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Transcriptional Mapping of the Varicella-Zoster Virus Regulatory Genes Encoding Open Reading Frames 4 and 63

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Four of the 68 varicella-zoster virus (VZV) unique open reading frames (ORFs), i.e., ORFs 4, 61, 62, and 63, encode proteins that influence viral transcription and are considered to be positional homologs of herpes simplex virus type 1 (HSV-1) immediate-early (IE) proteins. In order to identify the elements that regulate transcription of VZV ORFs 4 and 63, the encoded mRNAs were mapped in detail. For ORF 4, a major 1.8-kb and a minor 3.0-kb polyadenylated [poly(A)$^+$] RNA were identified, whereas ORF 63-specific probes recognized 1.3- and 1.9-kb poly(A)$^+$ RNAs. Probes specific for sequences adjacent to the ORFs and mapping of the RNA 3′ ends indicated that the ORF 4 RNAs were 3′ coterminal, whereas the RNAs for ORF 63 represented two different termination sites. S1 nuclease mapping and primer extension analyses indicated a single transcription initiation site for ORF 4 at 38 bp upstream of the ORF start codon. For ORF 63, multiple transcriptional start sites at 87 to 95, 151 to 153, and (tentatively) 238 to 243 bp upstream of the ORF start codon were identified. TATA box motifs at good positional locations were found upstream of all mapped transcription initiation sites. However, no sequences resembling the TAATGARAT motif, which confers IE regulation upon HSV-1 IE genes, were found. The finding of the absence of this motif was supported through analyses of the regulatory sequences of ORFs 4 and 63 in transient transfection assays alongside those of ORFs 61 and 62. Sequences representing the promoters for ORFs 4, 61, and 63 were all stimulated by VZV infection but failed to be stimulated by coexpression with the HSV-1 transactivator Vmw65. In contrast, the promoter for ORF 62, which contains TAATGARAT motifs, was activated by VZV infection and coexpression with Vmw65. These results extend the transcriptional knowledge for VZV and suggest that ORFs 4 and 63 contain regulatory signals different from those of the ORF 62 and HSV-1 IE genes.

Varicella-zoster virus (VZV) is a human herpesvirus which causes two clinically distinct diseases. Varicella, which results from primary infection, is most commonly acquired in childhood. Zoster, on the other hand, results from virus reactivated from the latent state and is most frequently seen in adults, particularly the elderly. Although usually self limiting, both diseases are more serious in immunocompromised individuals, and zoster is becoming a problem in patients with AIDS (3). Despite the clinical significance, the biology of VZV has remained quite refractory to study, largely as a result of the poor growth of the virus outside the human host. In tissue culture, VZV remains highly cell associated and has high particle/infectivity ratios, and cell-free virus has low, unstable titers. Much of our knowledge of VZV has stemmed from predictions based upon the VZV DNA sequence (13), largely in the context of organizational and sequential similarities to better-characterized and related herpesviruses, particularly herpes simplex virus type 1 (HSV-1) (12, 13, 44).

Although not yet well defined, VZV is considered to regulate its viral growth cycle in a fashion similar to that of other herpesviruses. In general, herpesviruses transcribe their genes in a tightly regulated fashion, in which viral genes are temporally expressed in three kinetic classes during infection, depending upon the requirements for their transcription (27, 28; reviewed in reference 73). Immediate-early (IE) genes are transcribed upon infection without any de novo viral protein synthesis (27, 28, 61) and encode products that function primarily to control further transcriptional events (reviewed in reference 17). Early- and late-class genes, on the other hand, require functional IE proteins and/or DNA replication. HSV-1, which has served as the primary model for regulation, encodes five IE genes, designated ICP4, ICP0, ICP22, ICP27, and ICP47 (27, 28). For VZV, attention has focused upon proteins encoded by open reading frames (ORFs) 4, 61, 62, and 63, which are considered to be homologs of HSV IE genes (12, 13). In transient transfection-expression assays, all four ORFs encode proteins that influence transcription from VZV and other promoters, in both negative and positive manners (6, 14, 16, 29–31, 50, 52, 58, 59). ORF 62, which has 58% amino acid identity with the HSV-1 major regulatory protein ICP4, is sufficiently functionally conserved in comparison to ICP4 that it partially complements HSV ICP4-negative or temperature-sensitive mutants (19, 20) and viably replaces ICP4 in the context of the HSV-1 genome (15). ORF 61 has also been shown functionally to complement HSV-1 mutants with mutations in its respective homolog, ICP0 (50), despite some apparent different transcriptional regulatory properties compared with ICP0 as shown in transient transfection assays (52). ORF 4, the positional homolog of HSV-1 ICP27, has been shown to augment transcriptional activation by ORF 62 as well as to possess independent transactivation properties on some VZV and non-VZV promoters (14, 29). ORF 63, the homolog of HSV-1 ICP22, has been implicated to have functions in regulating ORF 62 and early gene transcription (31).

Although they are considered to be homologous to HSV-1 IE genes, the temporal transcriptional class of ORFs 4, 61, 62, and 63 and the regulatory elements that govern their expres-

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sion remain to be fully defined, largely because of the difficulty in conducting a high-multiplicity synchronized VZV infection. Evidence from the analysis of immune-precipitated, phosphorylated polypeptides made under classical IE conditions have suggested that at least four or five VZV polypeptides, ranging from 45 to 200 kDa, are synthesized (39, 67) (although the identified polypeptides from the two studies are not in full agreement). Evidence obtained by using specific antibodies to the ORF 62 protein in immunofluorescence studies to identify individual cell-free virus-infected cells has indicated that this protein is synthesized under conditions of cycloheximide reversal (21). However, similar experiments to define the temporal class of ORFs 4, 61, and 63 have not yet been described.

As an alternative approach to addressing the regulation of these genes, we and others have set out to establish information regarding the sequences that regulate their expression. This requires definition of the transcriptional initiation sites of the encoded RNAs. To date, the initiation sites for ORFs 61 and 62 have been described and their respective promoters have been examined (45, 46, 69). Particular significance was placed upon the presence or absence of a *cis*-acting sequence, the TAATGARAT motif, within the promoters (40, 60). In HSV-1, this sequence lies within the consensus sequence 5′-GyATGnTAATGArATTCyTTGnGGG NC-3′, which is found in the promoters of all HSV-1 IE genes (40, 54, 56, 60). It is the target for a strong transcriptional transactivating complex composed of the virion protein Vmw65 (also known as αTIF and VP16) (5, 7, 41, 47) and several cellular transcription factors, including the ubiquitous factor OCT-1 (also known as αH1, NFIII, and OTF-1) (4, 22, 36, 47, 54, 62; reviewed in reference 65). Binding occurs only when a match to the motif is present (22, 36, 55). The motif is also found within the regulatory sequences of the IE genes of several alpha herpesviruses, including equine herpesvirus type 1 (26) and pseudorabies virus (8), suggesting a conserved role. For VZV, the regulatory region of ORF 62 contains two copies of the TAATGARAT motif, which can actively bind and be activated by HSV-1 Vmw65-transcription factor complexes (45, 46). However, no such motif is apparent in the regulatory sequences upstream of the ORF 61 transcriptional start site (69).

In this work, we have extended these studies to include analyses of the mRNAs and their transcriptional initiation sites for ORFs 4 and 63. We also present evidence suggesting that the promoters of these genes do not contain any functional TAATGARAT motifs upstream of their transcriptional initiation sites.

MATERIALS AND METHODS

Cells and virus. The VZV strains used in this study, strains Scott and Ellen, and their propagation in human foreskin fibroblast cells have been described previously (34, 35, 70). Virus for RNA preparation (strain Scott) was grown on a human melanoma cell line (MeWos; a kind gift of C. Grose, University of Iowa) or on human foreskin fibroblast cells in minimal essential medium supplemented with 4% fetal calf serum and 4% Serum Plus (JRH Biochemicals). The two cell lines yielded RNAs that gave similar and consistent results. All cells for RNA preparation were grown at 35°C in 175-cm^2 flats, and four to six flats were used per RNA preparation. For transient analyses of promoter function, Vero cells were grown at 37°C in medium 199 supplemented with 10% heat-inactivated fetal calf serum.

RNA preparation, isolation, and Northern (RNA) blot analyses. For infection, confluent monolayers of MeWo cells were overlaid with VZV-infected human foreskin fibroblast cells at an infected/uninfected cell ratio of approximately 1:40. Twenty-four hours later, the cells were trypsinized and allowed to reattach to the same flask. After 24 to 36 h of further incubation, cells showing approximately 10 to 20% cytopathic effect were washed in ice-cold phosphate-buffered saline and lysed directly on the flask at 4°C in an RNA lysis buffer as described previously (9, 10). Total RNA was subsequently prepared from VZV-infected and uninfected cells by the guanidinium isothiocyanate-phenol extraction procedure (10). From the extracted total RNA, polyadenylated [poly(A)$^+$] RNA was prepared by using the Polytract mRNA Isolation System (Promega Corporation, Madison, Wis.). For Northern blot analyses, 0.5 μg of poly(A)$^+$-selected RNA per lane was electrophoresed on formaldehyde-denaturing agarose gels and transferred to GeneScreen (NEN-Dupont Nemours, Boston, Mass.) or Nytran (Schleicher and Schuell, Keene, N.H.). RNA was fixed to the membranes by UV cross-linking. The two membranes gave comparable results.

TABLE 1. DNA sequences of oligonucleotides used to map VZV RNAs

ORF	Oligonucleotide		
	Sequence (5′→3′)	Initial position	Final position
3	CCACGCCAGTGGCGGTATAACTTGTG	2343	2370
4	ACTTCCAATGCAAAGTCATCCGA	4038	4060
5	TGTTCAAACTTTACACGAGCGGTATA	5171	5196
63	AGTCGCCCCGCGTAGCCGGTGAGGTGCA	110587r[a]	110614r
64	CGTGTGCAGTAAAGTCCGGCATATTC	111604r	111629r
4t	TTACAAGCATGAAATAGGTAT	2676	2697
63TI	CCCAAACGTTTACATCTATGAATAAG	110464r	110489r

[a] r, DNA sequence of the oligonucleotide is reverse and complementary to the sequence of coordinates given.

RNA blots to be probed with random-primed-labeled DNA probes were prehybridized and hybridized at 42°C in a buffer containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS), 50 mM sodium phosphate buffer (pH 7.6), 5× Denhardt's solution, and 200 μg of sheared, denatured salmon sperm DNA per ml. RNA blots to be probed with oligonucleotides were prehybridized and hybridized in the same buffer without formamide and with 25 μg of yeast tRNA per ml. Double-stranded DNA probes were prepared by random oligonucleotide-primed repair synthesis with radiolabeled [α-^{32}P]dCTP (3,000 Ci/mmol; NEN-Dupont Nemours) and a hexanucleotide DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Following hybridization for 6 to 14 h, excess probe was removed by washing twice in 2× SSC–0.1% SDS at room temperature, twice in the same buffer at 65°C, and once at room temperature in 0.1× SSC. Oligonucleotide probes (Table 1) were made from 200 ng of oligonucleotide labeled to high specific activity with T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol; NEN-Dupont Nemours). Probes were hybridized to the blots for 6 to 14 h at 42°C, and excess probe was removed with 6× SSC–0.1% SDS at 10°C below the calculated T_m of the oligonucleotide. For all blots, the RNA size was estimated from a 0.24- to 9.5-kb RNA ladder (Life Technologies, Inc., Rockville, Md.) run under the same conditions as the test RNA.

S1 nuclease protection and primer extension analyses. S1 nuclease analysis was carried out as described elsewhere (66) with modifications (37, 38). Briefly, 10 μg of total RNA was hybridized to end-labeled DNA probes at 44°C for 4 to 16 h.

RNA-DNA hybrids were subsequently digested with various amounts of S1 nuclease (Life Technologies, Inc.) for 30 min at 37°C. Products were resolved under native conditions on Tris-borate-buffered agarose gels or under denaturing conditions on sequencing gels as previously described (37, 38). Fragment sizes were estimated by using a 1-kbp ladder and a φX174 *Hae*III digest (Life Technologies, Inc.) labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP or by using an M13 sequencing ladder as supplied and made as described in the Sequenase version 2 kit (U.S. Biochemical Corp.).

Primer extension was carried out by using synthetic oligonucleotide primers predicted to hybridize to RNAs encoded by the genes under analysis (Table 1). The methodology and buffers were essentially similar to those outlined previously (37, 38, 66). Briefly, oligonucleotides were labeled to high specific activity by using T4 polynucleotide kinase and [γ-^{32}P] ATP, and 10 to 50 ng of primer was hybridized to 10 μg of total RNA or 0.5 μg of poly(A)$^+$ RNA. Annealed RNA-primer hybrids were allowed to form over 2 to 4 h at 42°C and were subsequently reverse transcribed by using 50 U of Superscript (Life Technologies, Inc). Products were separated on sequencing gels, and sizes of products were verified by comparison with sequencing ladders generated by using appropriate cloned DNA fragments with the same primers.

Plasmids and constructs. The cloned sequences used for studies of RNAs from ORF 63 were derived from a *Kpn*I R fragment cloned from VZV Scott in pUC19 (pKR-2) and an *Eco*RI E fragment derived from VZV Scott and cloned in pBR325, representing sequences from 109893 to 111204 and 100441 to 112758, respectively (coordinates are given in base pairs with respect to the left end of the sequenced VZV genome [13]). For ORF 4 RNA analyses, DNA sequences were derived from a cloned *Eco*RI C fragment of VZV Scott in pBR325 (VZV nucleotides 444 to 14726) or from pGI4 (a kind gift of J. Ostrove [29]), which carries a *Pvu*II fragment containing VZV ORF 4 and a large part of its upstream region (VZV nucleotides 2046 to 5036). Plasmid pMC1, which includes the HSV-1 gene encoding Vmw65 under its cognate promoter, was a kind gift of C. M. Preston (7). The plasmids containing the fragments tested for promoter activities in transient transfections (p4CAT, p61CAT, and p63CAT) have been described previously (14). Plasmid p4CAT contains a 611-bp *Nco*I-*Bst*EII fragment, representing sequences from 2 to 613 bp upstream of the ATG, driving the chloramphenicol acetyltransferase (CAT) gene in pSV0CAT. Similarly, p61 CAT contains a 2,259-bp *Nco*I-*Hin*dIII fragment driving the CAT gene, representing sequences from 2 to 2,261 bp upstream of the ATG, and p63CAT contains a 1,747-bp *Sca*I-*Sty*I fragment driving the CAT gene, representing sequences from 8 to 1,754 bp upstream of the ATG. Plasmid p140CAT (45, 46), a kind gift of C. M. Preston, contains sequences from 15 to 1,218 bp upstream of the ATG. Plasmid p4CAT(1), which contains sequences from 192 bp within ORF 4 to 895 bp upstream of the ATG, was a kind gift of J. Ostrove (29, 52). All plasmids were prepared by alkaline lysis and purified by CsCl-ethidium bromide equilibrium gradients or by Qiagen column fractionation (Qiagen Inc., Chatsworth, Calif.).

Transfection and transient expression analyses. Vero cells were used for transfection studies to analyze the expression of the VZV promoter-CAT fusion constructs. Cells plated at a density of 3×10^5 per 35-mm-diameter well were seeded the day prior to transfection. DNA transfection was performed by calcium phosphate precipitation (24) or by using cationic lipid vesicles of DOTAP (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's instructions and as described previously (18, 68). The two procedures gave comparable results. For the latter procedure, various amounts of target construct and effector plasmid DNAs were mixed in 50 μl of Hanks buffered saline, with the total amount of DNA in each experiment remaining constant at 5 μg by addition of sonicated herring sperm DNA, unless stated otherwise. A 10-μg/ml solution of DOTAP was added to the DNA, and the mixtures were kept at 22°C for 10 min before being deposited on the cells. When infected, cells were overlaid at 24 h posttransfection with VZV-infected cells at a 1:4 ratio. Cells were harvested 44 to 48 h posttransfection, assayed for expression of the reporter gene as described previously (23, 53), and equilibrated on the basis of total protein content. CAT activity was quantitated by estimation of the initial rates of conversion, and each transfection was repeated three times. Results were equivalent to those obtained by standard thin-layer chromatography techniques (23).

RESULTS

Mapping of ORF 4 and 63 RNAs by Northern blot analyses. Previous transcriptional analyses had indicated RNAs of 1.8 and 1.4 kb mapping to the ORF 63 region, whereas several species, including 3.1- and 1.8-kb RNAs, mapped to DNA including the ORF 4 region (57, 63). Our first aim was to expand these results by using ORF-specific probes to determine the transcriptional patterns of ORFs 4 and 63 with respect to adjacent sequences. As in previous work (37, 38), RNA was derived from heterogeneously infected cells, because the low titers of cell-free VZV obtainable precluded conducting efficient single-step cell-free virus infections. However, we routinely harvested cells prior to the onset of extensive cytopathic effect, with the intention of enriching for IE and early RNA.

Figures 1 and 2 show representations of the regions of the VZV genome containing ORFs 4 and 63, respectively, and indicate the relative positions of the probes used in the mapping studies. Map coordinates for enzyme sites refer to the published DNA sequence for VZV strain Dumas (13).

mRNAs specific for ORFs 4 and 63 were identified by probing Northern blots of poly(A)$^+$-selected RNA with the antisense oligonucleotide probes and DNA fragments shown in Table 1 and Fig. 1 and 2. Representations of the RNAs detected for ORF 4 are shown in Fig. 3A. An ORF 4-specific oligonucleotide identified a minor 3.0-kb and a major 1.8-kb RNA species (Fig. 3A, lanes e). RNAs with similar sizes were detected by a double-stranded DNA probe (the *Ssp*I-*Nco*I fragment shown in Fig. 1) representing the entire ORF 4 (Fig. 3A, lanes d). ORF 63 RNAs are shown in Fig. 3B. An ORF-specific oligonucleotide (Fig. 3B, lanes b) and an *Hpa*I-*Nco*I DNA fragment representing all of ORF 63 except the first 23 amino acids (Fig. 3B, lanes d) both recognize RNAs of 1.9 and 1.3 kb. No additional RNAs were detected for either ORF in experiments using RNA from VZV-infected cells showing less than 10% cytopathic effect or from cells showing greater than 70% cytopathic effect (data not shown), indicating that these are the only species transcribed from the respective ORFs in a productive infection. A 3.9-kb RNA and a 1.9-kb RNA were also detected with some oligonucleotide probes (e.g., lanes a, b, and e in Fig. 3A), but they were also found in uninfected-cell RNA. We therefore conclude that detection of these species is likely due to nonspecific interaction of the oligonucleotides with trace amounts of rRNA in the poly(A)$^+$-selected RNA preparations.

Additional probes were used to determine the relationship of the two RNAs for each ORF with respect to adjacent sequences. With respect to ORF 4, ORF 5 lies upstream and

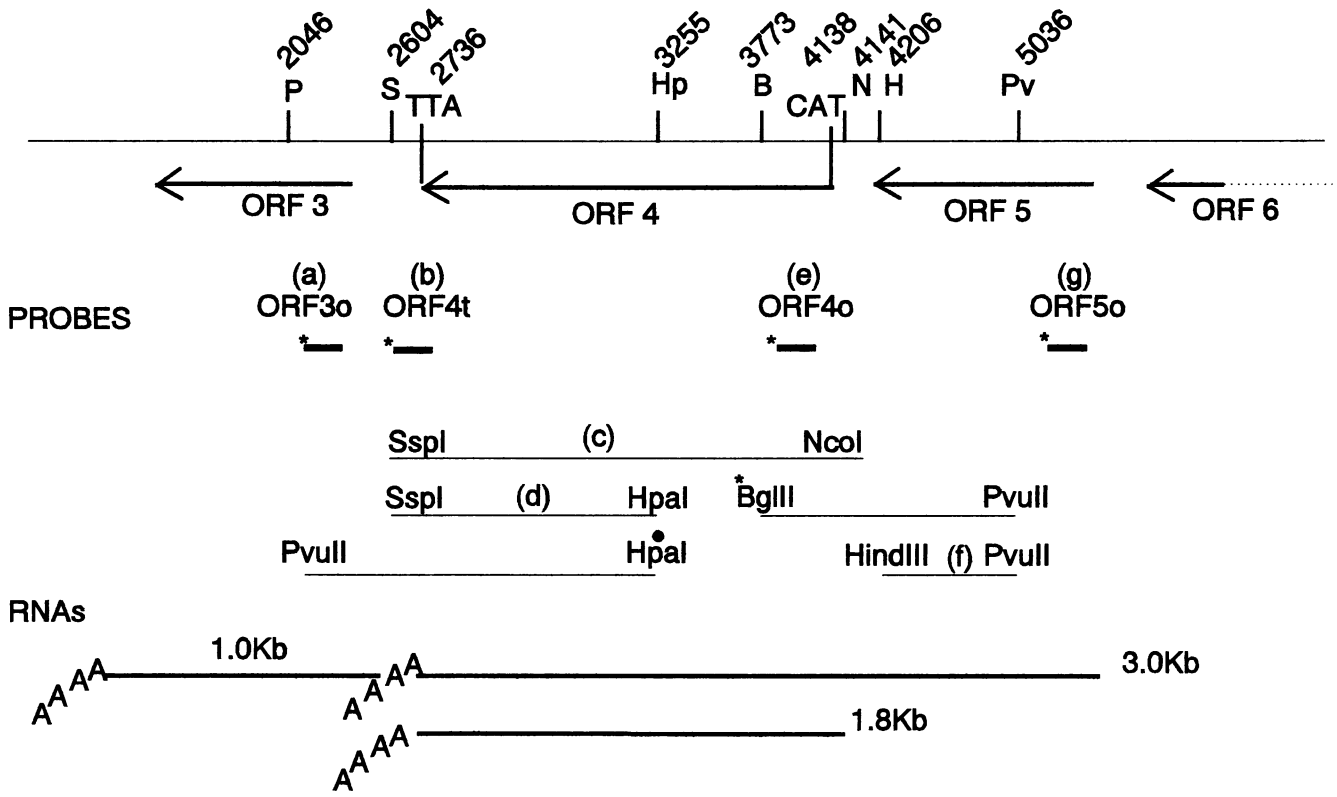

FIG. 1. Diagrammatic representation of the genomic organization of VZV ORFs 3, 4, and 5 and the probes employed to map the ORF 4-encoded RNAs. The top part of the figure represents the genome, the direction of the ORFs, and the positional locations of restriction enzymes and codons of ORF 4, given in base pairs from the left end of the sequenced VZV genome (13). CAT and TTA represent the complements of the start and stop codons for ORF 4. Oligonucleotide probes (o, t) (Table 1) are represented by bars at their approximate positions, and DNA probes are represented as lines with the restriction enzymes used in their generation. The letter designations of specific probes correspond to the respective lanes shown in Fig. 3A. Asterisks indicate uniquely labeled 5′-end-labeled probes, and ● represents a uniquely 3′-end-labeled probe. The relative positions of the RNA species detected for the ORFs are also shown, with AAAA representing the polyadenylated tails at the 3′ end of the RNAs. P, *Pvu*II; S, *Ssp*I; Hp, *Hpa*I; B, *Bgl*II; N, *Nco*I; H, *Hin*dIII; Pv, *Pvu*II.

ORF 3 lies downstream, and all three ORFs are read in the same direction (Fig. 1). An oligonucleotide probe antisense to part of the N-terminal region of ORF 3 recognized a novel 1.0-kb RNA (Fig. 3A, lanes a) but failed to recognize either of the ORF 4-specific RNAs, suggesting that the latter terminated before ORF 3. On the other hand, an *Ssp*I-*Hpa*I DNA probe representing the 3′ end of ORF 4 (Fig. 1) and an oligonucleotide derived from the sequences downstream of the ORF 4 termination codon both recognized the ORF 4-specific RNAs and failed to recognize the 1.0-kb ORF 3 RNA (Fig. 3A, lanes c and b, respectively). This result implies that the ORF 4 RNAs terminate close to each other, upstream of ORF 3. Upstream of ORF 4, an oligonucleotide to ORF 5 recognized the 3.0-kb RNA but failed to recognize the 1.8-kb ORF 4-specific RNA (Fig. 3A, lanes g). In addition, a *Hin*dIII-*Pvu*II DNA probe, derived from sequences 62 bp upstream of ORF 4 and part of ORF 5, detected the same RNA species as the ORF 5 oligonucleotide but failed to recognize the ORF 4 1.8-kb RNA (Fig. 3A, lanes f). The most direct explanation for the observed results is shown in Fig. 1, in which the 3.0-kb ORF 4-specific transcript originates upstream of ORF 5 and possesses redundant 3′ sequences derived from ORF 4. We conclude that the smaller RNA originates just upstream of ORF 4 and is most likely controlled by the ORF 4-specific promoter.

Similar studies were carried out to confirm the organization of ORF 63-specific transcripts. Downstream of ORF 63 lies ORF 64, and upstream lies an origin of replication. Previous studies suggested that the larger ORF 63 RNA extended 3′ to ORF 64 (57, 63). No additional RNAs were detected by using a *Kpn*I-*Mlu*I probe (Fig. 2) covering the upstream portion of ORF 63 and the origin of replication (Fig. 3B, lanes c). However, an ORF 64-specific oligonucleotide recognized a 1.9-kb RNA also detected by ORF 63 probes (Fig. 3B, lanes e) and an additional novel 1.0-kb RNA. A DNA probe derived from the majority of ORF 63 failed to recognize the 1.0-kb RNA (Fig. 3B, lanes d), strongly suggesting that the 1.0-kb RNA originated downstream of ORF 63 and was specific for ORF 64 sequences. Figure 2 shows the concluded organizational pattern of these transcripts. The 1.3-kb species probably terminates between ORFs 63 and 64, and the larger RNA represents transcriptional bypassing of this transcription site and termination further downstream. The 1.0-kb ORF 64 transcript probably originates at an ORF 64-specific promoter. These results are consistent with previous mapping studies (57, 63).

Mapping of the 3′ ends of ORF 4 RNAs. The previous results suggested that the 3′ ends of the ORF 4-specific RNAs were close or coterminal. To confirm this, S1 nuclease protection was used to define the 3′ ends more precisely. To generate a probe specific for the 3′ end of ORF 4, cloned *Eco*RI-C DNA was restricted and labeled with T4 DNA polymerase on the

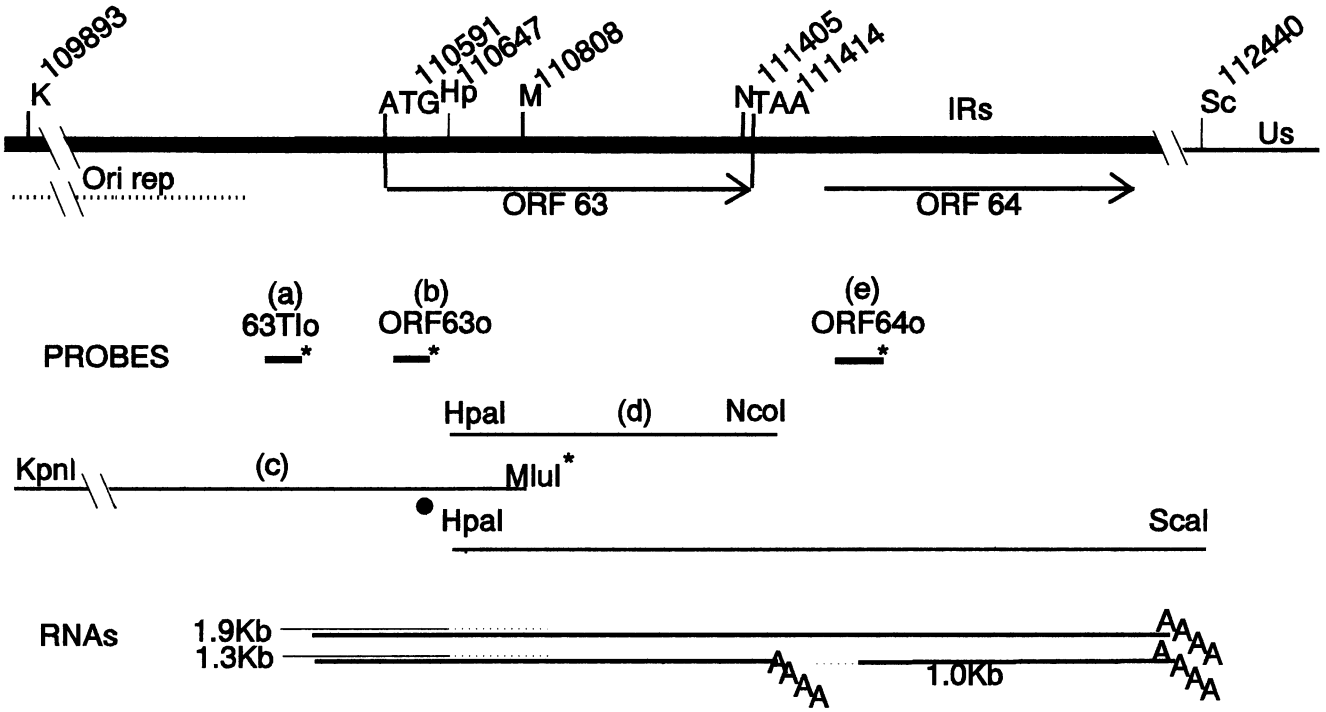

FIG. 2. Diagrammatic representation of the VZV genome containing ORFs 63 and 64 and the probes employed to map the ORF 63-encoded RNAs. Legend is as for Fig. 1, except that the start and stop codons are represented by ATG and TAA, and the relative position of the origin of replication (Ori rep) is indicated by a dashed line. In addition, the lowercase letter designations refer to the blots shown in Fig. 3B. IRs and Us, internal repeat region and unique short region of the VZV genome, respectively. K, *Kpn*I; M, *Mlu*I; Sc, *Sca*I.

strand complementary to the RNA at an *Hpa*I site (position 3255) (Fig. 1). After removal of unincorporated nucleotides, a fragment was generated by *Pvu*II digestion (position 2046), purified, and hybridized to RNA. Figure 4a shows the S1 nuclease-protected products resolved under denaturing conditions. With VZV RNA, a single fragment of 680 bases was found, which correlates to RNA termination at position 2575 (Fig. 4b). A similar-sized fragment was found under native electrophoresis conditions, suggesting that no splicing occurred in the region covered by the probe (data not shown). An oligonucleotide probe to sequences upstream of this termination site but downstream of the ORF 4 termination codon, when used in Northern blotting studies (Fig. 3A, lanes b), recognized both RNAs, supporting the conclusion that the single site identified represents the termination of both RNAs. The termination site is downstream of two consensus polyadenylation signals but, interestingly, represents RNA that has bypassed two upstream consensus AATAAA/AGTAAA polyadenylation motifs (Fig. 4b). Only downstream of the utilized AATAAA signals are found GT-rich elements similar to those suggested to be important for efficient transcriptional termination in many eukaryotic and viral genes, highlighting the importance of this element (42, 49).

Mapping of the ORF 63 RNA 3′ ends. The Northern blot analyses indicated two different ORF 63 RNA termination sites. To confirm this, a strand-specific DNA probe was created by radiolabeling the 3′ end at an *Hpa*I site with T4 DNA polymerase at position 110647 within ORF 63 (Fig. 2). After removal of nucleotides, digestion with *Sca*I, and electrophoresis, a 1,793-bp DNA probe was purified, hybridized to RNA, and digested with S1 nuclease. This probe includes the C-terminal region of ORF 63 and the entire ORF 64 and downstream sequences. Figure 5a shows the S1-protected labeled products identified after denaturing electrophoresis. Two radiolabeled products were detected from probe hybridized to VZV-infected RNA, one 770 to 780 base in size and the second approximately 1,450 to 1,500 bases. No fragments were generated from probe hybridized to uninfected-cell RNA, even upon extensive overexposure. These results confirm ORF 63-specific transcriptional termination at two positions, consistent with the Northern blot analyses. The sizes of the smaller fragments indicated a termination site for the 1.3-kb RNA close to a polyadenylation motif around position 111426 and upstream of a highly GT-rich region located around position 111468 (Fig. 5b).

Mapping of the ORF 4 RNA transcription initiation site. To enable identification of the ORF 4 promoter, S1 nuclease protection and primer extension analyses were carried out to map the 5′ end of the 1.8-kb RNA. DNA probes uniquely labeled on the strand complementary to the mRNA were generated by labeling the 5′ end of the *Bgl*II site located within the ORF at position 3773 (Fig. 1). After removal of residual nucleotides by gel exclusion, the DNA probe was generated by restriction at the *Pvu*II site at position 5036. The 1,265-bp fragment, which covers sequences from within 361 bp of the ORF to 896 bp upstream of the AUG, was denatured, hybridized to RNA, and digested with S1 nuclease. Figure 6 shows the protected fragments after electrophoretic separation. A single novel VZV-specific fragment of 410 to 420 bp was identified under native conditions (Fig. 6a), and a 405-base fragment was resolved under denaturing conditions (Fig. 6b). The fragment size corresponds to a transcriptional start site between 38 and 53 bp upstream of the ORF 4 AUG. Full-length probe was also found after S1 digestion and likely

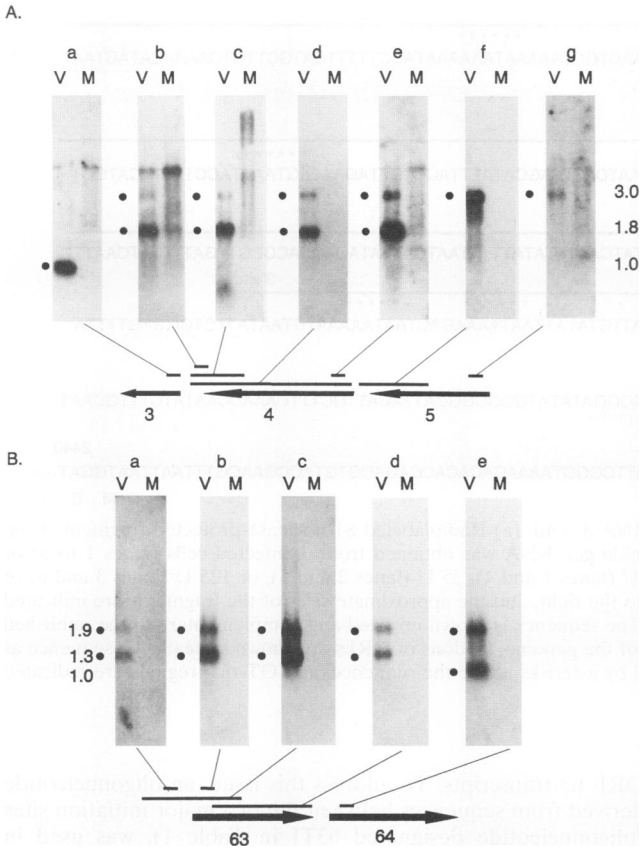

FIG. 3. Northern blot analyses of VZV-infected (V) and mock-infected (M) poly(A)$^+$-selected RNAs probed to analyze the RNAs for ORF 4 (A) and for ORF 63 (B). All RNAs were electrophoresed and blotted under similar conditions and probed with radiolabeled oligonucleotides and DNA as described in the text. The VZV-specific RNAs detected are indicated by ● to the left of each blot, and their approximate determined sizes are shown in kilobases. RNAs in blots b, c, and d in Fig. 3A and blot d in Fig. 3B show migrations slightly different from those for RNAs from adjacent blots because of different RNA preparations and minor variations in separate electrophoresis experiments. At the bottom of each panel is a diagrammatic summary of the locations of the probes with respect to each ORF. Lane designations are explained in the text.

protected complete probe (generated by complexing to the 3.0-kb RNA); however, it is also found in control digestions, suggesting that at least part of it originates from undigested, reannealed probe.

To define the ORF 4 start site more accurately, primer extensions were carried out with the ORF 4-specific oligonucleotide listed in Table 1. Figure 7a shows the product obtained for VZV-infected-cell RNA run alongside a sequencing ladder obtained by using the same primer with the plasmid pGI4. A product was identified that placed the transcription initiation start site 38 bp upstream of the ORF 4 AUG, in good agreement with that predicted by the S1 nuclease analysis. No extension products were detected in similar experiments using uninfected-cell RNA, but the same position was identified with a second ORF 4-specific oligonucleotide (data not shown). From these data in conjunction with the Northern blot results, we conclude that this site represents the 5′ end of the 1.8-kb transcript. A summary of the initiation site and the surrounding sequences is shown in Fig. 7b. At 38 bp, the leader sequence is shorter than those of most VZV genes described to date; only the gpV leader sequence is smaller (37 bp) (37). We also examined the upstream sequence for well-characterized transcriptional factor motifs such as the TATA box, which is responsible for binding the TATA-binding protein basal transcription factor (reviewed in reference 74). At −31 bp, a good consensus TATA box is present, and, although no strict consensus CCAAT box is apparent, a close-to-consensus element is present at position −53, overlapped by two possible consensus Sp1-binding motifs. However, extensive analysis of 1,000 bp upstream of this site revealed no motifs resembling the HSV-1 TAATGARAT element, and only a six-of-eight match for a possible octamer motif was found at position −132. This result is in contrast to the case for the promoter elements for ORF 62 and the HSV-1 IE genes.

Mapping of the ORF 63 transcription start sites. Similar approaches were used to identify the sites of transcriptional initiation of ORF 63 RNAs. A DNA probe complementary to ORF 63 RNA was uniquely labeled on the 5′ end of an *Mlu*I site, using T4 polynucleotide kinase (position 110808) (Fig. 1), and further restricted with *Kpn*I to generate a fragment spanning from 698 bp upstream of the ORF 63 AUG to 217 bp within the ORF. This fragment also includes the origin of replication. The labeled fragment was hybridized to RNA and S1 digested, and the resulting fragments were resolved. With VZV-infected-cell RNA, three novel protected fragments of 310, 380, and 460 bp were detected under native electrophoresis conditions (Fig. 8a), and similar-sized fragments of 310, 316, 385, and 460 bases were observed under denaturing conditions (Fig. 8b). These sizes reflect possible initiation start sites at approximately 85 to 90, 150, and 240 bp upstream of the ORF 63 AUG. A larger low-abundance fragment of approximately 580 bp was detected under native conditions, but lack of consistency in its detection suggested it to be a partially digested S1 fragment.

Primer extension analysis was carried out to substantiate the S1 data, and the extension products obtained from ORF 63 (Table 1) oligonucleotide-RNA complexes are shown in Fig. 9a. Two major VZV-specific extension products were resolved. The smaller set of products reflected an apparently heterogeneous start site 87 to 95 bp upstream of the AUG. Interestingly, multiple products correlating to this site (the 310- to 316-bp fragments [Fig. 8b]) were detected by S1 nuclease analysis. The second major set of products reflects a site 151 to 153 bp upstream of the AUG, correlating with the 385-base fragment detected by S1 nuclease analyses. Several minor primer extension products were also observed; since these do not correlate with S1 products, they are assumed to be premature termination products caused by pausing or secondary structure. However, one very minor extension product correlated to the 460-base S1-protected fragment (Fig. 9a, arrows), suggesting a possible site 244 to 250 bp upstream of the AUG. However, since the extension product was of very low abundance, we are tentative in the assignment of this as a transcription initiation site. Figure 9b shows the sequence surrounding these initiation sites. Upstream of the site proximal to the ORF 63 AUG lies a poor-consensus TATA box at position −25, but at positions −47 and −78 lie possible CCAAT box motifs. At 31 bp upstream of the major site distal to the AUG (located 65 bp upstream of the proximal site) is a good-consensus TATA box, but no consensus CCAAT box motif is apparent. A similar situation is found with the tentative third site 155 bp upstream of the major proximal site. In scanning the sequence for additional motifs, three possible SP1-binding motifs are found (Fig. 9b). However, like with the

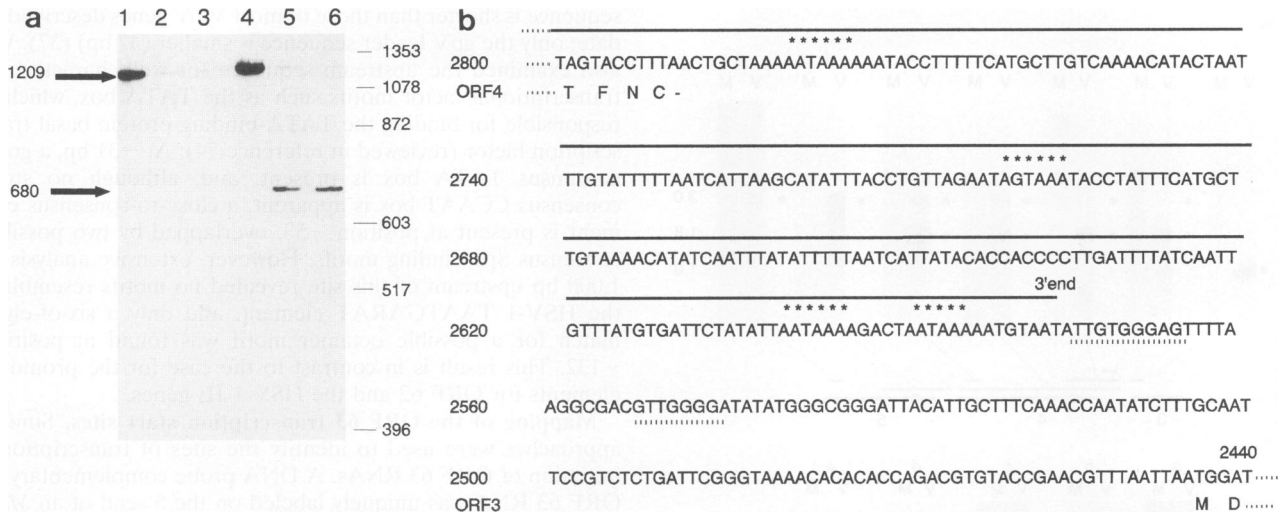

FIG. 4. Mapping of the transcriptional termination site of the ORF 4 RNA 3′ end. (a) Radiolabeled S1 nuclease-protected fragments from RNA-DNA complexes after separation upon a 4% denaturing polyacrylamide gel. RNA was obtained from uninfected cells (lanes 1 to 3) or VZV-infected cells (lanes 4 to 6), and the complexes were digested with 0 U (lanes 1 and 4), 25 U (lanes 2 and 5), or 125 U (lanes 3 and 6) of S1 nuclease. The positions of molecular size markers (bases) are indicated to the right, and the approximate sizes of the fragments are indicated by arrows. (b) DNA sequence surrounding the 3′ end of the ORF 4 RNA. The sequence is shown inverted and complementary to the published sequence, and the base pair positions are with respect to the left-hand end of the genome. Codons of ORFs are shown beneath the sequence as single-letter designations. Consensus polyadenylation motifs are highlighted by asterisks above the sequence, and GT-rich regions are indicated by dashed underlines.

promoter for ORF 4, no TAATGARAT-like motifs are apparent upstream of any of the transcriptional initiation sites.

In correlating the two RNAs detected by Northern blots with the two major start sites, two scenarios are possible: (i) each start site represents the initiation of only one of the two ORF 63 transcripts, or (ii) each site reflects the initiation of both ORF 63 transcripts. To address this issue, an oligonucleotide derived from sequences between the two major initiation sites (oligonucleotide designated 63TI in Table 1), was used in Northern blots. This oligonucleotide probe hybridized to RNAs with sizes similar to those detected with the ORF 63-specific oligonucleotide (Fig. 3, lanes a). While this result

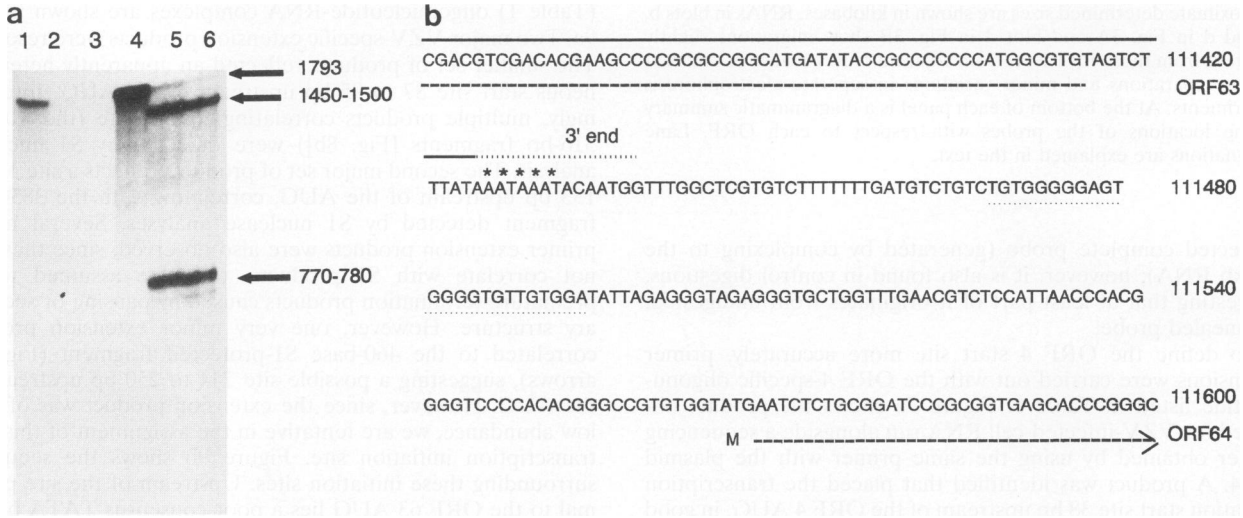

FIG. 5. Mapping of the transcriptional termination sites for ORF 63 RNA. (a) Radiolabeled S1 nuclease-protected fragments from RNA-DNA complexes separated on a denaturing 4% sequencing gel. RNA was derived from uninfected cells (lanes 1 to 3) or VZV-infected cells (lanes 4 to 6), and complexes were digested with 0 U (lanes 1 and 4), 10 U (lanes 2 and 5), and 100 U (lanes 3 and 6) of S1 nuclease. The estimated sizes of the major protected fragments and the undigested probe are shown in bases on the right. (b) DNA sequence surrounding the transcriptional termination site downstream of ORF 63. ORFs are represented by a dashed line below the sequence, and M indicates the initiation methionine of ORF 64. The 1.3-kb RNA for ORF 63 is represented by a continuous black line. The probable AT polyadenylation signal is marked with asterisks above the sequence, and a GT-rich region is underlined with a dotted line. The numbers to the right refer to the DNA sequence coordinates with respect to the left end of the VZV genome (13).

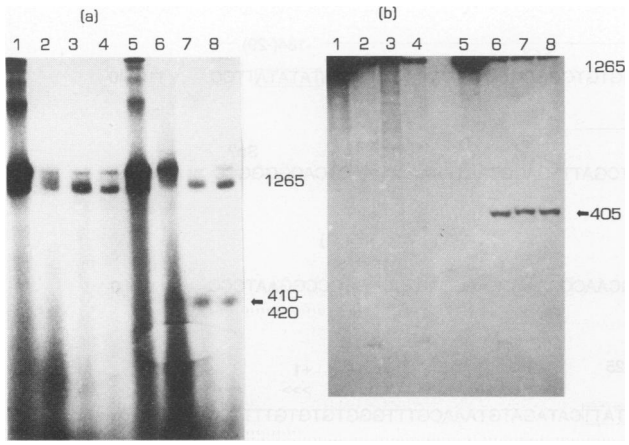

FIG. 6. S1 nuclease protection analyses of the ORF 4 RNA 5′ end. DNA-RNA complexes with RNA from uninfected cells (lanes 1 to 4) or VZV-infected cells (lanes 5 to 8) were digested with 0 U (lanes 1 and 5), 5 U (lanes 2 and 6), 25 U (lanes 3 and 7), and 125 U (lanes 4 and 8) of S1 nuclease. Samples were separated upon either native agarose gels (a) or denaturing sequencing gels (b). The sizes of the protected fragments, estimated from markers, and of the probe are indicated in base pairs (a) and bases (b).

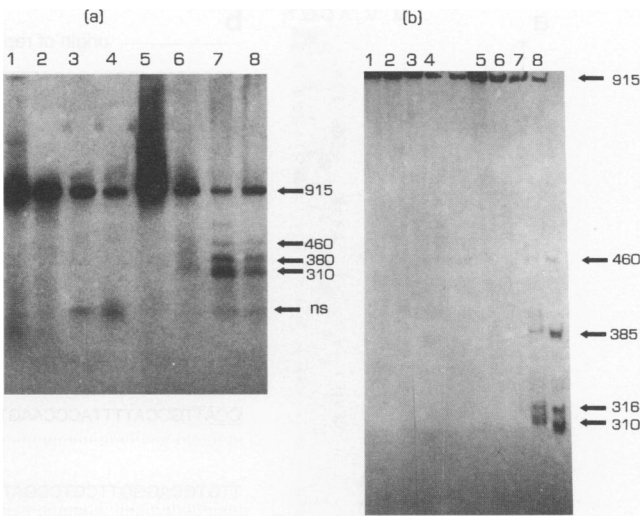

FIG. 8. S1 nuclease protection analyses of the ORF 63 RNA 5′ ends. Conditions and lane designations are as described in the legend for Fig. 6. ns, non-VZV-specific protected fragment.

does not confirm transcription from the most proximal site, it does confirm that the major distal site is used for both ORF 63 transcripts.

Functional analysis of the promoters of ORFs 4, 61, 62, and 63. Although ORFs 4 and 63 are considered to be homologs of HSV-1 IE genes, our analysis failed to reveal TAATGARAT motifs in the regulatory regions of these ORFs that are found in HSV-1 IE genes. The promoter for ORF 61 has also been reported to lack a TAATGARAT motif (69), while that of ORF 62 possesses two such elements (45, 46). Because of the importance of this motif in conferring the ability of a gene to be IE regulated in HSV-1, we conducted a functional assay for this element by testing the regulatory regions of ORFs 4 and 63 for responsiveness to the HSV-1 Vmw65 protein. Plasmids containing the promoter regions of ORFs 4 and 63 driving the assayable CAT reporter gene were transfected alongside plasmids containing sequences representing the promoters of ORFs 61 and 62 (14). All promoter fragments were large (>600 bp) so as to contain regulatory elements in the noncod-

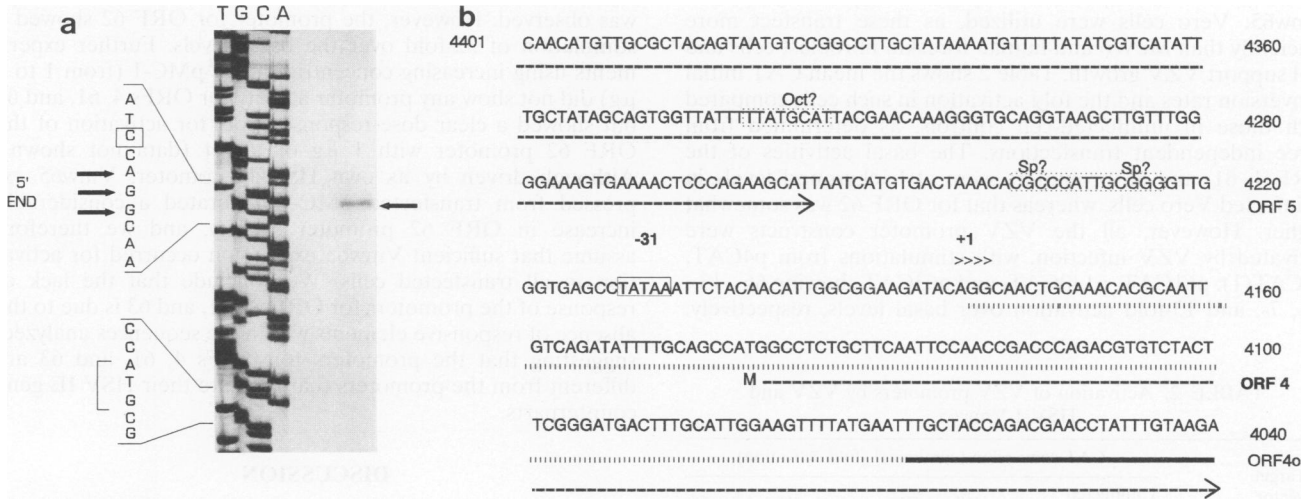

FIG. 7. (a) Primer extension analyses of VZV ORF 4 RNA. The primer extension product generated from VZV-infected cell RNA is shown alongside a DNA sequence ladder obtained by using the same primer and pGI4. The lanes representing each dideoxynucleotide are labeled above the sequencing ladder. The DNA sequence corresponding to the transcriptional start site and the starting AUG of the ORF (bracketed) are expanded. Arrows indicate the bases to which the transcription was concluded to initiate. A polymorphism found in pGI4, different from the published DNA sequence, is boxed. (b) VZV DNA sequence surrounding the ORF 4 transcriptional start site, indicated by >>>> and designated +1. The sequence is shown inverted and complementary to the published DNA sequence (13). ORFs are represented as dashed lines below the sequence. The oligonucleotide used to derive the primer extension product is shown as a heavy line below the respective sequence, and a dotted line represents the extension of the RNA. Possible TATA motifs are boxed, and possible SP1 (SP?), Oct1 (Oct?), and CAT box motifs are boxed with a dotted line.

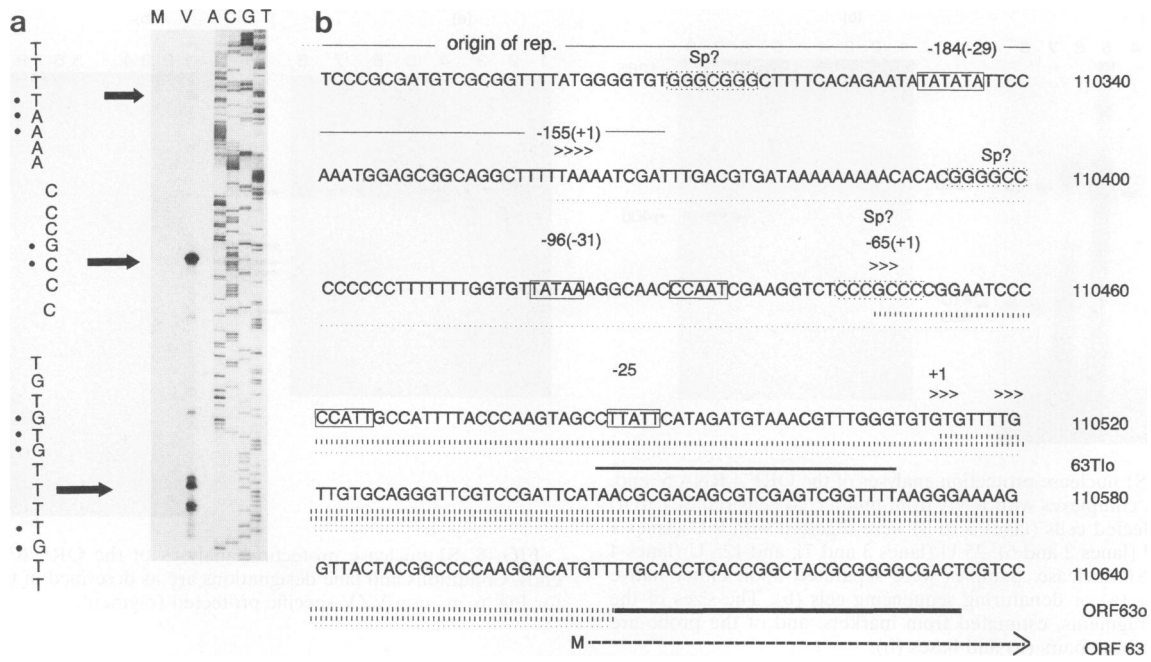

FIG. 9. Primer extension analyses of VZV ORF 63 RNA. The primer extension products generated from RNA derived from mock-infected cells (M) or VZV-infected cells (V) are shown alongside a sequencing gel derived by using the same primer with pKR-2. The dideoxynucleotide used to generate the sequence is shown above each respective lane. Arrows indicate primer extension products that correlate with results obtained from S1 nuclease analyses. Sequences which contain the concluded transcriptional start sites are expanded to the left, and the precise bases are indicated by ●. (b) DNA sequence surrounding the concluded transcriptional start sites of ORF 63. Legend is as for Fig. 7b, except that positions are given with respect to the G residue of the first start site, which is designated +1. The position with respect to the nearest start site is shown in parentheses. In addition, a line representing the oligonucleotide probe used in Fig. 3, lanes e (63TIo), is indicated below the sequence. Major RNA extension products are represented by dashed lines, and minor products are indicated by a dotted line below the sequence.

ing 5′ untranslated regions as well as upstream of the transcriptional initiation site (14). Each plasmid was either transfected alone or was superinfected with VZV, or it was cotransfected with pMC-1, a plasmid expressing HSV-1 Vmw65. Vero cells were utilized, as these transfect more efficiently than MeWo and human foreskin fibroblast cells and still support VZV growth. Table 2 shows the mean CAT initial conversion rates and the fold activation in such cells compared with those in uninfected-cell controls, as determined from three independent transfections. The basal activities of the ORF 4, 61, and 63 promoters were at background levels in uninfected Vero cells, whereas that for ORF 62 was somewhat higher. However, all the VZV promoter constructs were activated by VZV infection, with stimulations from p4CAT, p4CAT(1), p61CAT, p140CAT, and p63CAT showing 66-, 13-, 32-, 7-, and 13-fold activation over basal levels, respectively.

TABLE 2. Activation of VZV promoters by VZV and HSV-1 Vmw65

Target vector	CAT conversion (cpm/min)[a] (fold activation)		
	Uninfected cells	With VZV	With Vmw65
p4CAT	80	5,466 (66)	59 (<1)
p4CAT(1)	60	2,012 (13)	50 (<1)
p61CAT	61	996 (32)	81 (1.6)
p140CAT	1,375	14,378 (7.4)	42,925 (32.2)
p63CAT	61	418 (13)	43 (<1)

[a] Values indicated are the mean initial rates of CAT conversion derived from three independent experiments using assay of Neumann et al. (53).

Neither pSV0CAT or pCATBasic showed any significant activity in uninfected, VZV-infected, or cotransfected cells (data not shown). Upon coexpression with HSV-1 Vmw65, no significant activation of the promoters for ORFs 4, 61, and 63 was observed. However, the promoter for ORF 62 showed a stimulation of 32-fold over the basal levels. Further experiments using increasing concentrations of pMC-1 (from 1 to 5 μg) did not show any promoter activity for ORFs 4, 61, and 63 but showed a clear dose-response effect for activation of the ORF 62 promoter with 1 μg of target (data not shown). Although driven by its own HSV-1 promoter, Vmw65 expressed from transfected pMC-1 generated a considerable increase in ORF 62 promoter activity, and we therefore assume that sufficient Vmw65 expression occurred for activation in all transfected cells. We conclude that the lack of response of the promoters for ORFs 4, 61, and 63 is due to the absence of responsive elements within the sequences analyzed, suggesting that the promoters for ORFs 4, 61, and 63 are different from the promoters that regulate their HSV IE gene counterparts.

DISCUSSION

In this work, the RNAs encoding VZV ORFs 4 and 63 were mapped in detail to enable examination of the regulatory sequences governing their transcription. These ORFs are part of a group of genes (including ORFs 61 and 62) which encode proteins homologous to HSV-1 IE proteins (12, 13), and numerous transient expression studies concerning their regulation have been described (14, 16, 29, 31, 45, 46, 50, 51, 58, 59). However, interpretation of such studies is difficult without

knowledge of transcriptional initiation. For example, splicing or long leader sequences may have placed the promoters much further upstream of the ORFs; the long 420-bp leader sequence of VZV gene 36 highlights this problem (13). The present studies now provide a firm footing for such transient expression analyses.

Both ORFs appear to lie within complex transcriptional units and hybridize RNAs mapping to adjacent sequences. They are unlike ORFs 61 and 62, each of which encodes a single transcript (45, 69), but are reminiscent of the ORFs of the VZV short unique region, where both 5′ and 3′ overlapping transcripts are found (32, 38). For ORF 4, the transcription pattern appears to be straightforward; RNAs for two ORFs (ORFs 4 and 5) coterminate close to a typical eukaryotic termination signal composed of an AATAAA motif and a downstream GT-rich motif (42, 49). This is typical of transcription from many HSV-1 gene clusters, in which one polyadenylation signal may be shared by several genes (reviewed in references 43 and 72). The smaller 1.8-kb RNA is almost certain to encode the ORF 4 protein; the 3.0-kb RNA probably encodes the ORF 5 protein, since it is the only species hybridizing the ORF 5-specific probes. Although the 3.0-kb RNA contains the ORF 4 sequences, the monocistronic nature of eukaryotic RNAs implies that it is unlikely to translate into the ORF 4 protein.

Transcription from ORF 63 appears to be more complex. Like ORF 4 RNAs ORF 63 RNAs appear to be unspliced in their 5′ untranslated regions. This is in contrast to the HSV-1 counterpart of ORF 63, the gene encoding ICP22, which encodes a spliced transcript (64). However, the data suggest that at least four RNA species could encode the ORF 63 protein, originating at one of two major sites (65 bp apart) and terminating at one of two 3′ ends. Although RNAs from the two initiation sites were not resolved by blotting studies, the good correlation of both the S1 nuclease and primer extension analyses strongly support their existence. However, we do not yet know the explanation for the multiple initiation and polyadenylation sites of ORF 63. One likely hypothesis is that each site reflects different temporal events for this gene. The two start sites may reflect transcription from two promoter elements, each with signals required for expression at a particular stage of infection. Likewise, polyadenylation signals downstream of ORF 63 may become less efficient at later stages of infection. Heterogeneous 3′ ends have been described previously for VZV (32, 38). Resolution of these matters requires an RNA study with a single-step growth cycle, utilizing cell-free VZV; however, such experiments have proved to be very difficult because of the low titer of cell-free VZV and the inefficiency of infection.

The sequences upstream of each transcription initiation site show a canonical TATA-like motif found approximately 25 to 35 bp upstream. The motif upstream of the ORF 63 site proximal to the AUG is somewhat less typical (TTATT) and, interestingly, gives rise to transcripts with apparently heterogeneous 5′ ends, as evidenced by both S1 and primer extension analyses. However, unusual TATA boxes have been found for other VZV genes, including ORF 62 (37, 38, 45, 69). In addition, sequences reminiscent of CCAAT boxes and SP1 sites were found, but their identification was only tentative; such elements must be confirmed through further DNA-protein interaction studies.

The TAATGARAT motif, which confers IE regulation kinetics upon the HSV-1 IE genes, was not found upstream of the mapped initiation sites of these two VZV genes. The functional assay with HSV-1 Vmw65 confirmed the lack of any such elements and also supported the previously noted absence of this element upstream of ORF 61 (69). Although our functional assay may not necessarily reflect transcription from these genes within the context of the virus, similar transient assays were successfully used to indicate the presence of the TAATGARAT elements in VZV ORF 62 (Table 2) (45, 46). One possible reason for the negative response is that insufficient sequences were examined in such assays; however, all showed specific activation upon infection with VZV. Interestingly, the sequences examined for ORF 63 promoter activity contain both of the ORF 62 elements (45, 46) but in the wrong orientation, indicating directional or distance considerations for the effects of these elements.

The question remains as to the importance of the presence or absence of the TAATGARAT element in defining the transcriptional class of these four VZV genes. Although the kinetic class of ORFs 4, 61, and 63 remains to be defined, two hypotheses concerning their regulation can be made from current information. The first presumes that the highly conserved TAATGARAT element within the ORF 62 promoter mediates IE regulation kinetics, as in HSV-1 IE genes. In that case, the absence of this motif in sequences controlling ORFs 4, 61, and 63 implies that they are regulated other than as IE genes. Supporting this possibility is the recent demonstration of a weak ORF 10 transactivation activity (51). This is despite the apparent absence of the equivalent C-terminal 81 amino acids of Vmw65, which have been shown to be essential for that protein's transactivating activity (1, 2, 11, 48, 71). If ORF 10 does activate IE gene expression through TAATGARAT motifs, then ORFs 4, 61, and 63 may be regulated with different kinetics. This is reminiscent of the cases of pseudorabies virus and equine herpesvirus type 1, both of which have a single IE gene (the homolog of ORF 62 and HSV-1 ICP4) with upstream TAATGARAT elements (8, 25, 26). Although some homologs of other HSV-1 IE genes are found in these viruses, they lack TAATGARAT motifs and are regulated in a fashion other than IE (8, 25, 26).

A second hypothesis is that these VZV ORFs are IE regulated through sequences yet to be identified. It does appear that four to six IE phosphoproteins are made in VZV-infected cells (39, 67). It is unlikely that they are all products of the ORF 62 gene, even though this protein is highly susceptible to proteolysis (33). Additional indirect evidence can be drawn from features of VZV virion regulatory proteins. Clearly, the ORF 10 protein is much weaker in transactivating ability than HSV-1 Vmw65 (51) and thus may play a very minor role in activation. However, the VZV particle appears to contain significant amounts of the transactivator protein from ORF 62 (33). As this protein can upregulate the promoters of ORFs 4, 61, and 63 (14, 29, 52, 58) and, in some cells, its own promoter (13, 58, 59), it may act to stimulate IE gene expression in a novel mechanism of herpesvirus IE gene initiation. To address these questions further, the regulatory class of the ORF 4, 61, and 63 genes must be verified. We are attempting to use specific antisera to each gene in immunofluorescence studies similar to that previously shown to indicate the IE nature of ORF 62 (21).

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