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Transcription from Varicella-Zoster Virus Gene 67 (Glycoprotein IV)

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Three transcripts map to the varicella-zoster virus (VZV) open reading frame (ORF) 67, which encodes glycoprotein IV (gpIV). All of these transcripts are polyadenylated and are transcribed from left to right towards the genomic terminal short repeats. Previous Northern (RNA) blot analyses suggested that the most abundant of these transcripts (1.65 kb) might code for gpIV. We performed S1 nuclease protection and primer extension assays and determined that the 5′ terminus of the 1.65-kb transcript maps 91 bp upstream from the gpIV initiation codon. An AT-rich region (ATAAA), −28 bp from the cap site, is a potential TATA box, and at −71 bp there is a consensus CCAAT box motif. The 3′ end of the 1.65-kb transcript is 20 bp downstream of two overlapping polyadenylation signals, AATAAA and ATTAAA, and just downstream of the 3′ terminus is a GU-rich sequence. These results are reminiscent of data from our analysis of the VZV gpV gene, confirming that VZV appears able to use unusual TATA box motifs. Many canonical TATA sequences are present upstream from these VZV transcriptional start sites but, apparently, are not used. We tested sequences upstream from the gpIV cap site for promoter activity in transient expression experiments by cloning a DNA fragment (+63 to −343 bp) into pCAT3M, which contains a chloramphenicol acetyltransferase reporter gene. This clone showed little constitutive promoter activity but was activated more than 200-fold by infection with VZV and 5-fold with herpes simplex virus. The two known VZV transactivating genes (those for ORF 4 and ORF 62) were tested for their abilities to activate expression from the gpIV promoter by using their cognate promoters. The ORF 4 gene was minimally active, whereas the ORF 62 gene gave twofold induction; both genes, acting together, gave fivefold induction. However, replacement of the IE62 promoter with the immediate-early cytomegalovirus promoter in the ORF 62 construct gave over 40-fold induction of chloramphenicol acetyltransferase activity under the gpIV promoter in the same assay.

Varicella-zoster virus (VZV) is the causative agent of two clinical exanthems. Varicella, commonly called chickenpox, causes a mild disease with few complications and is usually contracted during childhood. Zoster, or shingles, caused by the reactivation of latent virus, is a painful disease normally found in older individuals or immunocompromised patients. Both chickenpox and zoster are occasionally serious or life-threatening, especially in immunocompromised individuals. While the general clinical picture of disease is well defined, our biochemical knowledge of the virus replication cycle is still quite limited, despite the determination of the entire nucleotide sequence of the VZV genome (8).

Five glycoproteins and their products have now been identified in VZV (7, 9, 21, 22). One of these, the product of open reading frame (ORF) 67, located in the unique short (U_s) segment of the genome, was initially defined as a likely glycoprotein on the basis of its predicted sequence (8) and, under the nomenclature for VZV glycoproteins, was designated gpIV (7). Antibodies, generated to a synthetic peptide derived from the sequence, identified glycoproteins in VZV-infected cells of 45,000 molecular weight (45K) and 55K (9). The ORF was found to have homology to herpes simplex virus type 1 (HSV-1) glycoprotein I (gI) (7, 8, 25, 30, 31), which has been implicated in complex formation with glycoprotein E (gE) to form an Fc receptor in HSV-1-infected cells (18). Fc receptor activity in VZV has recently been reported and it is likely, but not proven, that gpIV (like its gI counterpart) is involved (24).

Studies of VZV glycoprotein transcript structure have only recently been attempted. Generally, transcripts have been identified by Northern (RNA) blotting and, in a few cases, hybrid selected and translated in vitro to produce an immunologically reactive protein (19; for a review, see reference 12). However, little detailed analysis has been carried out. We have recently reported on the fine structure of transcripts encoding gpV (23) and defined two RNAs that are 5′ coterminal but have different 3′ termini. Examination of sequences upstream and downstream from these termini revealed that some of the consensus signals for eukaryotic transcriptional regulation are not present, except at large distances from the termini, indicating that they are not likely to be used (27, 33, 36). Thus, the presently available data imply that VZV may employ unusual regulatory elements to control its gene expression.

Recently, Inchauspe et al. (15) demonstrated that two VZV gene products (from ORF 4 and ORF 62) could activate thymidine kinase (TK; a probable early VZV gene) and gpI (a probable late VZV gene) promoters. Conversely, the product of ORF 61 apparently down-regulates these targets in the presence of ORF 4 and ORF 62 products, at least in Vero cells. Cabirac et al. (5) have also demonstrated TK promoter activation with the ORF 62 product but not with ORF 61, in transient assays. These VZV gene products are the homologous counterparts to HSV-1 regulatory genes ICP4/IE175 (ORF 62), ICP27/IE63 (ORF 4), and ICP0/IE110

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(ORF 61), that have all been demonstrated to be important for the regulation of HSV-1 promoters (10, 28, 37, 39, 41, 42, 44, 45; for a review, see reference 40). One striking difference between the two viruses is that the VZV ORF 61 product has negative effects on homologous promoter targets, whereas its HSV homolog, ICP0/IE110, is a strong general activator. Interestingly, HSV ICP0/IE110 is able to act synergistically with the VZV ORF 4 product to activate both the VZV and the HSV-1 TK promoters.

In this report, we have defined the termini of the major 1.65-kb transcript encoding gpIV and identified potential regulatory sequences for transcription. In addition, we have tested likely promoter sequences for this gene in transient assays and assessed their responses to viral transactivating genes.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblasts (HFF; strain USU 521) and VZV strain Scott were obtained from Monroe Vincent, Department of Pediatrics, Uniformed Services University of the Health Sciences. VZV strain Oka (ATCC VR-795) was purchased from the American Type Culture Collection. HFF cells were grown in 150-cm^2 flasks (Costar) or 850-cm^2 glass roller bottles (Bellco) in minimum essential medium (GIBCO) supplemented with 2% fetal calf serum (GIBCO), 8% Serum Plus (Hazelton Research Products), and 50 μg of gentamycin per ml at 37°C and 5% CO_2. When cell monolayers reached confluency, they were trypsinized (0.25% in buffered saline [Hazelton]), resuspended in growth medium, and seeded into new flasks or roller bottles. Virus was grown by trypsinizing infected cell cultures and adding them to uninfected cell monolayers at a ratio of between 1 and 5 infected cells to 10 uninfected cells. Infected cells were then incubated at 37°C for 3 to 7 days until the cytopathic effect reached approximately 80%. Cells were then harvested for experimental use or used to infect other monolayers.

RNA isolation. RNA was isolated as described previously (6, 13, 23). Briefly, VZV-infected cells showing approximately 80% cytopathic effect were washed three times with ice-cold phosphate-buffered saline, pH 7.0. The cells were then lysed with 4 M guanidinium isothiocyanate, scraped from the flask, and vortexed vigorously for 1 min. The solution was layered onto a 5.7 M CsCl cushion and centrifuged in an SW41 rotor at 33,000 rpm for 24 to 30 h. The supernatant was then aspirated, and the pellet was resuspended in 360 μl of guanidinium isothiocyanate–40 μl 3 M potassium acetate (pH 5.0) and precipitated in 3 volumes of absolute ethanol. The precipitate was pelleted in a microcentrifuge at 12,000 × *g* for 15 min. The pellet was washed several times with 70% ethanol, resuspended in H_2O, extracted with phenol-chloroform, and reprecipitated in ethanol. The final precipitate was then resuspended in H_2O, quantitated by reading its A_{260}, and used for experiments. Poly(A)$^+$ RNA was prepared by using oligo(dT)-cellulose (Collaborative Research) according to the protocol previously described (2).

Northern blot analysis. Northern blot analysis was performed essentially as described previously (23). The solid support used for binding and detection of RNA species was Nytran (Schleicher & Schuell), in place of nitrocellulose. In addition, DNA probes were generated by using a random-primed labeling system as described by Feinberg and Vogelstein (11). RNA sizes were estimated from the mobility of 18S and 28S rRNA and of the 0.24- to 9.5-kb RNA ladder (Bethesda Research Laboratories) run under the same conditions as the test RNA.

S1 nuclease analysis. Structural analysis of gpIV transcripts was performed by using the S1 digestion procedure of Berk and Sharp, with some modifications (3). 5′- or 3′-end-labeled probes were hybridized at 45°C to appropriate amounts of RNA for 16 h. The reaction mixture was then digested with 10 to 100 U of S1 nuclease (Bethesda Research Laboratories) for 30 min. The digestion products were then electrophoresed on denaturing polyacrylamide sequencing gels and detected by autoradiography.

Primer extension analysis. Primer extensions were performed essentially as described by Inoue and Cech (17).

Oligonucleotides were synthesized by using B cyanoethyl diester phosphoramidite chemistry on an ABS model 380A synthesizer (Applied Biosystems Inc., La Jolla, Calif.). Those used were OPL5 (5′CCTTGAAGATCAAAGC GTTG3′ for gpIV), OPL15 (5′ATCCAGTGATTTTTTTC TCC3′ for CAT), and OPL1 (5′TTGAGATTCAGTCG ATAATT3′ for gpV). Oligonucleotide (100 ng) was 5′ end labeled and purified through a G15 spin column. Labeled oligonucleotide (10 to 20 ng) was then incubated with 15 to 25 μg of RNA in 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 1 mM EDTA, 0.2% sodium dodecyl sulfate, and 0.4 M NaCl, at 37°C for 2 h. The reaction mixture was then precipitated in ethanol, and the pellet was resuspended in 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 40 mM KCl, and 10 mM dithiothreitol. The reaction mixture was then brought to 0.5 mM deoxynucleoside triphosphates–20 U of reverse transcriptase and incubated at 37°C for 1 h. The reaction was terminated by phenol-chloroform extraction and then by ethanol precipitation. Primer extension products were then electrophoresed on polyacrylamide sequencing gels and visualized by autoradiography.

Labeling of DNA probes. For 5′ labeling of DNA probes, appropriate DNA clones were digested with restriction enzymes, and the 5′ ends were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals). The fragments were end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase as described by Maniatis et al. (26). Uniquely end-labeled fragments were generated by digestion with a second restriction enzyme, and the resulting DNA was electrophoresed on a 1% agarose slab gel. The appropriate fragment was then excised from the gel, and the DNA was recovered by electroelution. Oligonucleotide (50 to 100 ng) was also 5′ end labeled by the procedure described by Maniatis et al. (26). 3′ end labeling was performed by digestion of appropriate DNA clones with restriction enzymes and filling-in recessed 3′ ends with [α-^{32}P]dNTPs and Klenow fragment as described by Maniatis et al. (26). The reaction was then digested with a second restriction enzyme, and the resulting uniquely end-labeled DNA was electrophoresed on a 1% agarose slab gel. The appropriate fragment was then excised from the gel, and the DNA was recovered by electroelution.

DNA sequencing. DNA sequencing was performed by using the dideoxy chain termination method of Sanger et al. (43) with the Sequenase sequencing kit (U.S. Biochemicals). The primer used was one employed for primer extension analysis (OPL 5 [see above]), and the template was a double-stranded DNA plasmid, PL1-7, containing the *Bam*K fragment of strain Scott in pUC19.

DNA transfections and CAT assays. dPK-CAT (tk-CAT), the chloramphenicol acetyltransferase (CAT) gene with the VZV pyrimidine deoxynucleoside kinase promoter, was a gift of G. Ostrove, National Institute of Allergy and Infec-

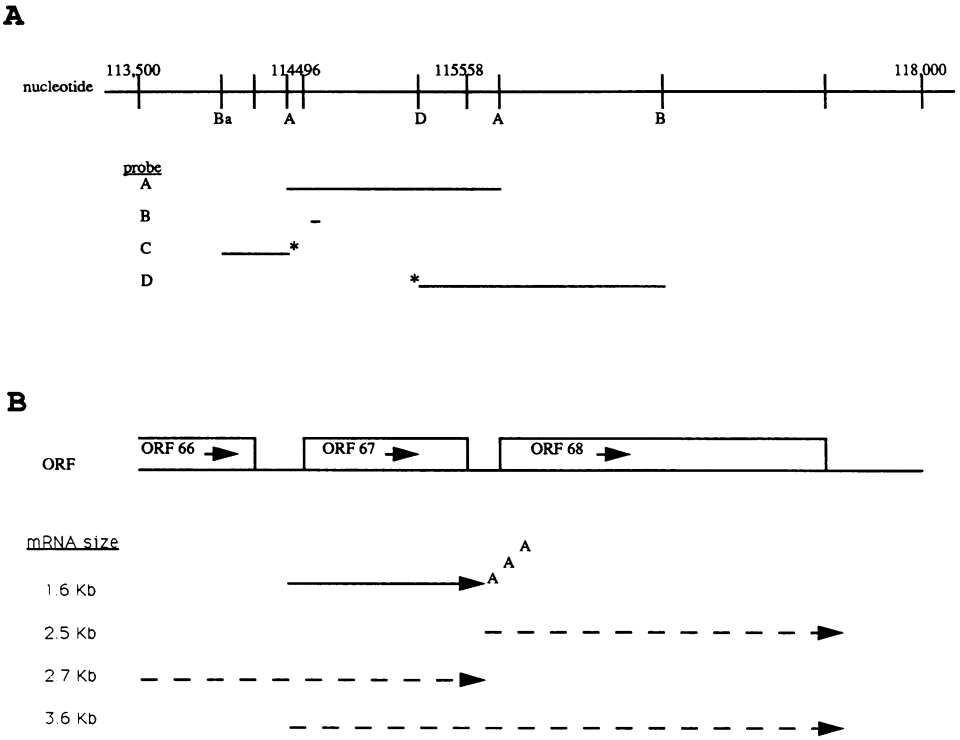

FIG. 1. Transcription mapping summary diagram. (A) DNA fragments used for both Northern hybridization (probes A and B) and S1 nuclease analysis (probes C and D). The uppermost line represents a portion of the U_s region of the genome, with the VZV genome nucleotide numbers above the line and restriction sites below. Ba, *Bam*HI; A, *Acc*I; D, *Dde*I; B, *Bst*EII. (B) On the same scale as panel A above is a representation of the ORFs found in the U_s region. In the lowest part of the figure is a diagram of transcripts as they have been defined in this paper, shown to scale in location and size.

tious Diseases. A plasmid containing the IE62 gene under the control of the cytomegalovirus (CMV) immediate-early (IE) promoter was constructed by using a CMV IE cassette kindly provided by E. Mocarski, Stanford University; the plasmid contains the appropriate VZV *Sca*I-*Bgl*II fragment encompassing IE62 coding sequences (20). DNA transfections were performed as previously described (1). Confluent HFF cells from one 150-cm^2 flask were split into five 10-cm^2 plates 1 day prior to transfection. Plasmid DNA was then brought to 10 mg of DEAE-dextran per ml in a volume of 0.160 ml in 1× TBS (10× TBS is 25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, and 0.5 mM $MgCl_2$). HFF cells were washed once in serum-free Eagle minimum essential medium and then covered in 4 ml of transfection media (Dulbecco minimal essential medium with 10% NuSerum; Collaborative Research Inc.). The DNA-dextran mixture was then added dropwise to each plate, bringing the final concentration of dextran to 400 mg/ml. Cells were then incubated for 4 h and subjected to a 2-min shock with 5 ml of 10% dimethyl sulfoxide in phosphate-buffered saline. After the shock, the dimethyl sulfoxide was removed and normal growth medium was replaced. Approximately 10 μg of target plasmid was routinely used to transfect 10-cm tissue culture plates. When the influence of VZV infection on expression from plasmids was to be assessed, cell monolayers infected with VZV were trypsinized and added to the transfected cells at a ratio of 1 infected to 10 uninfected cells.

After transfection, cell harvesting and CAT assays were carried out by the method of Gorman et al. (14). We used equal amounts of protein, as determined by the method of Bradford (4), for each extract in a given experiment. To quantitate levels of acetylation, acetylated and unacetylated spots were localized by autoradiography and excised from thin-layer chromatography plates before being counted in nonaqeous scintillation fluid in the Beckman LS9000 scintillation spectrometer.

RESULTS

Northern blot analysis of VZV gene 67 RNA. Previous Northern hybridization studies mapped three polyadenylated transcripts (3.6, 2.8, and 1.65 kb) to the gpIV ORF (19). Reinhold et al. (38) extended this Northern analysis by using single-stranded RNA probes and detected two RNAs of 3.6 and 1.65 kb that were transcribed from left to right (toward the terminal short repeat) in the prototype VZV genome arrangement. In order to clarify and extend these results, we used two probes in Northern hybridization experiments: a DNA fragment containing only gpIV ORF sequences and a single-stranded oligonucleotide specific for the gpIV ORF. The first of these, an *Acc*I fragment (Fig. 1A, probe A), when hybridized to poly(A)$^+$ or poly(A)$^-$ VZV RNA, recognized only transcripts in the poly(A)$^+$ tracks and was specific for infected cells. We estimate the transcripts to be 3.6, 2.7, and 1.65 kb in size (Fig. 2A), with the 2.7-kb species present in relatively small amounts. The oligonucleotide probe, which hybridizes to a 20-bp sequence within the gpIV ORF (Fig. 1B, probe B), detected the same transcripts as the *Acc*I probe; these results indicate that all three are transcribed from left to right (towards the TR_s) in the prototypic arrangement of the VZV genome (Fig. 2B). Further analyses, with a

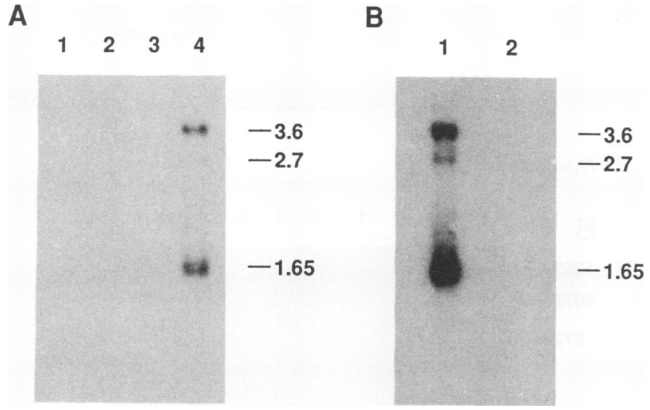

FIG. 2. Northern blot analyses. (A) Comparison of poly(A)$^+$ RNA and poly(A)$^-$ RNA from uninfected and VZV-infected cell RNA. Lane 1, uninfected-cell poly(A)$^-$ RNA; lane 2, uninfected-cell poly(A)$^+$ RNA; lane 3, VZV-infected cell poly(A)$^-$ RNA; lane 4, VZV-infected cell poly(A)$^+$ RNA. The blot was probed with probe A (whole ORF 67 [Fig. 1]). (B) RNA probed with the oligonucleotide OPL5 (close to the 5′ ORF 67 terminus [see Materials and Methods]). Lane 1 is VZV-infected cell RNA and lane 2 is uninfected-cell RNA. Numbers to the right of the blots are molecular sizes in kilobases.

probe downstream (toward ORF 68 [Fig. 1B]) from the gpIV ORF, detected only the 3.6-kb transcript plus a novel transcript of 2.5 kb (data not shown). Our conclusions, based on the above data and that of others, are (i) that the 1.65-kb transcript codes for gpIV specifically, (ii) that the 2.7-kb transcript is probably a read-through from ORF 66 that terminates close to the 1.65-kb transcript terminus, and (iii) that the 3.6-kb transcript is likely to initiate at or near the 1.65-kb 5′ terminus, but is probably read through, to terminate downstream of ORF 68 (gpI [Fig. 1B]).

S1 nuclease analysis of the 5′ end of the gene 67 transcript. In order to map the 5′ terminus of the major 1.65-kb transcript encoding gpIV, we performed S1 nuclease protection assays. Probe C, an *Acc*I-*Bam*HI fragment (Fig. 1A), was hybridized to VZV-infected cell RNA and digested with various concentrations of S1 nuclease. Two major protection products migrating at 63 and 64 bp are observed (Fig. 3, lanes 1 to 3) and indicate a 5′ terminus at 114,405 bp, 90 to 91 bp upstream of the proposed ORF 67 translational initiation codon at 114,496 bp. No protection products are seen when this probe is hybridized and digested with uninfected-cell RNA (Fig. 3, lanes 4 to 6). Bands at the top of Fig. 3, lanes 1 to 6, migrating at 426 bp, are either the result of probe-to-probe reannealing or indicate the presence of a transcript substantially larger than 1.65 kb. In lanes 1 to 3 (VZV-infected cell RNA), the amount of this larger band is much greater than that observed with probe and uninfected cell RNA (lanes 3 to 6), indicating that a transcript larger than 1.65 kb and with a 5′ terminus upstream from ORF 67 is present in the infected-cell RNA. This is probably the 2.7-kb species described above, which is able to protect the entire *Acc*I-*Bam*HI fragment (Fig. 1A), as would be predicted from our conclusions in the previous section.

Primer extension analysis of the 5′ end of gene 67 RNA and determination of the transcription initiation site. We hybridized an oligonucleotide (OPL5) (see Materials and Methods) to VZV-infected cell RNA and synthesized a cDNA product by using reverse transcriptase. A product of 162 to 163 bp was observed, which confirms the results of the S1 analysis

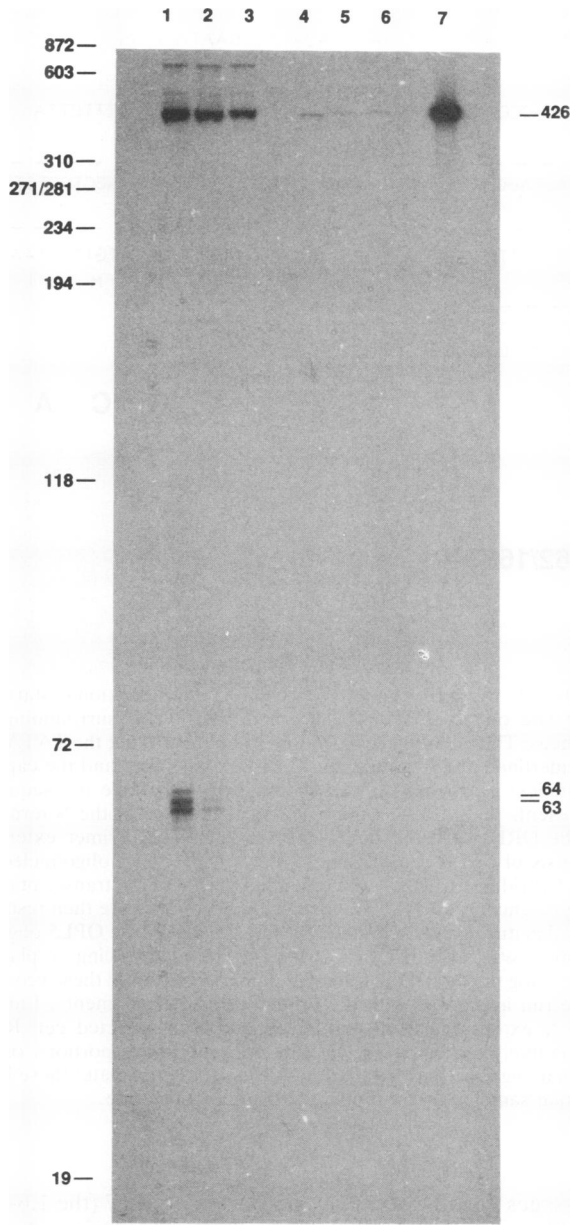

FIG. 3. S1 nuclease analysis of the 5′ end of gene 67 RNA. An *Acc*I-*Bam*HI fragment (Fig. 1, probe C), was hybridized with VZV-infected cell RNA (lanes 1 to 3) or uninfected cell RNA (lanes 4 to 6) and digested with S1 nuclease. Products were run on a 8% denaturing polyacrylamide sequencing gel. The following concentrations of S1 nuclease were used: lanes 1 and 4, 100 U; lanes 2 and 5, 10 U; lanes 3 and 6, 1 U; lane 7, untreated probe. Numbers to the left and right of the figure represent molecular sizes in bases.

(Fig. 4B). This primer extension product was analyzed in parallel with a sequencing ladder generated by using OPL5 as a primer on a VZV *Bam*HI K fragment DNA template (Fig. 4B) (see Materials and Methods). From this, it appears that the 5′ terminus of gene 67 RNA is most likely to be the G residue at position 114405, since most RNA polymerase II transcripts initiate at a purine nucleotide; however, we cannot rule out the possibility that the T at 114404 is also an initiation site for this VZV mRNA. It is possible that the two

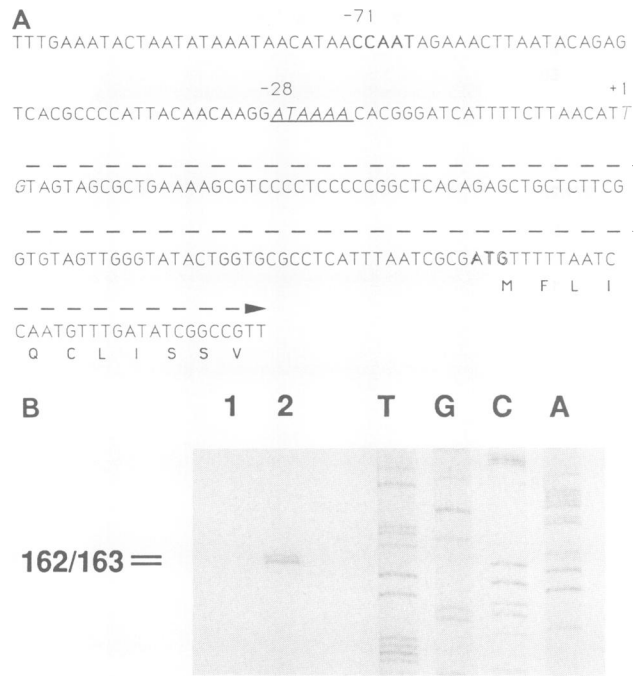

FIG. 4. 5′ end of gene 67 RNA. (A) Transcriptional start site start site of gene 67 RNA shown in relation to surrounding sequences. The initiating ATG codon is in bold outline; the TATA box is underlined and in italics; the CAT box is in bold and the cap site (in italics) is labeled as +1. The dashed line above the sequence represents the mRNA. Amino acid designations for the N terminus of the ORF are listed below the sequence. (B) Primer extension analysis of the 5′ end of gene 67 RNA. The OPL5 oligonucleotide was hybridized to RNA and extended with reverse transcriptase in the presence of dNTPs. The extension products were then resolved on a denaturing polyacrylamide sequencing gel. The OPL5 oligonucleotide was also used as a primer for sequencing a plasmid containing the *Bam*HI K fragment of VZV DNA, and these products were run in parallel with the primer extension fragments. Lanes 1 and 2, extensions from uninfected and VZV-infected cell RNA, respectively; lanes T, G, C, and A, appropriate portions of the sequencing reactions from the VZV *Bam*HI K template; these lanes contain samples that terminate with each nucleotide.

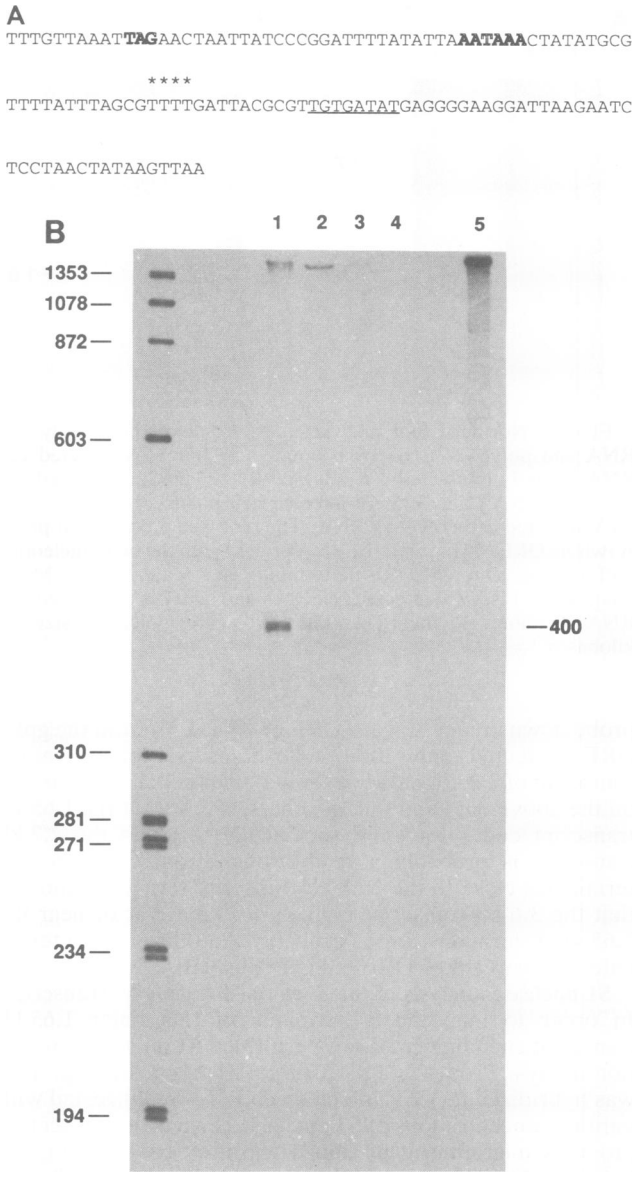

FIG. 5. 3′ end of gene 67 RNA. (A) The sequence surrounding the transcriptional stop site of VZV gene 67. The AATAAA polyadenylation signal is outlined in bold, the likely transcriptional stop site(s) is shown with asterisks, the GU-rich sequences are underlined, and the translational stop codon (TAG) is outlined in bold. (B) S1 nuclease analysis of the 3′ end of the gene 67 transcript. A *Dde*I/*Bst*EII fragment (Fig. 1, probe D) was hybridized with VZV-infected cell RNA (lanes 1 and 2) and uninfected-cell RNA (lanes 3 and 4). The hybridization reactions were digested with 10 U (lanes 1 and 3) and 1 U (lanes 2 and 4) of S1 nuclease. Lane 5, untreated probe. S1 digestion products were resolved on 4% denaturing polyacrylamide gels. Numbers to the left and right of the figure are molecular sizes in bases.

messages with 5′ termini at the start of gene 67 (the 1.6- and 3.6-kb transcripts) have different (by one base) 5′ ends, and our analyses are detecting these. Sequences around the transcriptional start site are shown in Fig. 4A. Upstream, at −28 bp from the cap site, is an AT-rich sequence (ATAAAA), that may serve as the TATA box; further upstream, at −85 bp, there is a TATAAA sequence, representing a more usual TATA motif, but its position makes it a less likely candidate. At −71 bp from the cap site is a consensus CCAAT sequence.

S1 nuclease mapping of the 3′ end of the gene 67 transcript. To determine the 3′ end of the 1.65-kb RNA, we performed S1 protection assays by using probe D (Fig. 1A) hybridized with VZV-infected cell RNA and digested with S1 nuclease. Protected hybrids were analyzed on denaturing polyacrylamide sequencing gels, and protected bands migrating at 400 bp were observed (Fig. 5B, lanes 1 and 2). No such bands are seen when probes were hybridized and digested with uninfected cell RNA (Fig. 5B, lanes 3 and 4). This result places the 1.65-kb RNA 3′ terminus approximately 23 bp downstream (at 115,617 bp) from ATTAAA and AATAAA consensus polyadenylation signals (Fig. 5A). In addition, a GU-rich sequence downstream of the poly(A) signal similar to that defined by McLauchlan et al. (34) is present and has been underlined in Fig. 5A. Thus, the combined data from S1 and primer extension analyses indicate that the body of the major gpIV transcript is 1,212 bp in length; this is consistent with the polyadenylated transcript of 1.65 kb detected in

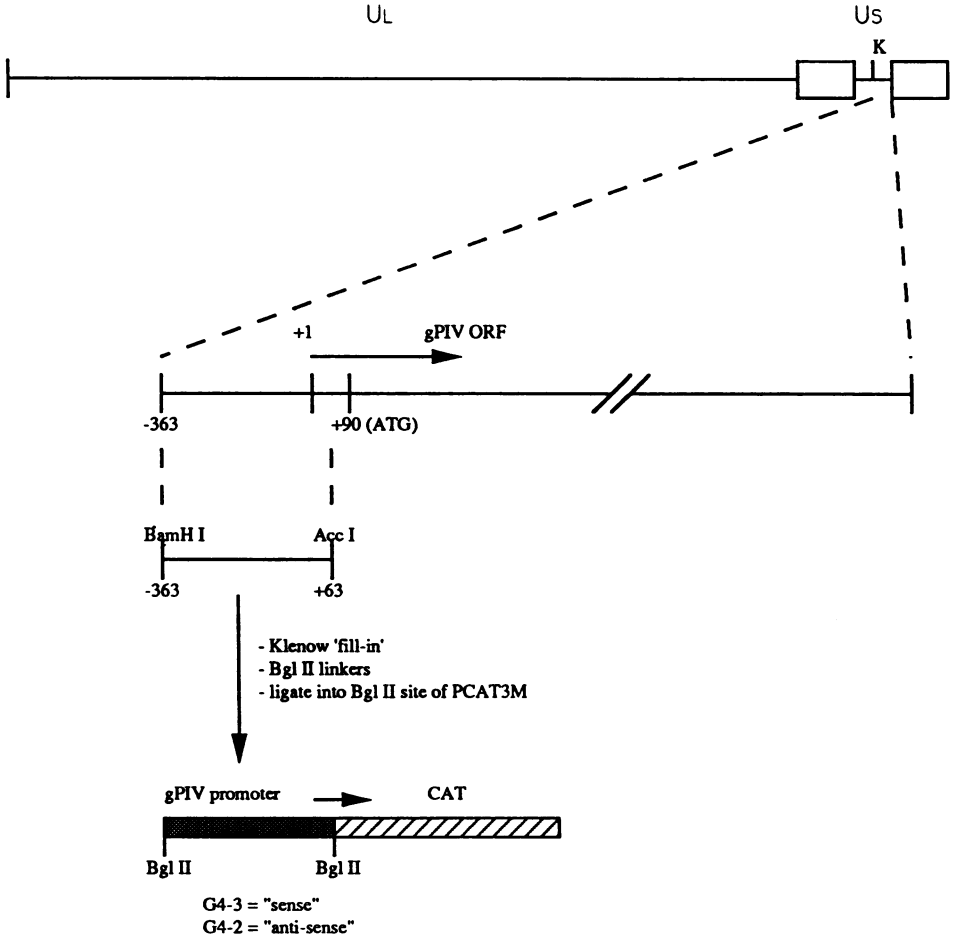

FIG. 6. Construction of a plasmid containing the CAT gene under the control of VZV gpIV promoter sequences. The top shows the VZV genome, with the *Bam*HI K region expanded below. A 426-bp fragment was cleaved from the *Bam*HI K fragment with *Bam*HI and *Acc*I. *Bgl*II linkers were added, and the DNA was cloned into the unique *Bgl*II site in the vector pCAT3M. The lower part of the diagram shows insertion into pCAT3M and the approximate location of the gpIV transcription initiation site with respect to the CAT gene (horizontal arrow). Two plasmids were constructed; G4-3 is shown in the diagram, while G4-2 has the promoter fragment cloned in the opposite (antisense) orientation from G4-3.

Northern blots. This implies a poly(A) tail of about 400 bases, somewhat larger than the normal (200- to 250-base) tails found in other eukaryotic messages. We find no indication of transcript splicing in the gene 67 region of VZV DNA; RNA splicing for homologous genes in the HSV-1 U_S region has also not been found.

Once again, as noted in the 5′ S1 analysis, we find that intact probe protection in infected-cell lanes is much greater than in uninfected-cell lanes (Fig. 5B, compare lanes 1 and 2 with 3 and 4). A reasonable interpretation is that a long transcript, most likely the 3.6-kb RNA, probably 5′ coterminal with the 1.65-kb RNA, hybridizes to the whole probe; this is consistent with our earlier conclusions.

Determination of promoter activity in gene 67 upstream sequences. Having mapped the 5′ terminus of the major gene 67 1.65-kb transcript, we were able to locate upstream sequences that might act as a promoter. Figure 6 shows the diagram of sequences that were cloned (to give plasmid G4-3) 5′ to a CAT gene in the plasmid pCAT3M, a conventional vector for testing putative promoter sequences in transient assays. As a control, an analogous construct containing gene 67 promoter sequences in the opposite orientation (G4-2) was made. These constructs were transfected into HFF cells and tested for CAT activity with and without VZV infection. Figure 7 shows a typical assay. The uninfected control and both the G4-3 and G4-2 plasmids had no detectable activity in uninfected cells, but infection with VZV gave a marked increase in CAT activity with the G4-3 construct (Fig. 7, lane 5). We conclude from this that the upstream sequences we selected can function as a promoter for gpIV and that these sequences require interaction with a VZV-induced factor(s) for activity.

In order to confirm that the observed increase in CAT activity was due to an increase in RNA levels and also that transcription initiation from the reporter gene-promoter fusion took place at the same point as in VZV, we carried out primer extension analyses on RNA derived from the CAT transient assays. No products were observed in transfected uninfected cells. In transfected VZV-infected cells, a 130-bp product was detected with a CAT gene-specific primer (OPL15) (see Materials and Methods). These data (not shown) indicate both that CAT transcript levels are raised after VZV infection and that the G4-3 transcript levels are raised after VZV infection. The G4-3 transcript is initiating

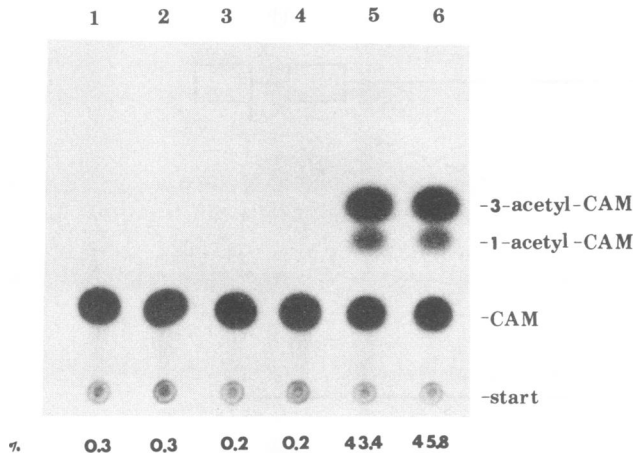

FIG. 7. Transactivation of the gpIV promoter by VZV infection. G4-3 (lanes 3 and 5) and G4-2 (lanes 2 and 4) plasmids were transfected into HFF cells and 3 days later were harvested. Cell extracts were then assayed for CAT activity, spotted on silica gel plates, and chromatographed. Lanes 4 and 5, cells were infected 24 h posttransfection with a ratio of VZV-infected to uninfected cells of 1 to 10; lane 6, CAT activity of an extract made from pSV_2-transfected cells; lane 1, untreated cells. The percent acetylation is listed below the appropriate lanes.

faithfully (at the same point as in gpIV), since our G4-3 CAT construct contains 63 bp of leader from VZV gpIV and 46 bp from pCAT3M and the oligonucleotide OPL15 is 20 bp in size (the predicted extension product is therefore 129 bp).

Activation of the gpIV promoter by ORF 4 and ORF 62. Recent work by Ostrove and his colleagues has shown that the products of VZV ORF 62 and ORF 4 can transactivate the VZV pyrimidine deoxynucleoside kinase and gpI promoters (5, 15). Since we were able to demonstrate activation of the gpIV promoter by VZV infection, we used plasmids containing ORF 4 and 62, driven by their cognate promoters, in transient assays to define their role in gpIV expression. Table 1 shows G4-3, the gpIV promoter construct cotransfected with these ORF 4 and/or ORF 62 plasmids, in CAT assays. Little or no activity is seen with ORF 4 alone, while ORF 62 stimulates to a small extent. Maximum activity, however, was observed when both plasmids were present, although activation by these cognate promoter-driven genes was always much less than with virus infection (Table 1). Suspecting that at least part of the reason for this might be the weakness of the VZV cognate promoters, we constructed a plasmid in which the VZV ORF 62 is driven by the CMV IE promoter, a strong constitutive promoter. This plasmid (pCMVIE62 [Table 1]) stimulates the gpIV promoter to substantially greater levels than does the cognate promoter-IE62 plasmid. As a control, we also used a VZV dPK CAT construct in assays with pCMVIE62; considerable stimulation of this (early) promoter was also observed. Both VZV promoters employed in these assays showed stimulation dependent on the concentration of input activating plasmid (Table 1).

TABLE 1. Promoter activation by VZV proteins

Activator plasmid or virus (amt)	% Acetylated chloramphenicol[a]	
	pdPKC[b]	pGPIVC[c]
pCMVIE62[d]		
0 ng	3.8	0.2
16 ng	17.5	4.9
80 ng	12.2	5.9
400 ng	16.1	8.5
2 μg	10.2	6.3
10 μg	1.3	2.1
pCMV[e]		
25 ng	2.0	0.5
125 ng	1.8	0.4
500 ng	2.5	0.2
2 μg	2.1	0.4
10 μg	0.2	0.1
IE62,[f] 1 μg	ND[g]	1.0
ORF 4,[h] 1 μg	ND	0.5
62+4,[i] 2 μg	ND	2.5
VZV	36.1	18.7

[a] A positive control plasmid, pSV2CAT, gave 94% acetylation, while a negative control plasmid, pCATBASIC, gave 0.4% acetylation.
[b] VZV pyrimidine deoxynucleoside kinase promoter driving CAT.
[c] VZV gpIV promoter driving CAT.
[d] CMV IE promoter driving VZV IE62.
[e] CMV IE promoter plasmid without insert.
[f] VZV IE62 gene under its own promoter.
[g] ND, not determined.
[h] VZV ORF 4 gene under its own promoter.
[i] 62+4, a 1:1 mixture of plasmids IE62 and ORF 4.

DISCUSSION

Earlier work has defined transcripts mapping to the general region of the VZV genome encoding the ORF 67 gene product (19). In this article, we have defined the termini of the major 1.65-kb transcript encoding ORF 67 (gpIV). These data have interesting similarities and differences when compared with transcript data for the only other VZV glycoprotein gene studied in detail thus far (gpV). For example, both genes exhibit heterogeneous start sites, suggesting that initiation might take place at a pyrimidine nucleotide in addition to the more conventional purine nucleotide. Both genes also have atypical TATA box regions at −25 bp, whereas many other canonical TATA sequences are located in various sites upstream of the translational initiation codon (27, 33, 35, 36). Finally, putative promoters for both gpIV and gpV have CAT box motifs at approximately −60 to −80 bp upstream of the cap site.

In contrast to the 5′ regions, the 3′ termini of ORF 67 transcripts are much more conventional. The RNA mapping data show a distinct 3′ terminus 23 bp downstream of two consensus overlapping poly(A) signals at 115,617 bp (29, 34, 36). In contrast, the potential poly(A) signals observed for the gpV gene (and for the VZV dPK gene) are atypical, but have precedents in other systems (8, 23, 36). It is not clear why VZV appears to use atypical control sequences for some genes and conventional ones in others. However, since only four genes have been transcript mapped in VZV to date (9, 23, 32), more work needs to be done before reliable patterns for VZV transcription will emerge. One common feature, though, at this point, is that prediction of potential regulatory sequences for transcription initiation and termination from nucleotide sequence data in VZV may have limited validity, and any predictions must be experimentally confirmed.

The results described in Fig. 7 and Table 1 indicate that the gpIV promoter has very low basal levels of activity but that viral factors or virus-induced factors can significantly activate its ability to stimulate CAT expression. Primer

extension analyses on RNA from transient assays confirmed that CAT activity was associated with increased levels of correctly initiated RNA. Whether the increased levels of RNA are due to increased initiation of RNA synthesis or posttranscriptional stabilization is not known. These results are in agreement with recent work by Inchauspe et al. (15, 16), demonstrating that VZV infection significantly stimulates the VZV dPK promoter.

Having determined that VZV infection activated the gpIV promoter, we attempted to identify the specific transactivating proteins responsible. Two VZV genes, ORF 4 and ORF 62 (the analogs of the HSV ICP27/IE63 and ICP4/IE175 genes, respectively) have been shown to produce transcriptional activator proteins (15). In keeping with published results obtained by using other target promoters, we found that ORF 4 had little activity on its own, that ORF 62 had some activating capacity, and that maximum activity was observed when ORF 4 and ORF 62 were used together. The activation we observed, however, did not approach that reached with whole virus. One reasonable explanation is that the activator gene polypeptides are synthesized in smaller amounts from transfected plasmids than from infecting virus; another is that there are additional VZV-encoded gene products required for optimal activation of this promoter. The VZV ORF 61 product, which is the homolog to the general HSV transactivator, HSV ICP0/IE110, is a possible candidate. However, two independent reports suggest that the product of this ORF has generally negative effects on VZV promoters (5, 16). In order to address the first issue raised above, we attempted to increase the amount of ORF 62 product available for transactivation by utilizing the CMV IE promoter. When this was done, activation levels approached those seen with whole virus, and it therefore seems, as has been suggested by others (32), that the cognate ORF 62 promoter does not allow high levels of IE62 expression. We have since carried out analogous experiments with VZV ORF 4 and again find that its cognate promoter appears poorly active relative to the CMV IE promoter.

A computer search for transcriptional motifs in gpIV promoter sequences analogous to those seen in herpesvirus IE and early promoters 5′ to CAT and TATA elements has been negative. This is not unexpected, given the likely late nature of gpIV. Having established the general parameters of the gpIV promoter, we are now in the process of addressing such issues as the specific sequence requirements for gpIV promoter activity and the possibility that there exist in the VZV genome transcriptional activating activities other than those currently recognized.

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