Control of expression of the varicella-zoster virus major immediate early gene

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The cis-acting DNA sequences and trans-acting proteins that control the expression of the major immediate early (IE) gene of varicella-zoster virus (VZV) were investigated. The location of the IE mRNA 5' terminus was determined by primer extension and S1 nuclease analyses and the functional activities of DNA sequences upstream of this site were analysed by a transfection assay. The VZV IE promoter exhibited low activity in BHK and HeLa cells, but was trans-activated by the herpes simplex virus type 1 (HSV-1) virion protein Vmw65. DNA sequences between positions -131 and +57 were responsible for promoter activity, whereas sequences between -410 and -131 mediated the response to Vmw65. Two short elements in the -410 to -131 region formed protein-DNA complexes with HeLa cell nuclear proteins and formed a ternary complex when Vmw65 was added. One of the elements, ATGTAAATGAAAT, possessed a strong similarity to the HSV-1 TAATGARAT. The VZV homologue of Vmw65, encoded by open reading frame (ORF) 10, failed to trans-activate expression from HSV-1 or VZV IE promoters and did not form a ternary complex with functional TAATGARAT elements and HeLa cell proteins. Therefore, stimulation of VZV IE transcription by Vmw65 can occur by a mechanism similar to that employed by HSV-1, but VZV ORF 10 does not function as a trans-activator of IE gene expression.

Introduction

Varicella-zoster virus (VZV), an alphaherpesvirus, is the causative agent of two human diseases, chicken pox and shingles. Primary exposure to VZV results in chicken pox and, after an initial infection, the virus becomes latent in the dorsal root ganglia. Reactivation of latent virus results in shingles, a painful vesicular rash which is usually confined to the distribution of a single sensory nerve root. Understanding of the biology of VZV has been hindered by difficulties in propagating viable cell-free virus at high titre in tissue culture. The determination of the complete VZV DNA sequence (Davison & Scott, 1986) has enabled the structure and function of the products of the 71 major open reading frames (ORFs) to be investigated, thus bypassing, to some extent, the problem of virus isolation.

Regulation of herpesvirus gene expression has been most intensively studied in the case of herpes simplex virus type 1 (HSV-1), in which the genes can be classified as immediate early (IE), early or late, depending on their kinetics of expression and response to inhibitors of macromolecular synthesis (Honess & Roizman, 1974; Clements et al., 1977; Preston, 1979; Everett, 1984; O'Hare & Hayward, 1985a; Sacks et al., 1985). The HSV-1 IE genes possess one or more copies of the element TAATGARAT (where R is a purine), a cis-acting motif that mediates stimulation of IE transcription by the virion protein Vmw65 (otherwise named VP16 or α-TIF), between 100 and 600 base pairs (bp) upstream of the mRNA initiation sites (Post et al., 1981; Mackem & Roizman, 1982; Campbell et al., 1984; Preston et al., 1984). Vmw65 does not bind directly to DNA (Marsden et al., 1987), but interacts with one or more cellular factors to form a ternary complex, IEC, that binds to TAATGARAT (McKnight et al., 1987; Preston et al., 1988; O'Hare et al., 1988). One of the cellular components of IEC appears to be a ubiquitous protein with various names, including NFIII (Pruijn et al., 1986), OBPl00 (Baumruker et al., 1988; Sturm et al., 1987), OTF-1 (Gerster & Roeder, 1988), TRF (O'Hare & Goding, 1988) and α-H1 (Kristie & Roizman, 1987), that recognizes an octamer element of consensus ATG-CAAAT as well as TAATGARAT. Although NFIII binds to a range of target sequences, complex IEC is formed efficiently only when a match to TAATGARAT is present (Gerster & Roeder, 1988; O'Hare et al., 1988;
ApRhys et al., 1989). The formation of IEC upstream of IE mRNA initiation sites enables a strongly acidic activating domain, located within the C-terminal 80 amino acids of Vmw65, to interact with a transcription complex and thereby augment transcription (Dalrymple et al., 1985; Trierenberg et al., 1988; Sadowski et al., 1988; Cousens et al., 1989). At the biological level the stimulation of IE transcription by Vmw65 is important for virus replication after infection at low m.o.i. or in vivo (Ace et al., 1989).

Four VZV IE gene products have been detected in infected cells (Shiraki & Hyman, 1987), but little is known about the regulation of their expression. The product of VZV ORF 62 (and ORF 71, since this gene is diploid), a polypeptide of calculated Mr 139989, is thought to be equivalent to HSV-1 Vmw175, which is a major trans-activator of HSV-1 early and late gene transcription. This conclusion is based on their comparable locations in the short repeat region of the genome (Davison & Scott, 1985, 1986), sequence homology (Davison & Scott, 1986) and the ability to stimulate gene expression in transfection assays (Everett & Dunlop, 1984; Everett, 1984). Furthermore, Vero cells that contain integrated copies of ORF 62 (F114 cells) complement the growth of HSV-1 temperature-sensitive mutants with mutations in the coding sequences of Vmw175 (Felser et al., 1988). Interestingly, the transcription of ORF 62 in F114 cells is stimulated by HSV-1 infection and this effect occurs even in the absence of protein synthesis, suggesting that a component of the HSV-1 inoculum, possibly Vmw65, is responsible (Felser et al., 1988). A homologue of Vmw65, ORF 10, has been identified in the VZV genome (Dalrymple et al., 1985). The two proteins share homology over the N-terminal 410 amino acids but, strikingly, ORF 10 is 81 amino acids shorter, lacking the acidic C-terminal activating region of Vmw65 (Dalrymple et al., 1985).

Studies with HSV-1 indicate that the efficiency of IE gene expression has important implications for the biological properties of the virus (Ace et al., 1989). We have therefore investigated the cis-acting sequences that control the expression of VZV ORF 62 and have clarified the functional relationship between Vmw65 and VZV ORF 10.

**Methods**

**Plasmids.** Plasmid p140CAT contains the region from −1146 to +57 of the VZV ORF 62 gene inserted into the chloramphenicol acetyltransferase (CAT) vector pCAT, which was derived from pBLW2 (Gaffney et al., 1985) and contains restriction sites for HindIII, PstI, SmaI and BamHI upstream of the CAT coding region. The ORF 62 promoter region in p140CAT was derived from pVZVSstf (kindly provided by Dr A. Davison), which contains the terminal Sstf fragment from the short repeat region of VZV cloned into the Pstf site of pAT153 by GC tailing, thus effectively creating a Pstf site at the genome terminus. Plasmid p140 contains the VZV ORF 62 gene from the Cat site at −1146 to the genome terminus Pstf site of pVZVSstf inserted between the Acet and Pstf sites of a pUC9 plasmid from which the EcoRI site had been removed by cutting and filling in. Plasmid p140ET is a derivative of p140, in which an EcoRI site had been created at position +57 by oligonucleotide mutagenesis of nucleotide +57, changing the sequence AAATTCC to GAATTCC (Fig. 1). The new EcoRI site in p140ET was converted to a BglII site (p140Bgl) by cleaving with EcoRI, filling in and insertion of an oligonucleotide linker (GAGATCTC). Using this novel BglII site and the SmaI site in vector sequences immediately 5' of the VZV insert, the ORF 62 region from −1146 to +57 of p140Bgl was cloned between the SmaI and BamHI sites of pCAT to give p140CAT. Sequential 5’ deletions of p140CAT were produced by cleaving p140CAT at the unique HindIII and SalI (−410) or XhoI (−131) sites, filling in the 5’ overhangs with T4 DNA polymerase and all four deoxynucleoside triphosphates and religating, to yield p140ΔA10CAT and p140ΔA131CAT.

The ORF 10 of VZV was cloned by introducing an 8 bp EcoRI linker (GGAATTC) into a DsaI site 25 bp upstream of the ORF 10 initiating ATG, then subcloning a 1290 bp EcoRI/SphI fragment containing the entire ORF into pTZ18R (Pharmacia), to yield two apparently identical isolates, pTZORF10A and pTZORF10B. The 1290 bp EcoRI/SphI fragment from pTZORF10A was recloned between the EcoRV and SphI sites of pMC1 inl7 (Ace et al., 1988). Plasmid pMC1 contains the HSV-1 gene encoding Vmw65 (Campbell et al., 1984) and pMC1 inl7 contains a 12 bp insertion, very near to the C terminus of the coding sequences, that does not affect the activity of Vmw65 (Ace et al., 1988). The use of pMC1 inl7, as opposed to pMC1, is irrelevant to the work described here. The resultant plasmid, pMCORF10, contains the VZV ORF 10 coding sequences in place of the Vmw65 coding sequences.

Plasmids pGEMTIF and the insertion mutant pGEMTIF in15, described previously (Ace et al., 1988), were used for in vitro transcription and translation. Plasmid pJF3, containing the β-galactosidase gene controlled by simian virus 40 promoter and enhancer sequences was kindly provided by Dr F. J. Rixon. Plasmid pR55, containing the CAT gene under the control of human cytomegalovirus (HCMV) strain AD169 IE gene promoter and enhancer sequences (Fickenscher et al., 1989), was kindly supplied by Dr R. Rüger. Plasmid pJ3CAT, containing the CAT gene under HSV-1 IE gene 3 control (Stow et al., 1986), was kindly supplied by Dr T. Paterson.

**Cells.** Babyhamster kidney (BHK) cells were grown in Eagle’s medium containing 10% tryptose phosphate and 10% newborn calf serum. Human foetal lung (HFL) cells were grown in Eagle’s medium containing 10% foetal calf serum and HeLa cells were grown in Dulbecco’s medium containing 2.5% newborn and 2.5% foetal calf serum. Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all media.

**Preparation of VZV-infected cell RNA.** HFL cells were infected by the addition of a one-sixth quantity of VZV-infected HFL cells showing 70 to 80% c.p.e. When the culture reached 70 to 80% c.p.e. cytoplasmic RNA was extracted as described by Preston (1977).

**Primer extension.** A 29 base oligonucleotide (5‘GGGGTGTA-GAGCGCTGCATCAGGGCGGCGTA3‘), complementary to a region close to the N terminus of VZV ORF 62 (Fig. 1), was 5’ end-labelled with 32P using T4 polynucleotide kinase. Ten ng of cytoplasmic RNA was mixed with radiolabelled oligonucleotide in hybridization buffer (40 mM-KCl and 50 mM-Tris·HCl pH 8.3) and heated to 90 °C. The temperature was decreased to 45 °C over 1 h, MgCl2 was added to a final concentration of 5 mM and the four deoxynucleoside triphosphates were added at a concentration of 0.5 mM. AMV reverse
Before conducting CAT assays, extracts were adjusted to contain equal Galactosidase was assayed as described by Spaete & Mocarski (1985). and pUC18 DNA to give a total of 16 μg. HeLa cells were harvested assayed for CAT activity as described by Gorman by Corsalo & Pearson (1981). Precipitates contained 8 μg of CAT- transfected with calcium phosphate-precipitated DNA, as described transcribed and translated. A 13 bp sequence centred at -255 contains both octamer and inverted TAATGARAT motifs and a simple octamer motif in inverse orientation is present at nucleotides -346 to -339. Sequences from which oligonucleotides for use in primer extension and S1 nuclease analysis were constructed are underlined. The A residue at position +57 was changed to a G residue during the construction of p140CAT. transcriptase (10 units) was added and the reaction continued at 45 °C for 30 min. Reaction mixtures were extracted with phenol–chloroform and precipitated with ethanol. Precipitates were dissolved in 90% formamide and heated at 90 °C for 1 min. Samples were electrophoresed on a 12% polyacrylamide gel retardation assays. A 77 base oligonucleotide (5'-GAGTTTTCC- AAACGCTTTGCACTTAAAGGATTGGCGATTTGGTG- GTTAGGGAGTTCCACAGTTACCTAAAGACGT3') was synthesized and 5' end-labelled with 32P using T4 polynucleotide kinase. The oligonucleotide was designed to overlap the VZV major IE RNA 5' terminus predicted by primer extension analysis (Fig. 1). Radiolabelled oligonucleotide was hybridized with 10 μg of VZV-infected cell RNA at 42 °C for 16 h, the reaction mixture treated with nuclease Sl and the products were electrophoresed on denaturing 12% polyacrylamide sequencing gels, as described previously (Rixon & Clements, 1982; Preston et al., 1984).

**Transfection.** Monolayers of 3 x 10^6 BHK cells in 50 mm diameter Petri dishes were transfected with calcium phosphate-precipitated DNA, as described by Cordingley et al. (1983). Precipitates contained 5 μg of CAT-containing plasmid and 0·5 μg of pFJ3. Three μg of pMC1 was added, where appropriate, and the total amount of DNA was adjusted to 9 μg by the addition of pUC18 DNA. Cells were harvested after 18 h at 37 °C.

Monolayers of 10^6 HeLa cells in 50 mm diameter Petri dishes were transfected with calcium phosphate-precipitated DNA, as described by Corsalo & Pearson (1981). Precipitates contained 8 μg of CAT-containing plasmid, 1 μg of pFJ3, 5 μg of pMC1, where appropriate, and pUC18 DNA to give a total of 16 μg. HeLa cells were harvested after incubation at 37 °C for 40 h.

**CAT and β-galactosidase assays.** Cell extracts were prepared and assayed for CAT activity as described by Gorman et al. (1982). β-Galactosidase was assayed as described by Spaele & Mocarski (1985). Before conducting CAT assays, extracts were adjusted to contain equal β-galactosidase activities, to correct for variations in transfection efficiencies. In practice this resulted in dilutions never greater than threefold for BHK cell extracts and fivefold for HeLa cell extracts. To quantify CAT assays radioactive spots were cut out and the percentage of substrate acetylated was calculated after scintillation counting. The amount of extract was varied to ensure that the linear response range of the assay was used.

In vitro transcription and translation. Plasmids were cleaved with appropriate restriction enzymes and in vitro transcription and translation was carried out as described by Ace et al. (1988). Duplicate translations were performed in either the presence or absence of [35S]methionine. Radiolabelled samples were analysed by SDS-PAGE (Preston, 1979) and non-radioactive samples were used in gel retardation assays.

** Gel retardation assays.** HeLa cell nuclear extracts and extracts of HSV-1 particles were prepared as described previously (Preston et al., 1988). DNA fragments were radiolabelled by incubation with T4 DNA polymerase and two non-radioactive and two 32P-labelled deoxynucleotide triphosphates and purified from polyacrylamide gels. Binding reactions contained 10 mM-HEPES pH 7·9, 0·6 mM-dithiothreitol, 2·3 mM-MgCl2, 0·1 μg/ml bovine serum albumin, 4 μg poly(dI),poly(dC), approximately 0·2 ng radiolabelled DNA fragment, 5 μg HeLa cell nuclear extract and, where appropriate, HSV-1 extract (0·5 μg) or a sample containing Vmw65 or VZV ORF 10 synthesized in vitro. After incubation at 25 °C for 30 min reaction mixtures were loaded onto a 3% polyacrylamide gel, electrophoresis was carried out for 3·5 h at 160 V and the gel was dried and exposed for autoradiography. If assays involved the use of competitor oligonucleotides then a 100-fold molar excess was incubated with the reaction components for 15 min prior to the addition of a radiolabelled DNA fragment. Competitors used were all 36 bp, one containing a CCAAT sequence (Preston et al., 1988), one the adenovirus major late promoter (MLP) recognition sequence (Preston et al., 1988) and the other two containing ATGCAGAT- CATTG (octamer) or GGGTGAATGAGAT (TAATGARAT) in an otherwise identical sequence context.

**DNase I protection assays.** DNA fragments were uniquely 3' end-labelled and used for gel retardation analysis. Protein–DNA complexes were eluted from polyacrylamide gels, and DNase I protected regions were identified as described by Preston et al. (1988).

**Results**

**Mapping the 5' terminus of the VZV major IE RNA**

To analyse the regulation of VZV IE transcription it was first necessary to determine the location of the mRNA 5' terminus. Two techniques were used for this analysis. First, the length of the 5' untranslated region was determined by primer extension analysis using a 5' 32P-labelled synthetic oligonucleotide complementary to 29 bases of the N-terminal coding sequence of ORF 62. Fig. 2 shows that an extension product of 105 bases was produced from VZV-infected cell RNA, but not mock-infected cell RNA, indicating that the length of the 5' non-coding region is 71 bases. No extension products longer than 105 bases were detected (results not shown). To confirm the primer extension result and to exclude the existence of an intron within the 5' non-coding region,
(a) Primer extension analysis was performed on three RNA samples prepared from different batches of VZV-infected HFL cells (lanes 1, 2 and 3) and from mock-infected HFL cells (lane 4). Markers of the indicated sizes (bp) were run in parallel and the extended product is labelled (●). (b) Nuclease S1 analysis was performed on RNA prepared from VZV-infected HFL cells (lane 5) or mock-infected cells (lane 6). G and G + A lanes of the oligonucleotide used in the assay were included as size markers and appropriate exposures are shown (lanes 7 and 8). Undigested oligonucleotide (□) and digested product (■) are labelled.

Fig. 2. Mapping the 5' end of the mRNA. (a) Primer extension analysis was performed on three RNA samples prepared from different batches of VZV-infected HFL cells (lanes 1, 2 and 3) and from mock-infected HFL cells (lane 4). Markers of the indicated sizes (bp) were run in parallel and the extended product is labelled (●). (b) Nuclease S1 analysis was performed on RNA prepared from VZV-infected HFL cells (lane 5) or mock-infected cells (lane 6). G and G + A lanes of the oligonucleotide used in the assay were included as size markers and appropriate exposures are shown (lanes 7 and 8). Undigested oligonucleotide (□) and digested product (■) are labelled.

nuclease S1 analysis was carried out using a radiolabelled probe that spanned the 5' terminus predicted by primer extension. As shown in Fig. 2(b) a cluster of protected DNA bands of 45, 46 and 47 bases was observed when VZV-infected cell RNA, but not mock-infected cell RNA, was used, in agreement with the result of the primer extension analysis and excluding the possibility of splicing in the 5' untranslated region.

Inspection of the sequence (Fig. 1) reveals the element TTTTAA to be 25 to 30 bp upstream of the mRNA start site. This probably represents the TATA box, a cis-acting element known to be an important signal for the correct initiation of transcription by RNA polymerase II.

Functional activity of VZV IE gene control sequences

Having ascertained the location of the mRNA 5' terminus a plasmid, p140CAT, was constructed in which sequences from +57 to −1146 were inserted 5' to the CAT gene. A comparison of the activity of this plasmid with pIE3CAT and pRR55, in which the HSV IE3 and the HCMV IE control sequences, respectively, direct expression of CAT, is shown in Fig. 3. The activities of

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<th>Plasmid</th>
<th>p140CAT</th>
<th>pRR55</th>
<th>pIE3CAT</th>
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<tr>
<td>Dilution</td>
<td>−</td>
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<td>Lane</td>
<td>1 2 3</td>
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Fig. 3. Comparison of the activities of IE control sequences of VZV (lanes 1 and 2), HCMV (lanes 4 and 5) and HSV-1 (lanes 6 and 7) linked to the CAT gene. Lane 3 represents mock-transfection. In lanes marked + (lanes 2, 3 and 7) reporter plasmids were cotransfected with pMC1, whereas in lanes marked − (lanes 1, 4 and 6) pUC18 was added in order to equalize plasmid quantities.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>p140CAT</th>
<th>pΔ410CAT</th>
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Fig. 4. Activity of p140CAT and deletion mutants in BHK cells (lanes 1 to 9) and HeLa cells (lanes 10 to 16). To demonstrate the responsiveness of these sequences to Vmw65, cells for lanes marked + were cotransfected with pMC1 and those for lanes marked − with pUC18. Samples 10 to 16 were excised from the same autoradiograph and represent equal exposures.
the three plasmids were tested with and without cotransfected pMC1, a plasmid that expresses HSV-1 Vmw65. The VZV upstream sequences were much less active in directing CAT expression, since it was necessary to dilute extracts from cells transfected with pIE3CAT or pRR55 250-fold to obtain comparable enzyme levels. When pMC1 was cotransfected with pIE3CAT a fivefold stimulation of CAT activity was observed, whereas a small stimulation (not greater than twofold) of CAT expression from pRR55 occurred. Cotransfection of pMC1 with p140CAT resulted in a 15-fold stimulation of activity, suggesting that the transcription of the IE gene of VZV is stimulated by Vmw65. Therefore, the major VZV IE gene has a relatively weak promoter, but contains sequences that respond to activation by Vmw65.

To analyse in greater detail the DNA sequences involved in the response to Vmw65, a set of 5' endpoint deletions of p140CAT was produced. Plasmids were transfected into BHK or HeLa cells, with or without pMC1, and expression of CAT was assessed after correction for different transfection efficiencies by the use of pFJ3, an internal control plasmid that expresses β-galactosidase (Fig. 4). Deletion to −410 or −131 did not affect the expression of CAT in either cell type. When pMC1 was included in transfection mixtures, the expression of p140Δ410CAT was stimulated on average 21-fold in BHK cells and 15-fold in HeLa cells, whereas expression of p140Δ131CAT was not stimulated in either cell type. The apparent small stimulation of p140Δ131CAT in HeLa cells was not reproducible. Therefore, sequences that respond to Vmw65 are located between −410 and −131, but sequences that determine the basal expression of the VZV major IE gene are located within the 131 bp upstream of the mRNA 5' terminus.

Fig. 5. Gel retardation analysis of the region between −410 and −131. Fragments containing the octamer (Oct) motif (114 bp) and the octamer/TAATGARAT motif (49 bp) were incubated with HeLa cell nuclear extract. HSV-1 virion extract (VE) was present in lanes labelled + and absent from lanes labelled −. A 100-fold molar excess of oligonucleotides containing octamer (lanes 3, 4, 9 and 10), TAATGARAT (lanes 11 and 12), CCAAT box (lanes 5, 6, 13 and 14) or adenovirus MLP binding site (lanes 15 and 16) were added as competitors. Complexes HC3 and IEC are labelled.
Formation of protein–DNA complexes responsible for stimulation by Vmw65

Previous studies have shown that stimulation of transcription by Vmw65 depends on the presence of TAATGARAT in the control sequences of the gene in question. Vmw65 does not bind directly to TAATGARAT, but interacts with proteins that themselves recognize the octamer element ATGCAAAT as well as TAATGARAT. Analysis of the region upstream of the TATA box reveals two sites that match the octamer element closely. Site I (TTTTGCAT) is centred at −345 and matches the octamer element at seven out of eight positions in the opposite orientation. Site II (ATG-TAAATGAAAT) is centred at −256 and is a more complicated motif representing a combination of the octamer sequence (seven out of eight matches) and the TAATGARAT motif (eight out of nine matches). Both of these sites are within nucleotides −410 to −131, defined above as the region that determines the response to Vmw65.

Binding of cell proteins and Vmw65 to these sites was investigated by gel retardation assays (Fig. 5). Three radiolabelled fragments from the region −410 to −131 in the VZV IE promoter were tested in the assay. A 114 bp SalI/AccI fragment, containing TTTTGCAAT, gave a complex (lane 2) and, upon addition of virion extract, slightly more material that migrated slowly into the gel was observed (lane 1). Additional experiments confirmed that the major complexes comigrated with the previously described HC3 (Preston et al., 1988) (results not shown). Production of the radiolabelled complexes was competed for by the addition of oligonucleotide containing ATGCAAAT (lanes 3 and 4), but not by an oligonucleotide containing CCAAT (lanes 5 and 6). A 49 bp AccI/AflII fragment containing ATGTAAATGAAAT readily formed the HC3 complex (lane 8) and upon addition of virion extract a major band representing IEC was formed (lane 7). Oligonucleotides containing TAATGAGAT or ATGCAAAT competed efficiently (lanes 9 to 12), but oligonucleotides containing CCAAT or MLP recognition sites did not (lanes 13 to 16). An AflIII/XhoI fragment making up the remainder of the −410 to −131 region failed to form any protein–DNA complexes (results not shown). Thus two subfragments of the −410 to −131 region bind proteins to form an HC3-like complex, and the IEC forms efficiently on the AflII/AflIII fragment that contains an eight out of nine match to TAATGARAT.

The protein binding site on an AflI/XhoI fragment (Fig. 1) was determined by DNase I protection analysis (Fig. 6). The only footprint detected in the HC3 complex was a disturbance to the digestion pattern at ATG-TAAATGAAAT (lane 2) with a slight extension of the region of protection in the presence of Vmw65 (lane 1). By analogy with the well characterized HSV-1 system it is likely that the element ATGTAATGAAAT is of major importance for the observed stimulation of expression from the VZV major IE promoter by Vmw65.

Activity of VZV ORF 10

It was of interest to determine whether the VZV homologue of Vmw65, the ORF 10 product, could also
stimulate transcription from the VZV major IE gene. The coding sequences of Vmw65 in the plasmid pMC1 were replaced by those of ORF 10 to form the plasmid pMCORF10. When pMCORF10 was used in short term transfection assays it failed to stimulate expression from either p140CAT or pIE3CAT (results not shown). To determine whether the protein encoded by ORF 10 was able to form a complex analogous to IEC, the coding sequences were inserted into the polylinker of the plasmid pTZ18R, which allows the transcription of ORFs in vitro. Two separate clones of this construction, pTZORF10A and pTZORF10B, were isolated and transcribed, together with the plasmid pGEMTIF (Ace et al., 1988), which encodes Vmw65, using T7 RNA polymerase. In addition, pGEMTIF.in15 cleaved with BamHI was transcribed to provide a transcript lacking the sequences encoding the acidic C-terminal tail, but retaining the N-terminal 411 amino acids of Vmw65 that are homologous to ORF 10. The transcripts were then translated using rabbit reticulocyte lysate in the presence or absence of [35S]methionine. Samples of reactions in which [35S]methionine was incorporated revealed the presence of translation products of the expected sizes upon SDS-PAGE (Fig. 7); differences in the intensities of the bands produced by the VZV and HSV polypeptides are largely due to the occurrence of threefold more methionine residues in HSV-1 Vmw65 than in VZV.

Fig. 7. In vitro translation of Vmw65 and ORF 10 polypeptides. RNA transcribed from pGEMTIF.in15 (lane 1), pGEMTIF (lane 2), pGEM2 (lane 3) and pGEMORF10A and -B (lanes 4 and 5) were translated in vitro in the presence of [35S]methionine. Mr values of the proteins predicted from the DNA sequence are given. Comparison with Mr standards gives apparent Mr of 63000 (Vmw65) and 54000 (ORF 10).

Fig. 8. Gel retardation assay using in vitro synthesized Vmw65 and ORF 10 products. Radiolabelled probes used in binding reactions were a 49 bp Accl/AflIII fragment from the VZV major IE gene control sequences (lanes 1 to 7) and a 74 bp fragment from HSV-1 IE control sequences (lanes 8 to 12). Fragments were incubated with HeLa cell nuclear extract alone (lanes 1 and 8) and with addition of virion extract (lanes 2 and 9), or translated products of ORF 10 (lanes 3, 4 and 10), pGEM2 (lane 5), pGEMTIF.in15 (lanes 6 and 11), or pGEMTIF (lanes 7 and 12). The positions of complexes IEC and HC3 are indicated.

Discussion

The VZV major IE gene possesses two classes of control sequences, as measured in the transfection assays. A promoter responsible for basal gene expression lies...
within the region −131 to +55, whereas sequences that mediate stimulation of expression by Vmw65 are located further upstream, between −410 and −131. The identity of the promoter is unknown, but the elements TTTTGCAT and, particularly, ATGTAATGAAAT are strongly implicated in the response to Vmw65. The observation that expression of ORF 62 is stimulated by Vmw65 confirms and extends the work of Felser et al. (1988), in which the response of ORF 62 to infection by HSV-1 was first described. However, our candidate responding sequences differ from the predicted regulatory elements of the earlier study. Felser et al. (1988) assumed that the TATA box is located 374 bp from the first AUG of ORF 62 and consequently identified a number of potential signals upstream of this site. The primer extension and nuclease S1 analyses presented here result in an assignment of the element TTTTAA, 97 bp from the first AUG of ORF 62, as the TATA box and hence a different set of regulatory sequences.

The VZV IE gene upstream region was considerably less effective at directing CAT production than were equivalent control sequences of HSV-1 or HCMV. Thus, VZV does not possess an efficient promoter or enhancer that operates in the systems tested here, in contrast to HSV-1 (Lang et al., 1984; Preston & Tannahill, 1984), HCMV (Boshart et al., 1985) and, presumably, pseudorabies virus (PRV) (Campbell & Preston, 1987). This finding can be viewed in two ways. The alphaherpesviruses HSV-1 and PRV produce only limited amounts of Vmw175 and 180K, the respective IE proteins homologous to the product of VZV ORF 62, during normal infection because autoregulation by the HSV-1 and PRV IE proteins results in a rapid reduction in transcription of their genes (Watson & Clements, 1980; Ihara et al., 1983; O'Hare & Hayward, 1985b; Muller, 1987). This homeostatic mechanism stabilizes the levels of Vmw175 and 180K and thus the potential for high level synthesis, which occurs when autoregulation is prevented, is not realized. In the case of VZV, the ORF 62 product may accumulate more slowly, but reach intracellular levels comparable to those of Vmw175 or 180K. Alternatively, the low observed activity of p140CAT may be related to the cell types used in this study. Even the most permissive tissue culture cells, such as HFL cells, human foreskin fibroblasts or Vero cells, give low virus yields and could be viewed as only semi-permissive. Although the activity of p140CAT is low in Vero as well as BHK and HeLa cells (T. A. McKee, unpublished results), the possibility remains that the VZV major IE promoter would be stronger in a natural host cell type. Unfortunately, the tissue culture systems currently available do not accurately reproduce the efficient growth of VZV that occurs during infection in vivo.

The failure of VZV ORF 10 protein to stimulate expression from HSV-1 or VZV IE gene promoters was expected, since it lacks the C-terminal acidic region that is crucial for the activity of Vmw65 (Sadowski et al., 1988; Triezenberg et al., 1988; Cousens et al., 1989). It was more surprising to find that ORF 10 did not form a ternary complex with cellular factors and either homologous or heterologous TAATGARAT elements. The possibility that the acidic C terminus is required for formation of IEC was eliminated by the demonstration that the N-terminal 411 amino acids of Vmw65 are fully active in the gel retardation assay, in agreement with the recent report of Greaves & O'Hare (1989). Thus, the observed homology between Vmw65 and VZV ORF 10 protein reflects conservation of features required for assembly of virus particles rather than for structures involved in interaction with cellular factors that bind TAATGARAT. The HSV-1 protein Vmw65 therefore possesses two distinct functional characteristics that are absent from the latter, namely a binding site, as yet undefined, for cellular proteins and an acidic C-terminal activating region.

Functional analyses have now been performed on IE control regions from the alphaherpesviruses HSV-1 (Mackem & Roizman, 1982; Preston et al., 1984; O'Hare & Hayward, 1987), herpes simplex virus type 2 (HSV-2) (Gaffney et al., 1985), PRV (Campbell & Preston, 1987) and VZV. The HSV-1 protein Vmw65 and its HSV-2 homologue stimulate transcription from the IE promoters of all these viruses, but PRV particles do not contain an active counterpart and VZV ORF 10 is inactive. With the reservation that stimulation of VZV IE transcription may be carried out by another virion protein, it appears that HSV-1 and HSV-2 differ fundamentally from PRV and VZV in this respect. All four viruses possess elements that respond to Vmw65 in their IE control regions, but the reason that the sequences are retained in the absence of the effector protein is unclear at present. One possibility is that binding of cellular factors to TAATGARAT per se is sufficient to confer IE specificity to a gene, perhaps by displacing other proteins and rendering the promoter accessible to transcription factors. This hypothesis is compatible with the observation that the HSV-1 mutant int1814, which specifies a form of Vmw65 inactive for trans-induction, nevertheless transcribes IE genes during infection in the absence of protein synthesis (Ace et al., 1989). As previously suggested (Ace et al., 1989) TAATGARAT, rather than an active effector protein, may be the primary determinant of an IE gene. It is also possible that functional TAATGARAT elements are retained by VZV and PRV because certain cell types contain homologues of Vmw65 that replace its function. This explanation could account for the low activity of the VZV major IE gene promoter in tissue culture cells and suggests that higher activity...
could be attained in the appropriate host cells. It is a challenging future prospect to determine whether the intriguing variations in strategies used for the expression of IE genes is related to the biological properties of different herpesviruses.

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