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Noncoordinate Regulation of a Vaccinia Virus Late Gene Cluster†

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Identification of a tightly spaced and tandemly oriented late gene cluster within the central conserved region of the vaccinia virus genome suggested the possibility of coordinate regulation of the genes within this domain (S. L. Weinrich and D. E. Hruby, Nucleic Acids Res. 14:3003–3016, 1986). To test this hypothesis, the steady-state levels of transcripts derived from the individual late genes were examined. Cytoplasmic RNA was isolated from infected cells at hourly intervals throughout infection and was used in concert with 5′ S1 nuclease mapping procedures to detect transcripts from specific late genes. Among the set of six closely linked late genes, marked differences were observed in both the levels of transcription and the kinetic patterns of expression, providing direct evidence for the existence of differentially regulated gene subsets within the late gene class. Furthermore, these experiments identified one of the genes (encoding a 33,000-molecular-weight polypeptide) as being expressed both early and late postinfection. Interestingly, although transcripts from the constitutively expressed gene were initiated at the same start sites throughout infection, a discrete terminus for these transcripts was detected only at early times. These data suggest that the lack of *cis*-acting termination signals is not the reason for the late gene transcript heterogeneity observed in vaccinia virus-infected cells.

The best-studied member of the orthopoxviruses is vaccinia virus (VV), which is characterized by its complex morphology, linear double-stranded DNA genome, uninterrupted coding sequences, and cytoplasmic site of replication (21). Along the 185-kilobase-pair genome are distributed an estimated 200 genes, which are expressed under tight temporal regulation in vivo. Minutes after entering the cytoplasm of a susceptible host cell, immediate early genes are transcribed into mRNAs that are capped and polyadenylated by viral enzymes present within the viral core (1, 2, 22, 24, 35). A second subset of the early gene class is the delayed early genes, whose expression presumably requires one or more immediate early gene product(s) and hence is sensitive to inhibitors of protein synthesis. Dramatic changes in gene expression accompany the onset of viral-DNA replication. The VV late genes, including those encoding major structural components of the virion, are selectively expressed, while most early gene products are no longer synthesized. Unlike early transcripts, which usually terminate just distal to the coding sequences, late transcripts exhibit extreme 3′-terminal heterogeneity and may pass through one or more downstream genes (11, 17, 39). It is unclear whether changes in *cis*- or *trans*-acting elements are responsible for this unique feature of VV late gene transcription.

By analysis of pulse-labeled polypeptides synthesized in infected cells, it was inferred that at least two subsets of the late gene class could be defined on a temporal basis (23, 29). Since these initial studies, a number of late genes have been mapped and sequenced (10, 11, 13, 17, 30, 33, 38, 40–42), and their promoters have been identified (5, 39), but no evidence has been reported to support the notion of late gene subsets. Additionally, the previous pulse-labeling experiments defined a constitutive class of VV genes whose gene products were synthesized both before and after DNA replication. While this temporal class would appear to include several major viral genes, only for the gene encoding the 7,500-molecular-weight polypeptide (7.5K polypeptide) have data been obtained which clearly indicate early and late transcription of this gene, with separate RNA start sites and overlapping promoters (9).

Transcriptional (16, 25, 26) and translational (3, 7) mapping surveys indicated a lack of overall temporal organization of the VV genome. However, precise mapping and sequencing analyses of large segments of VV DNA have identified clusters of temporally related genes, suggesting local organization (11, 17, 18, 30). The identification of tandemly oriented and temporally related gene clusters suggested the possibility of coordinate regulation of genes within these domains (13, 20).

In this study, we addressed several questions of VV gene expression by examining the temporal patterns of steady-state transcripts from a tandemly oriented late gene cluster recently identified (37). Our results indicate that (i) the genes within this late gene cluster are not coordinately expressed, (ii) late gene subsets can be identified at the transcriptional level, (iii) a constitutive gene can be transcribed with the same 5′ RNA start, (iv) lack of discrete termination for late transcripts is not due to *cis*-acting signals, and (v) early RNA obtained from cycloheximide-treated infected cells may not accurately reflect authentic early RNA start sites.

MATERIALS AND METHODS

Cells and virus. Vaccinia virus strain WR was grown in suspensions of HeLa cells and purified by sucrose gradient centrifugation, and the infectious titer was determined by plaque assay on monolayers of BSC-40 monkey kidney cells as previously described (14). All media and sera were obtained from GIBCO Laboratories. Polypeptides synthesized in VV-infected BSC-40 cells were labeled with [^{35}S]methionine (1,145 Ci/mmol; New England Nuclear Corp.) as previously described (38).

HeLa cells, mouse L cells, and BSC-1 cells are commonly used as permissive hosts for studies of VV replication. Differences in the kinetics of viral replication are observed not only between these cell lines but also in the same cell line when similar experiments are performed on cells from different passages. In our hands, BSC-40 cells (selected from BSC-1 cells for their ability to grow well at 40°C) behaved

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consistently with regard to the biochemical aspects of VV replication within the first 20 to 25 passages. Hence, all experiments reported here utilized BSC-40 cells of no more than 15 passages. In addition, uniformity was maintained by utilizing the same stock of purified virus, and synchronous infection was favored by utilizing a high multiplicity of infection. Under these conditions, VV DNA replication is first detected at 1.5 h postinfection (p.i.) and peaks at 3.5 h p.i. (15); hence, an approximate division can be drawn between early and late gene expression.

RNA isolation and translation. Total cytoplasmic RNA was isolated from monolayers of BSC-40 cells infected with 10 PFU of purified VV per cell. Infected cells were placed on ice to retard enzymatic changes in RNA levels while cells were being harvested. Cycloheximide (100 μg/ml) or hydroxyurea (5 mM) was added to the medium, and infection was allowed to proceed for 4 h, after which cycloheximide-treated RNA or hydroxyurea-treated RNA was extracted. RNA was purified by using cesium chloride gradients containing sodium sarcosyl exactly as previously described (38). Rabbit reticulocyte lysates were prepared (28) and programmed with total cytoplasmic RNA at a final concentration of 250 μg/ml. Polypeptides synthesized de novo were labeled by the addition of 0.1 mCi of [^{35}S]methionine per ml.

S1 nuclease mapping. Plasmid or bacteriophage derivatives containing cloned fragments of VV genomic DNA (37, 38) were digested with appropriate restriction endonucleases. Fragments of interest were isolated by a combination of agarose gel electrophoresis and freeze-phenol techniques (4). The 5′ ends were labeled with polynucleotide kinase and [γ-^{32}P]ATP. The 3′ ends were labeled by using the large fragment of *Escherichia coli* DNA polymerase and [α-^{32}P]dCTP. In most cases, the end-labeled fragments were asymmetrically cut in subsequent restriction digests to isolate strand-specific probes. Probes were annealed to 20 μg of total RNA and subjected to S1 nuclease digestion under conditions exactly as previously described (38). S1-resistant hybrids were analyzed by electrophoresis in 8 M urea–6% polyacrylamide sequencing gels. Chemical sequencing reactions were carried out by the method of Maxam and Gilbert (19). All enzymes and radionucleotides were obtained from commercial suppliers (Bethesda Research Laboratories, Inc.; New England BioLabs, Inc.; Boehringer Mannheim Biochemicals; and New England Nuclear Corp.) and used according to the directions of the manufacturer.

RESULTS

RNAs isolated from infected cells at specific times throughout infection were assayed for transcripts initiating within a late gene cluster. The nucleotide sequences and exact locations of the RNA starts for these six genes were recently reported (37), enabling us to design specific probes to detect transcripts from each individual gene (Fig. 1B). An enigmatic characteristic of late transcription in VV is the lack of discrete termination and extreme 3′ heterogeneity of late mRNAs. For this reason, probe 01 (Fig. 1A, gel 01) detected the 5′ end of the open reading frame (ORF) 0 transcript (808-nucleotide [nt] protected fragment), detected the 5′ end of the ORF 1 transcript (615-nt fragment), and was fully protected (Fig. 1A, [FL]) by mRNA resulting from late transcriptional readthrough initiating upstream of ORF 0 (cf. lanes 2 and 3, Fig. 1A). Likewise, probes 2 through 5 detected specific RNA starts just upstream of their cognate ORFs, as well as nonspecific readthrough transcripts.

The kinetic patterns of steady-state transcripts for ORF 1 (Fig. 1A, gel 01, 615-nt fragment) and ORF 2 (gel 2, 458-nt fragment) were identical in that the signal was first detected at 2 h p.i., peaked at 3 h p.i., and tapered off but was still detectable at 12 h p.i. This pattern was apparently not a result of RNAs competing for limiting amounts of the probe, as can be seen by comparing the full-length protected signals at peak and later times. The kinetic patterns of steady-state transcripts for ORF 0 (gel 01, 808-nt fragment) and ORF 5 (gel 5, 88-nt fragment) were similar in that the transcripts were first detected at 3 h p.i. and continued to be detected at a constant level throughout, although the ORF 5 transcript showed a slight lag. Detection of the ORF 3 transcript (gel 3, 152-nt fragment) was particularly difficult, apparently because of the extensive full-length protection of probe 3. While the signal for the 152-nt fragment showed some fluctuation over time, this may have been due to the difficulty of detecting this specific transcript. Hence, we hesitate to assign a temporal pattern and simply conclude that this transcript was detectable after 3 h p.i. ORFs 3 and 5 were similar in that they appeared to be transiently utilizing upstream RNA start sites just before utilizing the downstream start sites (Fig. 1A, gels 3 and 5, 243-nt fragment; gel 5, 126-nt fragment). These transient RNA starts differed in distances from the downstream starts and in the times when they were first detected. The ORF 4 transcript was first detected at 0.5 h p.i., was estimated to peak at 1.5 h p.i., and tapered down but was still detectable at 12 h p.i. (gel 4, 149-nt fragment). High-resolution sequencing-gel analysis confirmed that RNA starts at early and late h p.i. were identical (data not shown). The onset of full-length protection of each probe and the onset of transcript detection for the upstream gene showed complete agreement, indicating the expected 3′ heterogeneity of late transcripts and the correspondence of RNA starts to transcripts which extend beyond their cognate orfs.

Because of differences in probe size, specific activity, and the hybridization conditions required for detection of specific 5′ ends amid abundant overlapping RNA, we made no attempt to quantitatively compare levels of expression between the genes. We stress the qualitative nature of these results and summarize the data in a simple fashion in Fig. 1B.

RNAs isolated from infected cells that were treated with cycloheximide or hydroxyurea were subjected to S1 mapping analyses to distinguish between immediate early and delayed early transcripts. Both treatments resulted in a block of late transcription. By definition, cycloheximide treatment should also result in a block of delayed early transcription. The RNA start for ORF 4 was detected with each inhibitor (Fig. 1A, gel 4, lanes C and H), indicating that ORF 4 was transcribed as an immediate early gene. In no other case was an RNA start detected late in infection also detected with each inhibitor, a finding which confirms the designation of ORF 0, 1, 2, 3, and 5 as late transcripts. To our surprise, when cycloheximide-treated RNA was used, transcriptional starts were detected in the vicinity of the late starts for ORF 2 (gel 2, lane C) and ORF 3 (gel 3, lane C). Since these RNA starts had no counterpart detected in hydroxyurea-treated RNA or in early RNA from untreated cells, the starts appeared to be dependent on the presence of cycloheximide and might not represent biologically relevant transcripts.

By using 3′ S1 nuclease mapping, the locations of discrete termini for early mRNAs but not for heterogeneously terminating late mRNAs can be determined. Several different 3′-end-labeled probes covering sequences from the coding region of ORF 4 into the coding region of ORF 5 were used

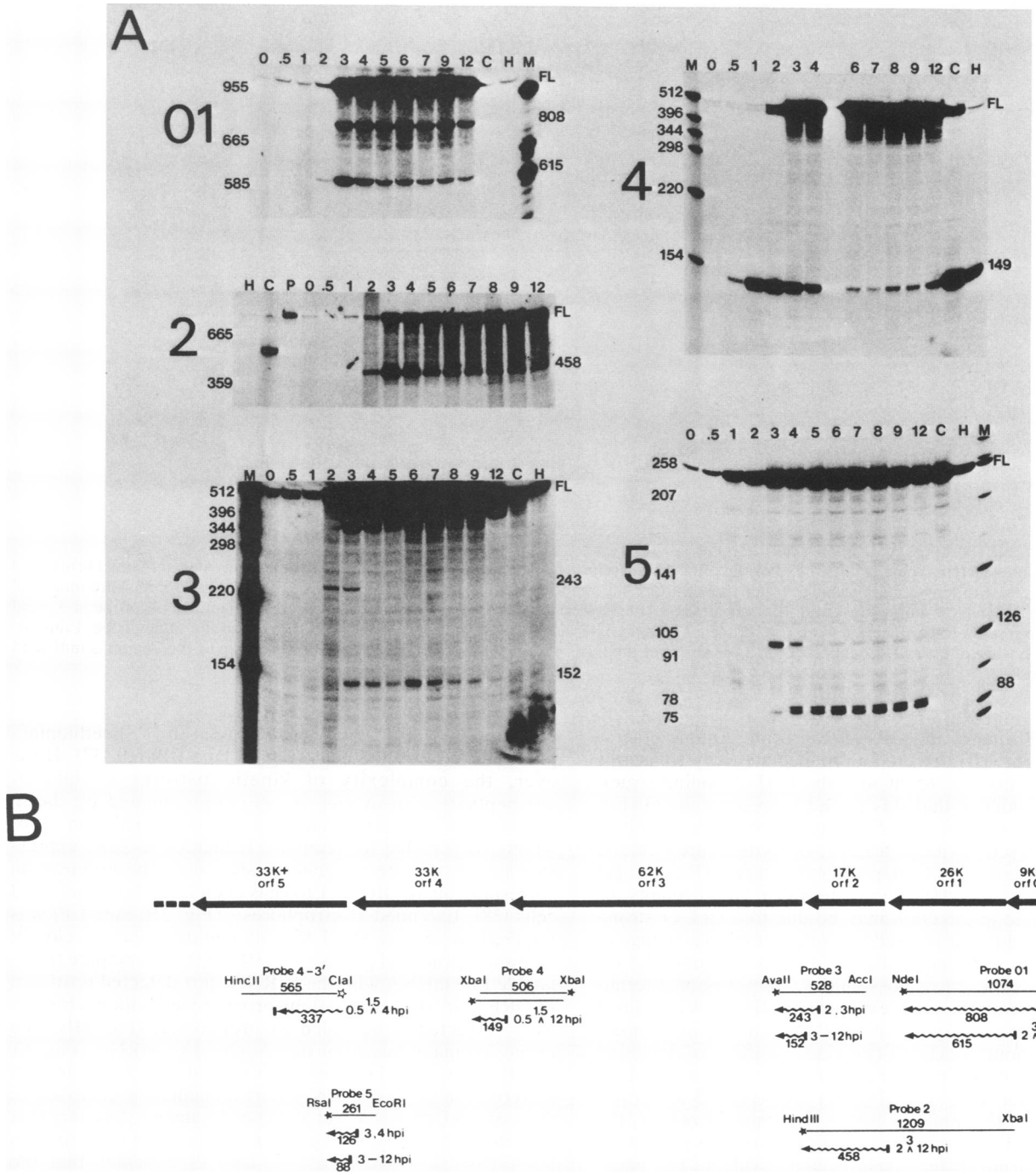

FIG. 1. (A) Steady-state transcript analyses by 5′ S1 nuclease mapping. Gel numbers 01, 2, 3, 4, and 5 refer to the corresponding 5′ S1 probe, as described in panel B. Lanes 0 through 12, C (cycloheximide treatment) and H (hydroxyurea treatment) indicate, respectively, the hours p.i. or treatment of RNA isolation from infected cells. On the right of each gel, FL indicates full-length protection of the probe, and the numbers represent the sizes (in nucleotides) of S1 nuclease-protected fragments. Lanes M, Markers for the sizes (in nucleotides) which appear on the left of each panel; Lane P (gel 2), probe in the absence of RNA or S1 nuclease. Each panel is an autoradiograph (3- to 24-h exposure) of an 8 M urea–6% polyacrylamide gel. (B) Bold arrows for P4b and ORFs 0 through 5 represent the organization of the *Hin*dIII D/A late gene cluster recently identified (37). Approximate polypeptide sizes of the predicted translated products appear above each ORF. Probes 01, 2, 3, 4, and 5 were designed to detect the RNA starts for transcripts initiating upstream of ORFs 0 and 1, 2, 3, 4, and 5, respectively. The relative position, restriction site, size, and 5′-end label (★) for each probe are indicated. ⟜, Transcripts which, when hybridized to their overlying probes, protected fragments (of the indicated sizes) of that probe from S1 nuclease digestion. To the right of each transcript is indicated the range of times during which the transcript was detected, as summarized from the data above. ∧, Peak time (in hours) of transcript signal. Probe 4-3′ was designed to detect the 3′ terminus of the ORF 4 transcript. The 3′-end label is designated (☆). The transcript and protected fragment are indicated, and the times of detection are summarized from data presented in Fig. 2.

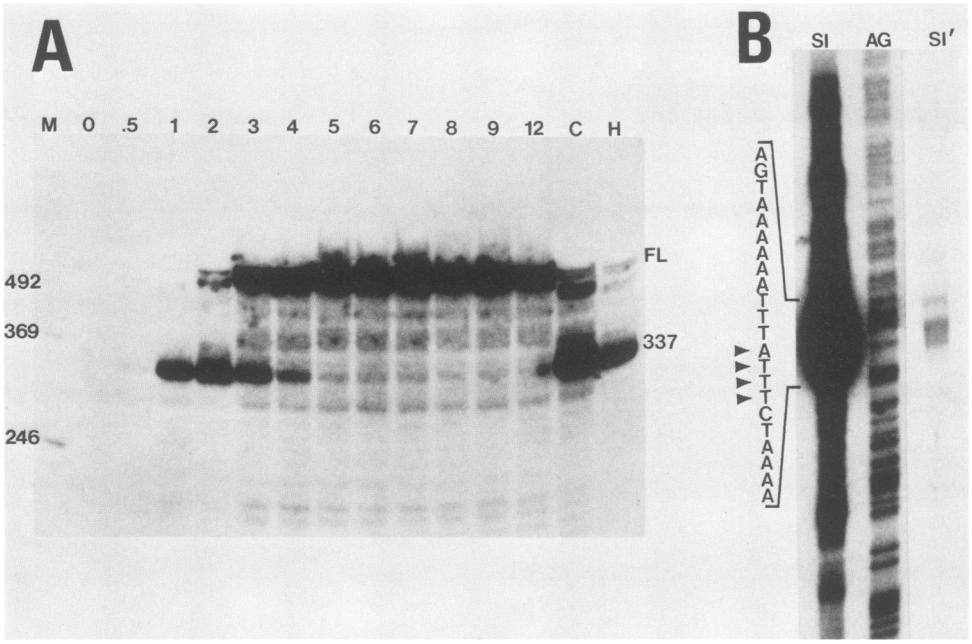

FIG. 2. S1 nuclease mapping of the 3′ end of the ORF 4 transcript. (A) As described in the Fig. 1A legend, lane designations correspond to RNAs used, the full-length and protected fragments of probe 4-3′ are indicated on the right, and marker sizes (in nucleotides) are indicated on the left. (B) Diagram of probe and transcript. S1 nuclease-resistant fragments resulting from hybridization of probe 4-3′ and cycloheximide-treated RNA (lane SI) were run beside a Maxam-Gilbert A-plus-G reaction (lane AG) performed on the same probe. Lane SI′ is a shorter exposure of lane SI. Location of the 3′ terminus of the ORF 4 transcript within the nucleotide sequence of this region is indicated with arrowheads.

to map the 3′ end of the ORF 4 transcript. Among others, probe 4-3′ (Fig. 1B) detected an immediate early transcript which terminated at 365 nt into the ORF 5 coding region (Fig. 2B). Evidence that this 3′ end represents the ORF 4 transcript was supported by full-length protection of probe 5 when early cycloheximide-treated, or hydroxyurea-treated RNAs were used in 5′ S1 mapping experiments (Fig. 1A, gel 5, lanes 1, C, and H). The 3′-terminal heterogeneity of transcripts late in infection may be due to a lack of strong termination signals at the end of late genes or to a change in transcriptional termination factors late in infection. To directly address these two possibilities, transcriptional termination of the constitutively expressed ORF 4 was examined by 3′ S1 nuclease mapping with RNAs isolated at different times throughout infection. The kinetic pattern of detection for the 3′ end of the ORF 4 transcript (Fig. 2A) was identical to that seen for the 5′ end (Fig. 1A, gel 4) at 0.5 to 4 h p.i. when either of the inhibitor RNAs was used. However, the discrete 3′ end was no longer detected after 4 h p.i., when the full-length protected signal became evident. The background signal that comigrated at 337 nt at later times had the same intensity as several other background signals seen in this figure. These results are supported by previous work in which DNA fragments downstream of ORF 4 were able to hybrid-select late mRNA encoding a 32K polypeptide (38). Since any *cis*-acting early termination signal would still be present, these data indicate that a lack of strong termination signals for late genes is not the explanation for late transcript heterogeneity.

To assess the translational activity in vitro of the RNAs used in the S1 nuclease mapping analyses, total RNA from each time point was translated in reticulocyte lysates, and labeled polypeptides were analyzed by gel electrophoresis (Fig. 3). The standard gel (panel A) resolved a spectrum of polypeptides very similar to those seen in [^{35}S]methionine-pulse-labeled infected cells (Fig. 3, lane LP) (29, 37). However, the complexity of kinetic patterns of labeled polypeptides seen in vivo is only approximated by the in vitro results, indicating that transcriptional regulation is not wholly responsible for temporal regulation of gene expression in VV. Of the six-gene cluster, only the polypeptide corresponding to ORF 3 (L65) has been identified in infected cells (38). Extended electrophoresis (Fig. 3B, lane LP) was required to resolve the L65 polypeptide from the closely migrating structural polypeptide P4b (33). To compare translatable L65 mRNA with the 5′ RNA start detected upstream of ORF 3, in vitro translations programmed with each RNA were subjected to extended electrophoresis (Fig. 3B). The onset and maintenance of translatable L65 mRNA (Fig. 3A and B, lanes 3 to 12) closely correlated with the detection pattern seen for the 5′ RNA start immediately upstream of the L65 ORF (Fig. 1A, gel 3, 152-nt fragment). The fact that no detectable L65 polypeptide was translated in vitro with RNA from 2 h p.i. (Fig. 3B, lane 2) indicates that the transient RNA start upstream of ORF 3 (Fig. 1A, gel 3, 243-nt fragment) detected at 2 h p.i. was truncated or otherwise unable to be translated into the L65 polypeptide.

DISCUSSION

Quantitation of specific mRNAs at late times in VV-infected cells is complicated by overlapping or complementary transcripts which result from the lack of discrete termination. This phenomenon is clearly apparent in Northern blot and 3′ S1 nuclease mapping analyses which readily detect complex mixtures of complementary late mRNA species with no obvious size classes. Furthermore, the full-length protection of 5′ S1 probes in the data presented

here and the lack of success in confirming late RNA starts by primer extension analyses (data not shown; 33, 37) can probably also be ascribed to terminal heterogeneity. We therefore chose a temporal 5′ S1 mapping approach as a simple and sensitive method for determining the in vivo levels of steady-state transcripts throughout infection for specific late genes.

The suggestive organization of this temporally related gene cluster and the detection of high levels of transcriptional readthrough warrant consideration of two distinct transcriptional strategies for attaining noncoordinate expression. First, transcription for the entire cluster could begin at a single upstream promoter. RNA polymerase would transcribe a large primary transcript containing 5′ processing sites which may be recognized with various kinetics yielding temporally distinct mRNAs. Second, each gene could contain sequences responsible for promoting its own transcription at the specific time when that gene product is required, and transcript termination mechanisms relaxed late in infection. To distinguish between these possibilities, direct 5′-cap labeling by the addition of [β-^{32}P]GTP with subsequent identification of labeled mRNAs (36) and target size determination for UV-induced premature termination of specific (12) or nonspecific (6, 27) transcripts are techniques which have been applied. In all of these cases, the data support an interpretation of each mRNA being synthesized from its own individual promoter site. These methods suffer from technical limitations when applied in vivo on a cytoplasmically replicating virus and have only been successful with purified virions, which contain the VV RNA polymerase and can be induced to transcribe early genes in vitro. Additionally, direct 5′-cap labeling could be misleading if transcript processing included scavenging of 5′-cap structures. Consequently, all information available to date concerning RNA start sites for VV late genes and much of the information on early RNA start sites have been the result of 5′ S1 nuclease analyses. An alternative means for testing the validity of S1 mapping results is to identify transcriptional promoter sequences. This can be accomplished by linkage of putative promoter sequences of late genes to reporter genes for transient expression analysis (8) and by direct analysis of early promoter sequences in runoff transcription assays with a virion-derived, template-dependent in vitro transcription system (32). Using these techniques, we identified promoters residing upstream of each of the genes in this tandem cluster (manuscript in preparation). Additionally, further analysis of the present data is consistent with monocistronic transcription. If individual mRNAs are derived from a primary transcript, then the primary transcript (or its processed remains) must be present at the earliest time that any of the processed mRNAs is detected. The transcript for ORF 4 was first detected at 0.5 h p.i. and strongly detected at 1 h p.i. If this transcript was processed from a primary transcript that had initiated well upstream of ORF 4, then probes 01, 2, and 3 would be fully protected at 1 h p.i. This is clearly not the case, a fact which argues against polycistronic transcript processing.

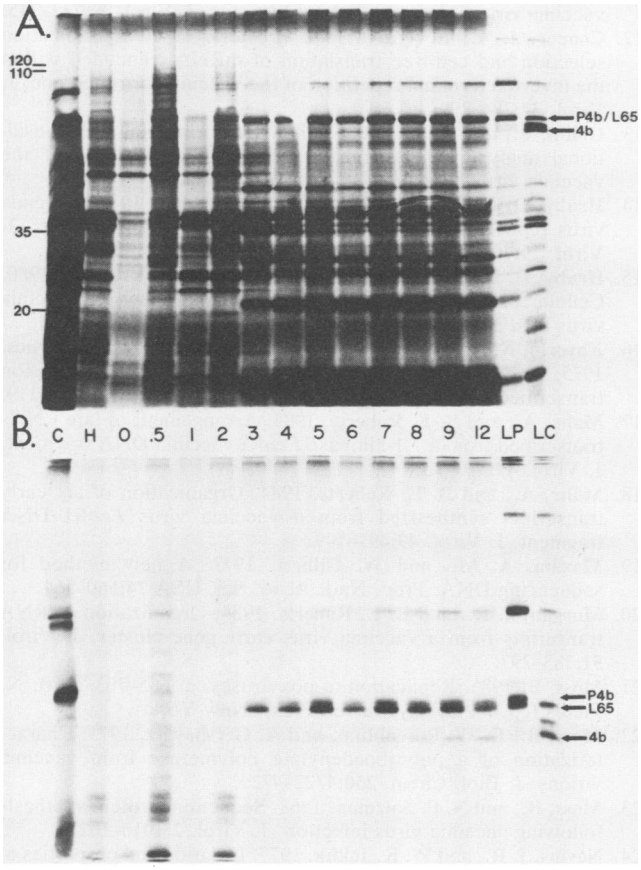

FIG. 3. In vitro translation products of RNAs as indicated by sodium dodecyl sulfate-polyacrylamide gel analysis of [^{35}S]methionine-labeled products of reticulocyte lysate translations. RNAs used were isolated from infected cells at 0 to 12 h p.i. (lanes 0 to 12) or in the presence of cycloheximide or hydroxyurea (lanes C and H). Lanes LP, Products from [^{35}S]methionine pulse-labeled infected cells at 6 h p.i.; lanes LC, the same as lanes LP except the cells were chased with excess cold methionine for an additional 4 h. (A) 12% polyacrylamide gel run at 150 V for 4 h; (B) 10% polyacrylamide gel run at 150 V for 18 h. Distinctive late proteins L65, P4b, and 4b are indicated on the right. Migration positions and molecular masses (in kilodaltons) of marker proteins are indicated on the left.

A limitation of the measurement of steady-state levels of transcripts is that no distinction can be made between de novo transcription and posttranscriptional stabilization or degradation. Previous studies indicated that VV RNA has a 2-h half-life at early times and a much shorter half-life (13 min) at late times (25, 34). Recent studies confirmed an increasing level of mRNA degradation as infection proceeds (31). The transient RNA starts detected for ORF 3 (Fig. 1A, gel 3, lanes 2 and 3) and ORF 5 (Fig. 1A, gel 5, lanes 3 and 4) and RNA starts detected for strict early genes at 0.5 and 1 h p.i. (J. N. Miner, S. L. Weinrich, and D. E. Hruby, submitted for publication) are not detected in RNA isolated a few hours later. It would thus appear that de novo transcription was predominantly responsible for the different patterns of steady-state transcripts reported here. Despite the limitations of this analysis, it is clear that the onset of transcription for the genes within this tandem cluster was not simultaneous and that the qualitative patterns of transcript levels were not identical. We conclude that, in contrast to their suggestive organization, these genes are differentially regulated at the transcriptional or postranscriptional level.

The points raised above supporting the role of de novo transcription in the patterns of mRNAs detected also support the identification of the ORF 4 locus as a constitutive gene. While the 5′-end signal for the ORF 4 transcript remained steady at late times (Fig. 1A, gel 4), the 3′-end signal was no longer detected after 4 h p.i. (Fig. 2A), indicating a rapid turnover of the discretely terminated early

mRNA for ORF 4. If the 5'-end signal was a result of increased transcript stability, then the 3'-end signal should still be detected after 4 h p.i.

For every VV late transcript that has been mapped to date, it has been directly demonstrated or inferred that, while the 5' ends are uniform, the 3' ends are not, resulting in extreme size heterogeneity for transcripts of a specific late gene. This characteristic has been attributed to a lack of *cis*-acting termination signals for late genes or to a change in *trans*-acting factors involved in termination or processing of late transcripts. It was shown that mRNA encoding a specific late polypeptide extended through early genes residing downstream, a fact which is inconsistent with the former model, since any early termination signals were not recognized (39). The results reported here are similar in showing that a constitutively expressed gene was transcribed as a distinctly terminated mRNA at early but not at late times.

Cycloheximide is typically used to block the expression of late genes, and it has the additional technical benefit of boosting transcription of immediate early genes (Fig. 1A, gel 4, lane C). For this reason, cycloheximide-treated RNA has been utilized as early RNA in many studies of VV gene expression. However, the results reported here indicate that cycloheximide may have additional effects which alter the recognition specificity of the transcriptional machinery. These findings suggest that authentic early RNA or RNA that has been isolated with a different inhibitor of late transcription should be used to confirm results with cycloheximide-treated RNA.

In an attempt to identify further relationships between those genes which exhibited similar patterns of steady-state transcripts, the first 100 nt upstream of the RNA start site were compared for homology and alignment at a stringency lower than that previously utilized (37). Included in this analysis were upstream sequences for the transiently utilized RNA starts for ORFs 3 and 5. No significant homologies with respect to location from the RNA start were found. While it has been shown that *cis*-acting control signals lie just upstream of the RNA starts for late genes (5, 39), no apparent consensus with eucaryotic or procaryotic signal sequences or among the sequenced late genes was identified (data not shown). The available nucleotide sequences, RNA start sites, and temporal regulatory schemes of this late gene cluster should now facilitate a directed approach to determining the control signals responsible for regulating late gene transcription in VV.

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