjected to electrophoresis on a 12% polyacrylamide gel, and labeled polypeptides were visualized by fluorography. The lane number indicates the hour p.i. Unlabeled lanes are times on the half hour. The migration position and molecular weights (in thousands) of four brome mosaic virus proteins used as markers are indicated on the left. Two representative late polypeptides (94K and 65K) are indicated on the right.

were separated by electrophoresis in a 0.6% agarose gel, and the 16-kbp VV D fragment was isolated by electroelution (11). The D fragment was digested with either *Eco*RI or *Bam*HI, and the digestion products were ligated to pUC13 (32) cut with *Eco*RI, *Eco*RI plus *Hin*dIII, *Bam*HI, or *Bam*HI plus *Hin*dIII. *E. coli* JM83 was transformed by the method of Kushner (17), and plasmid DNA from ampicillin-resistant, β-galactosidase-negative colonies was characterized. Plasmids were prepared by a scaled-up version of the method of Holmes and Quigley (13), purified by extractions with phenol and ether, twice ethanol precipitated, and analyzed by digestion with the appropriate endonuclease.

Recombinant plasmids containing VV *Hin*dIII A subfragments were cloned in pUC13 as described above with VV *Sal*I-J cloned in pUC13 as starting material.

Isolation and purification of VV RNA. Suspension cultures of HeLa cells at a concentration of 5.5×10^5 cells per ml were collected by centrifugation at 2,000 rpm for 5 min at 25°C in a Beckman J-21 centrifuge and then suspended at a concentration of 10^7 cells per ml in adsorption medium (1 part Pucks saline, 1 part minimal essential medium plus 5% heat-inactivated fetal calf serum). Purified VV (10 PFU per cell) was added, and the cells were swirled gently at 37°C for 30 min. The infected cells were then diluted to a concentra-

tion of 5×10^5 cells per ml and shaken at 37°C for 6 h before late RNA was isolated. To isolate early RNA, 100 μg of cycloheximide per ml was added to the medium and incubation was for 4 h. Infected cells were pelleted by centrifugation at 2,000 rpm for 10 min at 4°C and then washed three times with ice-cold phosphate-buffered saline containing 1 mM magnesium chloride. The cells were swollen in hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.6], 10 mM NaCl, 1 mM magnesium chloride) and then broken with 20 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 2,000 rpm at 4°C for 10 min. The resulting supernatant was removed, and the pellet was rehomogenized and centrifuged as described above. The supernatants were pooled, made 2% (wt/vol) in *N*-lauroylsarcosine and 1 g/ml in cesium chloride, and warmed to 25°C. This mixture was layered over a 2-ml cushion of 5.7 M cesium chloride–0.1 M EDTA (in hypotonic buffer containing 2% *N*-lauroylsarcosine). Then the mixture was overlaid with hypotonic buffer (12). Centrifugation was in a Beckman SW40 rotor at 25,000 rpm and 25°C for 12 to 18 h with a Beckman model L8-70 M ultracentrifuge. The resulting RNA pellet was dissolved in H_2O and twice ethanol precipitated in the presence of 0.15 M potassium acetate (pH 5.3). mRNA was selected by affinity chromatography on oligo(dT)-cellulose (grade T-3; Collaborative Research, Inc., Waltham, Mass.). Poly $(A)^+$ RNA was eluted, twice ethanol precipitated, and stored at −70°C until used for hybrid selection or translation.

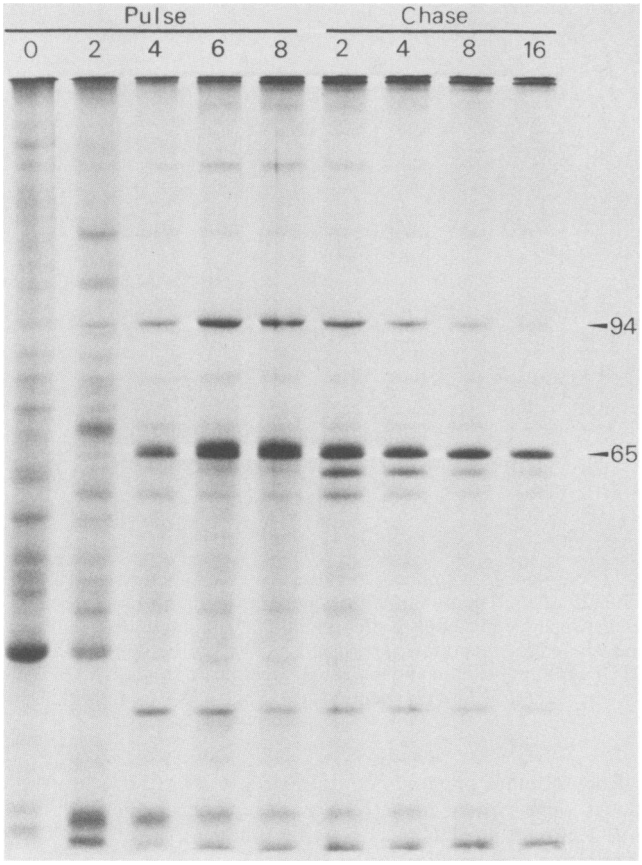

FIG. 2. Pulse-chase analysis of polypeptides synthesized in VV-infected BSC-40 cells. Infected cells were pulse-labeled in medium containing 0.1× the normal amount of methionine at the indicated times p.i. At 8 h p.i., pulse-labeled infected cells were chased by replacing the radioactive medium with medium containing 100× the normal amount of methionine. Chase times indicated are in hours after the pulse at 8 h p.i. Samples were analyzed by gel electrophoresis as described in the legend to Fig. 1. Approximate molecular weights (in thousands) are indicated on the right.

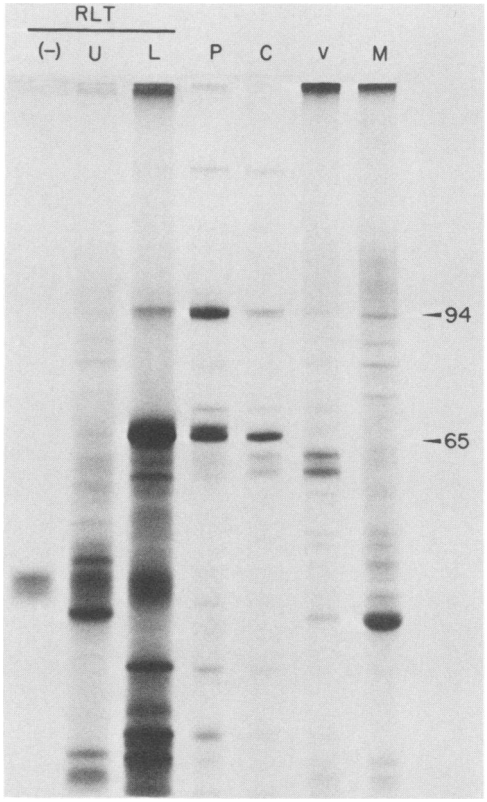

FIG. 3. Comparison of polypeptides synthesized in vitro with those synthesized in vivo. Equivalent amounts of VV-infected BSC-40 cell lysates, purified VV, or reticulocyte lysates programmed with different RNAs were subjected to extended electrophoresis on a 12% polyacrylamide gel. RLT heads the reticulocyte lysate translation lanes in which labeled polypeptides resulting from H_2O (lane [−])-, uninfected BSC-40 RNA (lane U)-, or late RNA (lane L)-programmed cell-free translations were resolved. Infected cell lysates were resolved in lane P (8 h p.i. pulse), lane C (8 h p.i. pulse plus 8-h chase), and lane M (mock-infected pulse). Partially purified VV polypeptides were resolved in lane V. Approximate molecular weights (in thousands) are indicated on the right.

Hybrid selection of VV mRNA. Binding of recombinant plasmid DNA to nitrocellulose filters was carried out as previously described (26) with 20 μg of each recombinant plasmid. Plasmid DNAs were digested to completion with the appropriate endonuclease and spotted onto 3-mm squares of nitrocellulose. Hybridization solutions contained 10 μg of poly$(A)^+$ RNA isolated from VV-infected HeLa cells at 6 h after infection. Hybridization conditions and elution of bound mRNA were as previously described (21).

Reticulocyte lysate reactions. Rabbit reticulocytes were obtained from Green Hectares, Oregon, Wis., and were used to prepare translation lysates (28). In a final volume of 15 μl, the cell-free translation reaction mixtures contained the following: 50% nuclease-treated reticulocyte lysate, 0 to 100 μg of VV mRNA per ml, 10 mM Tris hydrochloride (pH 8.2), 25 μg of creatine phosphokinase (Sigma Chemical Co., St. Louis, Mo.) per ml, 1.6 μM hemin, 125 mM potassium

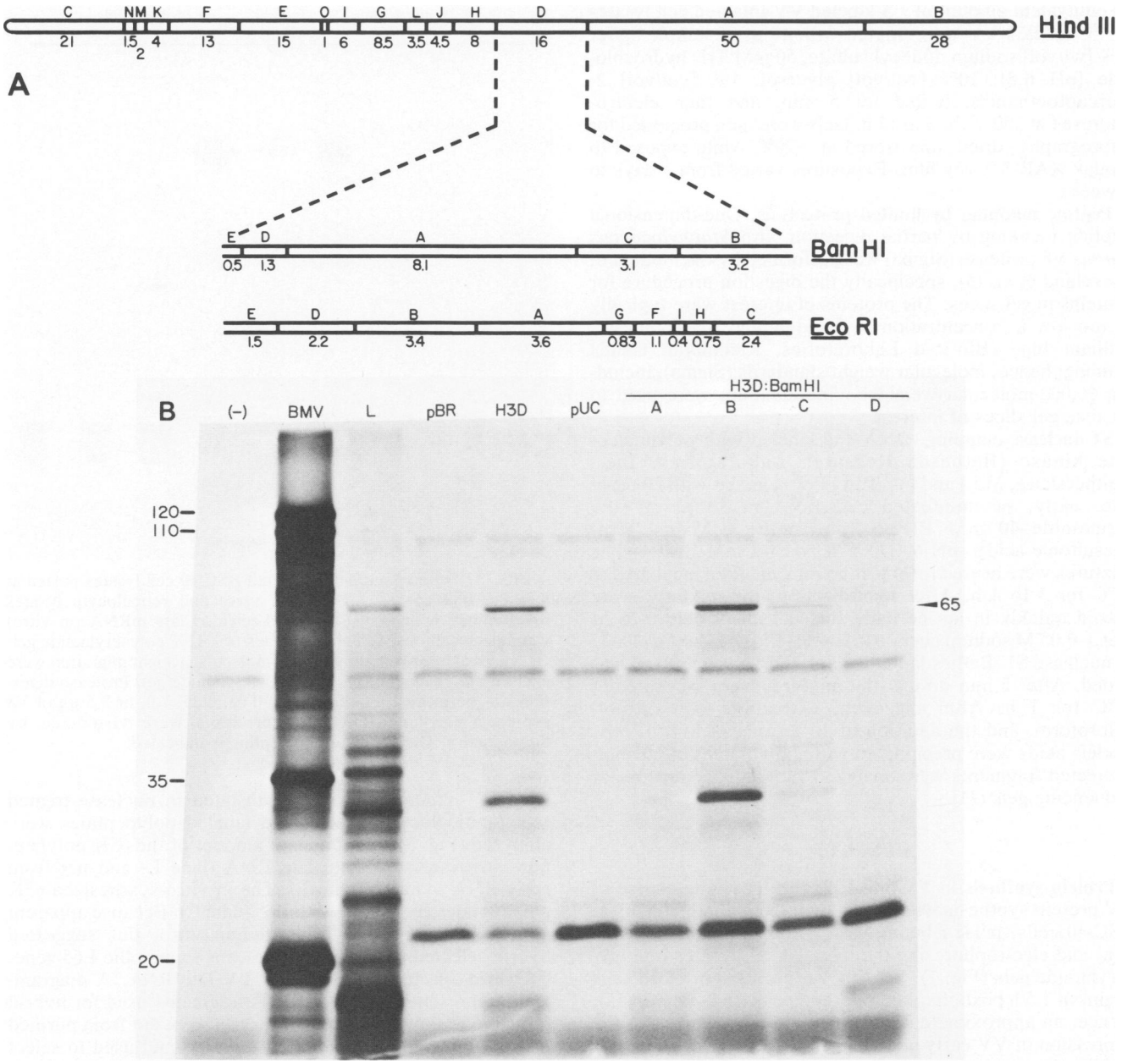

FIG. 4. (A) Diagrammatic representation of the *Hin*dIII restriction map of the VV genome and restriction maps of the *Hin*dIII D fragment as generated with *Bam*HI and *Eco*RI endonucleases. *Bam*HI and *Eco*RI fragments were cloned in pUC13. The number below each restriction fragment designates approximate size in kbp of DNA. (B) Polypeptides synthesized in reticulocyte lysates programmed with VV late RNA hybrid selected by *Hin*dIII-D *Bam*HI restriction fragments. In vitro translation products of late RNA hybrid selected by pBR322 (lane pBR), *Hin*dIII-D (lane H3D), pUC13 (lane pUC), *Bam*HI-A (lane A), *Bam*HI-B (lane B), *Bam*HI-C (lane C), and *Bam*HI-D (lane D) are shown. In lane (−), no RNA was added to the reticulocyte lysate; in lane BMV, brome mosaic virus RNA was added to the reticulocyte lysate; and in lane L, unselected late RNA was added to the reticulocyte lysate. Four proteins used as markers are indicated on the left. The 65K late polypeptide is indicated on the right.

acetate, 0.6 mM magnesium acetate, 10 mM creatine phosphate (Calbiochem-Behring, La Jolla, Calif.), 100 μg of calf liver tRNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml, a mixture of 19 amino acids (minus methionine) at a concentration of 100 μM each, 5 mM dithiothreitol, and 0.1 mCi of [^{35}S]methionine (1,145 Ci/mmol; New England Nuclear) per ml. To prevent double-stranded RNA-mediated inhibition of protein synthesis, 4.5 mM cyclic AMP was included (7). Reaction mixtures were incubated at 30°C for 90 min. Protein synthesis was measured by spotting 2.0-μl samples of the reaction mixtures on Whatman 3MM filter paper disks and processing them to determine hot-trichloroacetic acid-precipitable radioactivity as measured by liquid scintillation counting.

Polypeptide analysis. Gel electrophoresis in the presence of sodium dodecyl sulfate was performed on 10 or 12% polyacrylamide slab gels by the method of Studier (29). A 4-μl portion of ^{35}S-labeled cell-free translation products and

an equivalent amount of ^{35}S-labeled VV-infected cell lysates or ^{14}C-labeled VV were diluted with 40 μl of sample buffer (1% [wt/vol] sodium dodecyl sulfate, 50 mM Tris hydrochloride [pH 6.8], 10% [vol/vol] glycerol, 1% [vol/vol] 2-mercaptoethanol), boiled for 5 min, and then electrophoresed at 150 V for 5 to 15 h. Gels were then processed for fluorography, dried, and stored at −70°C while exposed to Kodak XAR-5 X-ray film. Exposures varied from 3 days to 3 weeks.

Peptide mapping by limited proteolysis. One-dimensional peptide mapping by partial digestion with *Staphylococcus aureus* V8 protease (Sigma) was performed by the method of Cleveland et al. (5), specifically the digestion procedure for proteins in gel slices. The proteins of interest were typically at too low a concentration to be identified by Coomassie brilliant blue (Bio-Rad Laboratories, Richmond, Calif.) staining; hence, molecular weight standards (Sigma), including 66,000-molecular-weight bovine albumin, were used to localize gel slices of interest.

S1 nuclease mapping. DNA end labeled with polynucleotide kinase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and [γ-^{32}P]ATP was mixed with 20 μg of late, early, or uninfected cell RNA in 30 μl of 80% formamide–40 mM PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) (pH 6.4)–0.4 M NaCl–1 mM EDTA. The mixtures were heated to 90°C for 5 min and then incubated at 42°C for 3 to 4 h. After hybridization, the mixtures were placed quickly in an ice bath, and 0.3 ml of cold 0.28 M NaCl–0.05 M sodium acetate (pH 4.6)–4.5 mM $ZnSO_4$–400 U of nuclease S1 (Bethesda Research Laboratories) per ml was added. After 5 min on ice, the mixtures were incubated at 25°C for 1 h. After digestion, extraction with phenol-chloroform, and the addition of 10 μg of calf liver tRNA, nucleic acids were precipitated with 2.5 volumes of ethanol. Protected fragments were analyzed with alkaline agarose or sequencing gels (21).

RESULTS

Protein synthesis in VV-infected cells. The time course of VV protein synthesis was shown by infecting monolayers of BSC-40 cells, pulse-labeling with [^{35}S]methionine every 30 min, and electrophoresing the infected cell lysates on polyacrylamide gels (Fig. 1). VV DNA replication in these cells begins at 1.5 h postinfection (p.i.) and peaks at 3.5 h p.i. (15). Hence, an approximate division can be drawn between the expression of VV early and late genes. While close scrutiny of Fig. 1 reveals many different kinetic patterns of gene expression, obvious representatives of major late gene products appeared at 94,000 and 65,000 molecular weights. When similar lysates were electrophoresed on polyacrylamide gels for increased periods, two closely migrating 65K polypeptides were resolved (Fig. 2). Pulse-chase analysis showed that the larger of the two polypeptides was apparently processed while the smaller of the two remained intact throughout the chase period. It is likely that the former corresponds to p4b, the precursor to the core structural protein 4b (22). We designated the latter L65 to indicate that it is present in infected cells late in infection and has an approximate molecular weight of 65,000. Not only was the fate of L65 different from that of p4b, but L65 also appeared to be expressed with different kinetics from that of p4b; L65 was apparent at 2.5 h p.i., whereas the synthesis of p4b was not switched on until 3.5 h p.i. (Fig. 1). The amounts of these polypeptides in infected cells at 5 to 8 h p.i. were similar.

In vitro translation products of VV mRNA. mRNA isolated from VV-infected cells was translated in nuclease-treated rabbit reticulocyte lysates, and labeled polypeptides were analyzed (Fig. 3). An abundant amount of the 65K polypeptide was translated from late RNA (lane L) and not from early RNA (data not shown). The in vitro-synthesized 65K polypeptide comigrated with L65 (lane C). Because apparent viral polypeptides are translated in vitro, this suggested hybrid-selected translation as a means to map the L65 gene.

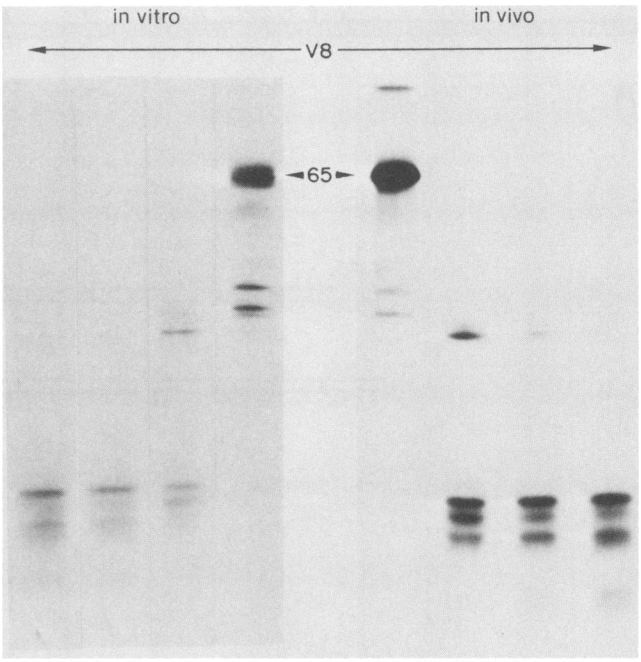

FIG. 5. Peptide mapping. Infected BSC-40 cell lysates pulsed at 8 h p.i. and chased for 8 h (in vivo) and reticulocyte lysates programmed with *Eco*RI-C hybrid-selected late mRNA (in vitro) were electrophoresed in several lanes of a 12% polyacrylamide gel. Gel slices at approximately 65,000-molecular-weight migration were excised and loaded into a 15% polyacrylamide gel. Protease digestion was performed in the gel with 0.05, 0.25, 1.0, or 2.5 μg of V8 protease per lane. Digestion products were visualized by fluorography. The 65K late polypeptide is indicated.

Hybrid-selected translation of VV late RNA. A diagrammatic representation of the DNA fragments used for hybrid selection is shown in Fig. 4A. Inserts were cut from purified plasmids, bound to nitrocellulose filters, and used to select complementary species from poly(A)$^+$ RNA isolated late in infection. Hybrid-selected RNA was eluted from the filters and translated, and [^{35}S]methionine-labeled polypeptides were analyzed.

By using 12 of the 15 *Hin*dIII restriction fragments of the VV genome, a number of late polypeptides have been translationally mapped (1; data not shown). The *Hin*dIII D fragment selected RNA that encoded a 65K polypeptide (Fig. 4B, lane H3D). None of the other 11 *Hin*dIII restriction fragments used selected RNA that encoded a 65K polypeptide.

To further define the map position of the sequences selecting RNA that encoded a 65K polypeptide, *Bam*HI and *Eco*RI restriction fragments of the *Hin*dIII D fragment were cloned (Fig. 4A) and used in hybrid selection. Sequences that hybridized to RNA encoding the 65K polypeptide apparently spanned the *Bam*HI site between the B and C fragments (Fig. 4B, lanes B and C) and perhaps extended into *Bam*HI-A. This map position was confirmed by hybrid

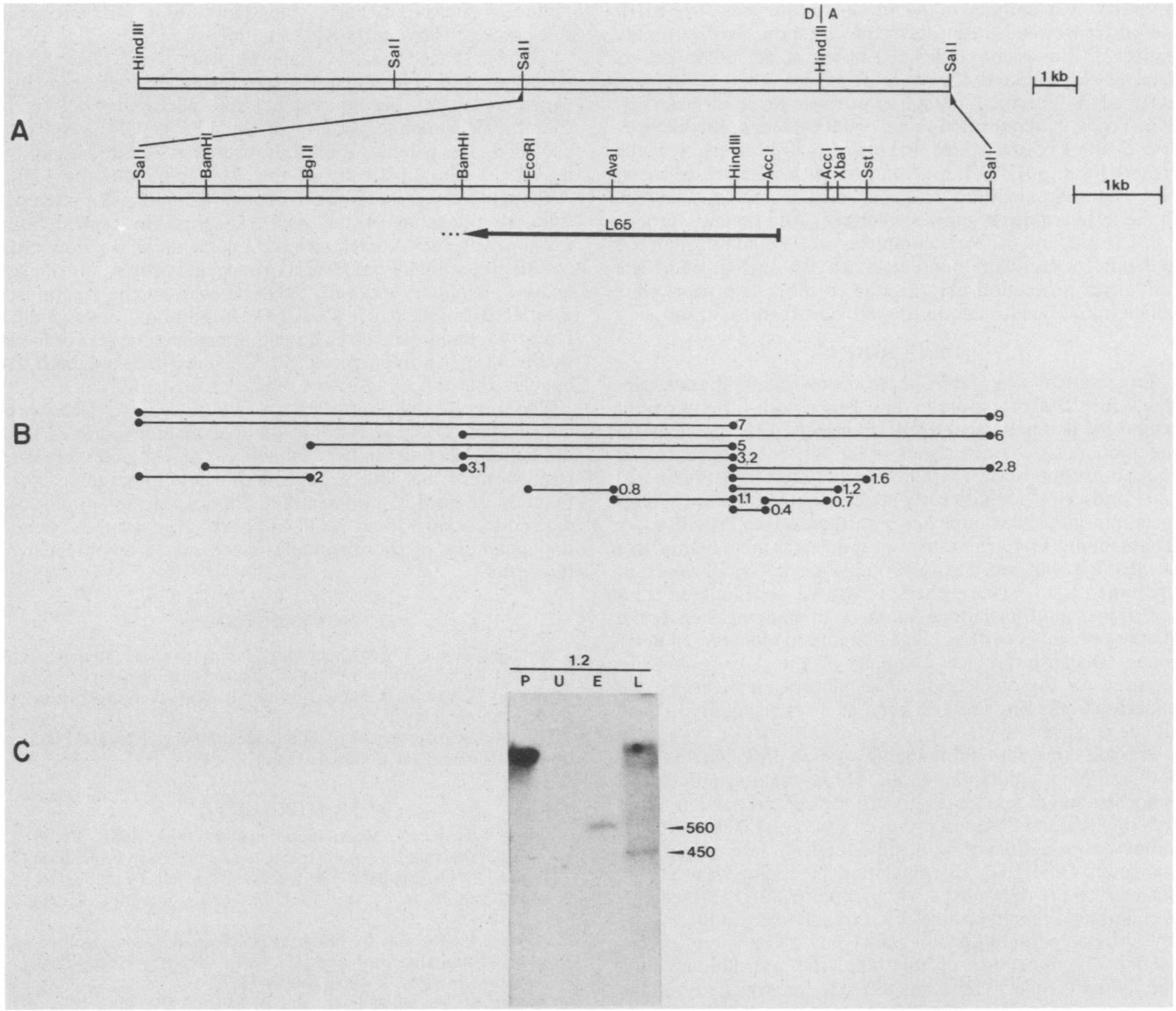

FIG. 6. Locating the 5′ end of a late transcript by nuclease S1 mapping. (A) Restriction map of the genomic region of interest. The 9-kbp *Sal*I J restriction fragment is expanded. The map position and direction of transcription of the mRNA for polypeptide L65 are indicated. (B) Various DNA fragments isolated and end labeled (●) with [γ-^{32}P]ATP and polynucleotide kinase for use as S1 probes. (C) Probe 1.2 was hybridized to 20 μg of uninfected BSC-40 cell RNA (lane U), 20 μg of early RNA (lane E), or 20 μg of late RNA (lane L); single strands were digested with nuclease S1; and the resistant hybrids were analyzed on sequencing gels. Lane P is undigested probe. Sizes of protected fragments in bases are indicated.

selection experiments in which *Eco*RI fragments C, H, and F selected RNA encoding the 65K polypeptide.

The identity of the hybrid-selected 65K translation product was determined by partial proteolytic peptide mapping. In this experiment, the in vitro peptide source was the 65K polypeptide excised from gel lanes in which *Eco*RI-C hybrid-selected translation products were resolved. For the in vivo polypeptide, 65,000-molecular-weight migrating material was excised from gel lanes in which polypeptides from infected cells pulsed at 8 h p.i. and chased for 8 h were resolved (Fig. 3, lane C), allowing isolation of labeled L65. *S. aureus* V8 protease digestion products of the gel-isolated material indicated identity between the in vitro and in vivo 65K polypeptides (Fig. 5).

Transcriptional mapping. *Bam*HI fragments A, B, C, and D were gel isolated and nick translated for use as probes in a Northern analysis of VV early and late RNAs. Several distinct early transcripts were detected; late RNA produced a heterodisperse mixture of transcripts which ranged from 6 to 0.5 kb in size (data not shown).

Translational mapping data did not dismiss the possibility that the L65 gene could span the *Hin*dIII site at the *Hin*dIII D-A junction. Hence, leftmost fragments of *Hin*dIII A were cloned from the *Sal*I J fragment which spans that junction (Fig. 6A). These plasmids along with those described above were digested with appropriate restriction enzymes, and fragments were gel isolated and then radioactively labeled at the 5′ ends for use in S1 mapping experiments (Fig. 6B).

An analysis of the late-RNA-protected fragments of probes 9, 7, 6, 5, 3.2, 3.1, and 2 indicated that a major late

transcript was initiated in the leftmost sequences of *Hin*dIII-A and transcribed in the leftward direction, protecting the entire 3.2-kbp probe and 3,600 bases of the 6-kbp probe. Late RNA protected 450 bases of probes 2.8, 1.6, and 1.2; early RNA protected 560 bases of these three probes (Fig. 6C). Probe 0.7 confirmed these results. Hence, the likely 5′ end of the L65 transcript mapped at 450 base pairs to the right of the *Hin*dIII D-A junction. At 100 base pairs upstream of the late start site the 5′ end of an early transcript, also read in the leftward direction, was mapped. Additionally, probes 2.8, 1.6, 1.2, and 0.7 were completely protected by late RNA and not by early or uninfected cell RNA (Fig. 6C). The full-length protection may be due to other late transcripts which initiate upstream on the leftward-reading strand.

DISCUSSION

Triggered by VV DNA replication, the viral transcriptional mechanism expresses late genes with little apparent regard for distinct transcript termination. This may explain the anomalous characteristics of late mRNA biogenesis. (i) Late transcripts show extreme size heterogeneity (7, 20). (ii) Late transcripts include early sequences (24) and are able to form intermolecular duplexes with themselves or with early RNAs (3, 6). (iii) Early transcript sequences are detected late in infection, but most early proteins are not synthesized at this time (2, 25). These characteristics have hindered transcriptional and translational methods of mapping; hence, the locating of late genes has lagged behind the locating of early genes. Because precise mapping of the VV genome is required for the elucidation of regulatory mechanisms, we undertook mapping the L65 gene as a representative of the late-gene class.

Previous translational mapping studies provided substantial preliminary data (1, 4, 8). Those results indicated a concentration of late genes toward the central portion of the 185-kbp genome. Among these was a 65,000-to-70,000-molecular-weight polypeptide-encoding late gene that mapped to *Hin*dIII-D. By translational mapping we localized this gene to the rightmost 4.5 kbp of the *Hin*dIII D fragment. We identified this hybrid-selected translation product as the VV late polypeptide L65. Because only 2 kbp is required to encode a polypeptide of this size, the translational data undoubtedly reflect the expected size heterogeneity of the L65 transcripts. The *Hin*dIII-A-proximal *Hin*dIII D sequences most strongly selected L65 mRNA (Fig. 4). Assuming that length heterogeneity was due to the 3′ end of the transcript and that RNA sequences were equally available for DNA hybridization, this suggested that the L65 gene was transcribed in the leftward direction, as are most of the late genes which have been precisely mapped (9, 20, 36–38).

A variety of 5′-end-labeled DNA fragments was used in S1 mapping experiments to locate the likely 5′ end of the L65 transcript at 450 base pairs to the right of the *Hin*dIII D-A junction. The direction of transcription was leftward. A Northern analysis of late RNA with the rightmost 3.2 kbp of *Hin*dIII-D as a probe detected a complex mixture of complementary RNA species. Combined, these results suggest that the 3′ end of the L65 transcript is the source of heterogeneity. In parallel experiments, the 5′ end of an early leftward-reading transcript was detected at 560 base pairs to the right of the *Hin*dIII D-A junction. Northern analysis of early RNA with the rightmost *Hin*dIII-D probe detected several minor transcripts and one major transcript of approximately 2 kb. We are examining the possibility that L65 is expressed early in infection. Similarly, the p4b late-specific transcript is initiated about 35 base pairs downstream of the initiation site of an early transcript (38). The biological relevance of these early RNAs is unclear.

Tightly spaced and overlapping transcripts have been described in previous mapping studies of VV early and late genes (9, 19, 20), but no evidence for splicing has yet been found. The genomic vicinity of the L65 gene is similarly crowded. The p4b late-specific transcript is initiated approximately 3 kbp upstream of the L65 initiation site (38). Although 2 kbp is adequate to encode the 65K p4b polypeptide, 3′ extension of the p4b transcript (in typical late fashion) appears to reach into the L65 gene, as is evidenced by full protection of the *Hin*dIII-A-specific probes. Northern analysis maps several early RNA species to the rightmost *Bam*HI B fragment of *Hin*dIII-D. In addition, several different VV temperature-sensitive mutants are marker rescued by the *Bam*HI B fragment (E. G. Niles, unpublished data). It appears that this is an active region of the genome.

The precise map position of VV late gene L65 was determined. Our current line of investigation pursues the dissection of the control sequences responsible for the regulation of this late gene and the determination of the function of the L65 polypeptide. The increasing amount of data concerning VV early and late genes may soon lead to an understanding of the regulatory mechanisms employed by this virus.

ACKNOWLEDGMENTS

We thank Dennis T. Brown, in whose laboratory these studies were initiated, Rich Condit for the Oregon State University-State University of New York connection, and Bill Dougherty for critically reading the manuscript.

This work was supported by Public Health Service grant AI-21335 from the National Institutes of Health.

LITERATURE CITED

1. **Belle Isle, H., S. Venkatesan, and B. Moss.** 1981. Cell-free translation of early and late mRNAs selected by hybridization to cloned DNA fragments derived from the left 14 million to 72 million daltons of the vaccinia virus genome. Virology **112**:306–317.
2. **Boone, R. F., and B. Moss.** 1978. Sequence complexity and relative abundance of vaccinia virus mRNA's synthesized in vivo and in vitro. J. Virol. **26**:554–569.
3. **Boone, R. F., R. P. Parr, and B. Moss.** 1979. Intermolecular duplexes formed from polyadenylylated [sic] vaccinia virus RNA. J. Virol. **30**:365–374.
4. **Chipchase, M., F. Schwendimann, and R. Wyler.** 1980. A map of the late proteins of vaccinia virus. Virology **105**:261–264.
5. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
6. **Colby, C., C. Jurale, and J. R. Kates.** 1971. Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. J. Virol. **7**:71–76.
7. **Cooper, J. A., and B. Moss.** 1979. *In vitro* translation of immediate early, early, and late classes of RNA from vaccinia virus-infected cells. Virology **96**:368–380.
8. **Cooper, J. A., and B. Moss.** 1979. Translation of specific vaccinia virus RNAs purified as RNA-DNA hybrids on potassium iodide gradients. Nucleic Acids Res. **6**:3599–3612.
9. **Cooper, J. A., R. Wittek, and B. Moss.** 1981. Extension of the transcriptional and translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J. Virol. **39**:733–745.
10. **Dales, S., and B. G. T. Pogo.** 1981. Biology of poxviruses. Springer-Verlag, New York.
11. **Gailbert, F., J. Sedal, and E. Ziff.** 1974. Direct determination of DNA nucleotide sequences: structure of a fragment of bacteriophage ϕX174 DNA. J. Mol. Biol. **87**:377–407.

12. **Glisin, V., R. Crkuenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**:2633–2637.
13. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**: 193–197.
14. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for the host-cell nucleus. J. Virol. **29**:705–715.
15. **Hruby, D. E., D. L. Lynn, R. C. Condit, and J. R. Kates.** 1980. Cellular differences in the molecular mechanisms of vaccinia virus host range restriction. J. Gen. Virol. **47**:485–488.
16. **Hruby, D. E., R. A. Maki, D. B. Miller, and L. A. Ball.** 1983. Fine structure analysis and nucleotide sequences of the vaccinia virus thymidine kinase gene. Proc. Natl. Acad. Sci. U.S.A. **80**:3411–3415.
17. **Kushner, S. R.** 1978. An improved method for transformation of *Escherichia coli* with Col E1-derived plasmids, p. 17–23. *In* H. B. Boyer and S. Nicosia (ed.), Genetic engineering. Elsevier/North-Holland Publishing Co., Amsterdam.
18. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
19. **Mahr, A., and B. E. Roberts.** 1984. Organization of six early transcripts synthesized from a vaccinia virus *Eco*RI DNA fragment. J. Virol. **49**:497–509.
20. **Mahr, A., and B. E. Roberts.** 1984. Arrangement of late RNAs transcribed from a 7.1-kilobase *Eco*RI vaccinia virus DNA fragment. J. Virol. **49**:510–520.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 329–333. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Moss, B., and E. N. Rosenblum.** 1973. Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. J. Mol. Biol. **81**:267–269.
23. **Munyon, W., E. Paoletti, and J. T. Grace, Jr.** 1967. RNA polymerase activity in purified infectious vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. **58**:2280–2287.
24. **Oda, K., and W. K. Joklik.** 1967. Hybridization and sedimentation studies on "early" and "late" vaccinia messenger RNA. J. Mol. Biol. **27**:395–419.
25. **Paoletti, E., and L. J. Grady.** 1977. Transcriptional complexity of vaccinia virus in vivo and in vitro. J. Virol. **23**:608–615.
26. **Parnes, J. R., B. Velan, A. Felsenfeld, L. Ramanathan, U. Ferrini, E. Appella, and J. G. Sidman.** 1981. Mouse β_2 microglobin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. Proc. Natl. Acad. Sci. U.S.A. **78**:2253–2258.
27. **Puckett, C., and B. Moss.** 1983. Selective transcription of vaccinia virus genes in template dependent soluble extracts of infected cells. Cell **35**:441–448.
28. **Shih, D. S., C. T. Shih, D. Zimmern, R. R. Rueckert, and P. Kaesberg.** 1979. Translation of encephalomyocarditis virus RNA in reticulocyte lysates: kinetic analysis of the formation of virion proteins and a protein required for processing. J. Virol. **30**:472–480.
29. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
30. **Venkatesan, S., B. M. Baroudy, and B. Moss.** 1981. Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. Cell **25**:805–813.
31. **Venkatesan, S., A. Gershowitz, and B. Moss.** 1982. Complete nucleotide sequences of two adjacent early vaccinia virus genes located within the inverted terminal repetition. J. Virol. **44**:637–646.
32. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
33. **Weir, J. P., and B. Moss.** 1983. Nucleotide sequence of the vaccinia virus thymidine kinase gene and the nature of spontaneous frameshift mutations. J. Virol. **46**:530–537.
34. **Weir, J. P., and B. Moss.** 1984. Regulation of expression and nucleotide sequence of a late vaccinia virus gene. J. Virol. **51**:662–669.
35. **Wittek, R.** 1982. Organization and expression of the poxvirus genome. Experientia **38**:285–297.
36. **Wittek, R., J. A. Cooper, and B. Moss.** 1981. Transcriptional and translational mapping of a 6.6-kilobase-pair DNA fragment containing the junction of the terminal repetition and unique sequence at the left end of the vaccinia virus genome. J. Virol. **39**:722–732.
37. **Wittek, R., M. Hänggi, and G. Hiller.** 1984. Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. J. Virol. **49**:371–378.
38. **Wittek, R., B. Richner, and G. Hiller.** 1984. Mapping of the gene coding for the two major vaccinia virus core polypeptides. Nucleic Acids Res. **12**:4835–4848.