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Replacement of the herpes simplex virus type 1 Vmw175 DNA binding domain with its varicella-zoster virus counterpart results in a protein with novel regulatory properties that can support virus growth

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The alphaherpesviruses encode major immediate early transactivator proteins that are essential for the expression of later classes of viral genes. We have previously shown that the extensive sequence similarity between the herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) members of the family (proteins Vmw175 and VZV140k) extends to function, since a virus which expresses VZV140k in place of Vmw175 is able to grow, albeit at reduced efficiency. We have also shown that the DNA binding characteristics of the isolated DNA binding domains of Vmw175 and VZV140k are related but distinct. In order to assess whether the different DNA binding properties of the two proteins are responsible for the differences in their individual transcriptional regulatory functions, we constructed a plasmid and an HSV-1 virus in which the VZV140k DNA binding domain coding sequences replace those of Vmw175. The characteristics of the resultant hybrid protein in transfection assays and during virus infection suggest that the nature of the DNA binding domain plays a significant role in the transactivation and repression properties of the Vmw175 family of proteins.

Introduction

The alphaherpesviruses comprise an extensive family of neurotropic viruses with representatives which infect a wide range of hosts. A characteristic feature of these viruses is the expression of a major transcriptional transactivator protein, of which the prototype is Vmw175 from herpes simplex virus type 1 (HSV-1) (McGeoch *et al.*, 1986). Vmw175 (also known as ICP4) is one of five immediate early (IE) genes of HSV-1 and is essential for the expression of later classes of viral genes (for reviews, see Fields *et al.*, 1996). Intensive study of Vmw175 and its varicella-zoster virus (VZV) counterpart, VZV140k, has established that both are DNA binding proteins with related, but distinct DNA binding properties (Faber & Wilcox, 1986; Michael *et al.*, 1988; Everett *et al.*, 1991; Wu & Wilcox, 1991; Tyler & Everett, 1993). However, controversy has surrounded the contribution of DNA binding to the mechanism of transcriptional activation by Vmw175 since sequences which conform to its high affinity binding consensus are either absent from, or not required in, its target viral promoters (Faber & Wilcox, 1986; Michael *et al.*, 1988; Michael & Roizman, 1989; Imbalzano *et al.*, 1990; DiDonato *et al.*, 1991; Flanagan *et al.*, 1991; Smiley *et al.*, 1992; Gu & DeLuca, 1994). More recent evidence highlights the requirement for DNA binding for Vmw175 action since a tripartite complex of Vmw175, TFIID and TFIIB forms cooperatively on promoter sequences containing both a Vmw175 binding site and a TATA box (Smith *et al.*, 1993). Furthermore, we have shown that a single amino acid substitution within the DNA binding domain of VZV140k that results in loss of DNA binding function also destroys the ability of the mutant VZV140k protein to transactivate (Tyler *et al.*, 1994).

It has been established that the sequence similarity between Vmw175 and VZV140k is reflected in partial functional equivalence, since cell lines expressing VZV140k complement Vmw175 mutant HSV-1 viruses (Felser *et al.*, 1988), and a virus (HSV140) which expresses VZV140k in place of Vmw175 grows (with reduced efficiency) in tissue culture (Disney & Everett, 1990). Among the many potential explanations as to

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why HSV140 has reduced growth potential is the interesting possibility that the differing DNA binding properties of Vmw175 and VZV140k might contribute to their individual transcriptional regulatory properties. For example, it has been established that, in transfection assays, VZV140k is a more powerful and promiscuous transactivator than Vmw175 (Everett, 1984; Inchauspe & Ostrove, 1989; Cabirac *et al.*, 1990). It is possible that the efficient transactivation by VZV140k may be a consequence of its relaxed DNA binding specificity compared to that of Vmw175 (Tyler & Everett, 1993).

In addition to their roles in transactivation, the major regulatory proteins of the alphaherpesviruses also repress specific target promoters. For instance, Vmw175 autoregulates its own IE3 transcription unit by binding to sequences which overlap the transcriptional initiation site of that gene (DeLuca & Schaffer, 1985; O'Hare & Hayward, 1985; Muller, 1987; DeLuca & Schaffer, 1988; Roberts *et al.*, 1988; Michael & Roizman, 1993). This repression function of Vmw175 appears to be a consequence of Vmw175 positioned over the IE3 cap site, therefore blocking communications between the upstream activator proteins and the pre-initiation complex (Gu *et al.*, 1995; Kuddus *et al.*, 1995). Intriguingly, VZV140k can also bind to the Vmw175 binding site spanning the HSV-1 IE3 cap site (Wu & Wilcox, 1991; Tyler & Everett, 1993), yet does not repress IE3 gene expression, although VZV140k does autoregulate expression from its own promoter (Disney *et al.*, 1990). Since the specificity and details of the Vmw175 interaction with sequences at the IE3 cap site differ from those of VZV140k (Wu & Wilcox, 1991; Tyler & Everett, 1993), it is possible that the distinction between the two proteins is a consequence of their differing DNA binding properties.

In order to investigate the contribution of the differing DNA binding characteristics of Vmw175 and VZV140k to their individual regulatory functions, we have constructed a hybrid gene which expresses a Vmw175 protein with its DNA binding domain replaced with that of VZV140k. The ability of the hybrid protein to repress the IE3 promoter and activate gene expression was compared to the activities of the parental proteins in transfection assays. In addition, a recombinant HSV-1 virus (HSVR2DS) was constructed which expresses the hybrid protein instead of Vmw175. The results showed that replacement of the Vmw175 DNA binding domain with the VZV140k equivalent destroyed the ability of Vmw175 to autoregulate its own expression in both transfection and infection experiments. In addition, the VZV140k DNA binding domain conferred stronger transactivation properties on Vmw175 in transfection assays. Interestingly, the increased transactivation activity of the hybrid protein was not reflected in more efficient virus growth. Indeed, HSVR2DS gave lower yields than wild-type HSV-1. The simplest interpretation of our data is that the DNA binding domain of VZV140k confers altered regulatory properties to Vmw175 that now more closely resemble those of full-length VZV140k. These data support and extend the view that the DNA binding activities of the alphaherpesvirus major regulatory proteins make a significant contribution to their specific transcriptional regulatory activities that in turn help define the characteristics of the virus life cycle.

Methods

■ **Plasmids and bacteria.** The following plasmids have been described previously: plasmid p175 contains the complete HSV-1 IE3 transcription unit linked to the SV40 early promoter/enhancer region (Everett, 1986); plasmid pI9 is a derivative of p175 which contains a 12 bp *Eco*RI oligonucleotide linker inserted into codon 252 of the Vmw175 open reading frame (Paterson & Everett, 1988*a*); plasmid p140SV contains the complete VZV gene 62 coding region (which encodes VZV140k) and 3′ transcriptional control sequences, linked to the SV40 early promoter/enhancer region (Disney *et al.*, 1990); plasmid pLI10/11 is a derivative of p175 which contains a small deletion just 5′ of the DNA binding domain coding sequences and, more importantly for the experiments described in this paper, a point mutation which removes the second of two *Bam*HI sites just 3′ of the DNA binding domain coding sequence, but maintains the coding potential (Allen, 1993); p585T7b2 is a T7 expression vector essentially identical to p585T7a (Tyler & Everett, 1993) except for a frameshift in the coding region; plasmids pgDCAT, pIE3CAT and p140CAT are reporter plasmids used to assess the activation of gene expression (Everett, 1986; Paterson & Everett, 1988*a*; Disney *et al.*, 1990). All plasmids were propagated in *E. coli* strain DH5 and purified by density gradient centrifugation.

■ **Construction of hybrid plasmid p175R2DS.** A PCR primer was synthesized with 17 bases homologous to VZV140k sequences (codons 417–421), linked to an *Eco*RI site and 7 additional random bases at its 5′ end. A second primer included 17 homologous bases (VZV140k codons 668–673) linked to a *Bam*HI site followed by 6 random bases at its 5′ end. The primers were designed to amplify VZV140k codons 417–673 with the 5′ *Eco*RI and 3′ *Bam*HI sites able to link in frame to Vmw175 coding sequences. The PCR product was excised from a gel, cut with *Eco*RI and *Bam*HI, and ligated to a *Pst*I–*Eco*RI fragment of plasmid pI9 (see above), which contains part of the vector sequences and the promoter and the 5′ part of gene IE3 in plasmid p175, and the *Bam*HI–*Pst*I fragment of pLI10/11 (see above), which contains the 3′ part of the IE3 gene and the remainder of the vector sequences. The resulting plasmid, p175R2DS, is similar to the parent Vmw175 expression plasmid, p175, except that codons 253–521 of Vmw175 have been replaced by codons 417–673 of VZV140k. In addition, there are three additional residues (proline-arginine-isoleucine) between codons 251 and 417 of Vmw175 and VZV140k, respectively, which arise from the original *Eco*RI linker insertion into Vmw175 codon 252.

■ **Transfections and CAT assays.** HeLa cells were transfected by the calcium phosphate co-precipitation technique, and cell extracts were prepared for assay of chloramphenicol acetyltransferase (CAT) activity as previously described (Tyler *et al.*, 1994). Each experiment used several different amounts of activator plasmid, and the total amount of DNA in the transfection mixture was kept constant by addition of pUC9. The titration transfection experiments were independently repeated on a number of occasions to ensure that the relative effectiveness of each construct was reproducible.

■ **Cells and viruses.** Baby hamster kidney (BHK) cells were grown in Glasgow Modified Eagle's Medium (GMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 10% newborn calf serum (NBCS) and 10% tryptose phosphate broth. HeLa cells for transfection

assays were grown in Dulbecco's Modified Eagle's Medium supplemented with antibiotics, 2·5% NBCS and 2·5% FCS. Vero cells were grown in GMEM supplemented with 10% FCS and antibiotics as above. All viruses used in this study were derivatives of HSV-1 strain 17^+. Derivative D30EBA contains a large deletion within both copies of the IE3 gene (Paterson & Everett, 1990).

■ **Construction of recombinant viruses.** A mixture containing approximately 1 μg of plasmid p175R2DS (linearized by digestion with *Pst*I), 3 μg of D30EBA viral DNA and 5 μg of calf thymus DNA (as a carrier) was transfected into 2×10^6 BHK cells using the calcium phosphate method followed by glycerol shock. Following transfection, cells were incubated at 37 °C for 4 days. The cells and medium were harvested and sonicated, and progeny virus was isolated by three rounds of plaque purification. A number of isolates were screened by Southern blotting for the presence of an IE3 gene of the expected structure, after which large scale stocks of virus HSVR2DS were prepared. The titre of viable recombinant virus was determined by titration on BHK cells. A repaired version of HSVR2DS was constructed by preparation of HSVR2DS viral DNA followed by co-transfection with linearized p175. Progeny plaques were picked and screened by Southern blotting. The rescuant virus, HSVR2DSR, was purified by three rounds of plaque purification.

■ **Preparation and analysis of viral DNA.** Viral DNA was prepared from infected BHK cells and analysed by Southern blotting using probes labelled by the Megaprime method (Amersham), as detailed in the text.

■ **Analysis of viral polypeptides.** BHK or Vero cells (1×10^5 cells on a 10 mm Linbro well) were infected at an m.o.i. of 5 p.f.u. per cell in 0·3 ml medium. After appropriate incubation times, cells were washed with PBS and harvested in 100 μl SDS boiling mix. Extracts were stored at −20 °C prior to analysis by SDS–PAGE using a Bio-Rad Mini-PROTEAN II kit as recommended by the supplier. The gels were either stained with Coomassie blue for direct visualization of viral proteins, or proteins were transferred to nitrocellulose by Western blotting using the compatible Bio-Rad kit and the methods recommended by the supplier. The filters were blocked with 3% gelatin in TBS, then incubated with various monoclonal antibodies or monospecific rabbit sera as detailed in the text. Bound antibodies were detected by the Amersham ECL method, following the manufacturer's instructions. The antibodies used were as follows: 58S, anti-Vmw175 (Showalter *et al.*, 1981); r109, anti-VZV140k DNA binding domain (Tyler *et al.*, 1994); r106, anti-R1 (ICP6) (Conner *et al.*, 1993); 11060, anti-Vmw110 (Everett *et al.*, 1993); Z1F11, anti-UL42 (Schenk & Ludwig, 1988).

Results

Construction of plasmid expressing a hybrid Vmw175/VZV140k protein

The construction of a properly folded hybrid protein, even by using closely related sequences, is usually complicated by a lack of structural information to use as a guide. In this case, several criteria were relevant. (i) Vmw175 and VZV140k are highly related, especially in their DNA binding domains (McGeoch *et al.*, 1986). (ii) The Vmw175 DNA binding domain can be readily excised from the parent protein as a protease resistant and active polypeptide, implying that the sequences surrounding the domain are of open structure (Everett *et al.*, 1990). (iii) Both the Vmw175 and VZV140k DNA binding domains can be expressed in bacteria as separate and active

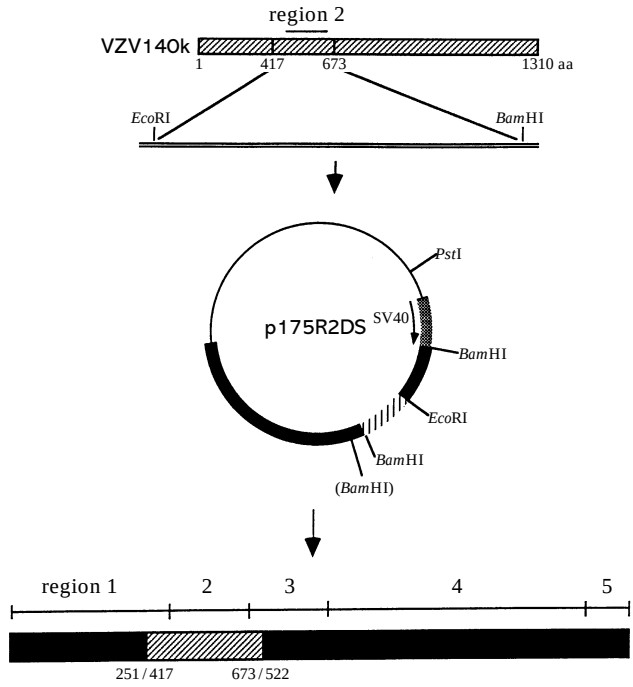

Fig. 1. Construction and structure of plasmid p175R2DS. The VZV140k open reading frame is depicted by the hatched bar at the top; the locations of region 2 (which is highly conserved throughout the Vmw175 family of proteins and which includes the DNA binding domain) and the section amplified by PCR are indicated. The *Eco*RI–*Bam*HI fragment resulting form the PCR was ligated to the *Pst*I–*Eco*RI fragment (derived from plasmid pI9) and the *Bam*HI–*Pst*I fragment (from pLI10/11) to create p175R2DS. The structure of the hybrid coding region is shown in the lower section, with HSV-1 Vmw175 sequences shown as solid black boxes and the VZV140k sequences shown as hatched boxes. The five regions of Vmw175, defined by relative sequence conservation, are indicated.

entities, implying that their correct folding does not require sequences from other parts of the protein (Wu & Wilcox, 1990, 1991; Everett *et al.*, 1991; Tyler & Everett, 1993). (iv) Mutations in Vmw175 flanking the DNA binding domain have little effect on Vmw175 activity. Specifically, insertion of a 12 bp *Eco*RI linker into codon 252 of Vmw175 has little effect on its activity either in transfection assays (Paterson & Everett, 1988*a*), or in the context of intact virus (Allen, 1993). (v) A Vmw175 polypeptide extending between residues 253 and 523 has a DNA binding specificity very similar to that of the intact protein in both gel retention and footprinting assays (Everett *et al.*, 1991; Tyler & Everett, 1993). A comparable VZV140k polypeptide encompassing residues 417–647 has similar properties (Tyler & Everett, 1993). On the basis of these considerations, PCR primers were designed to amplify the VZV140k coding sequence between residues 417 and 673 in such a way as to allow the replacement of Vmw175 residues 253–521. The details of this procedure are given in the methods section, and are depicted in Fig. 1. The VZV140k insert includes the whole of the highly conserved DNA binding domain. Like p175, the SV40 early promoter/enhancer

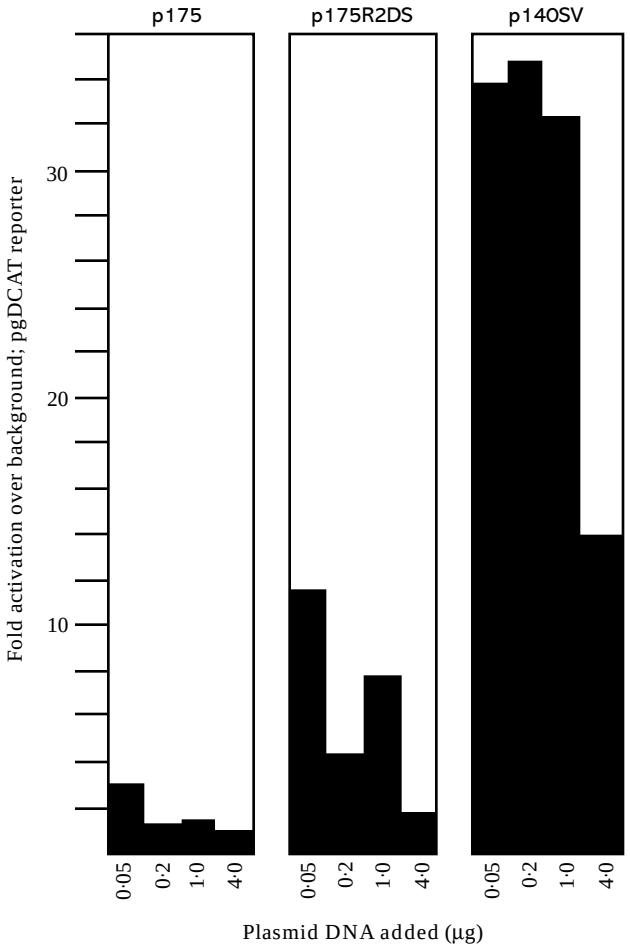

Fig. 2. Activation of the gD promoter by Vmw175, VZV140k and the hybrid 175R2DS protein in transfection assays. Increasing amounts of activator plasmids, as indicated, were co-transfected into HeLa cells with 4 μg of pgDCAT reporter. Forty hours after transfection, cell extracts were prepared and CAT activity was measured. The relative activity per unit of protein in the extracts is plotted as activation over background (a transfection with no activator plasmid). The results shown are from a single typical experiment; the complete titration set was repeated on a number of occasions with similar results.

region is present in p175R2DS instead of the HSV-1 IE3 promoter.

To ensure that the PCR did not introduce frameshift or deleterious point mutations within the amplified segment, the product was also cloned into the p585T7b2 expression vector, which enables high level expression of the inserted sequence in bacteria. The 175R2DS DNA binding domain polypeptide was expressed and partially purified as described (Tyler & Everett, 1993); it was found to be of the expected size and to have DNA binding properties indistinguishable from those of the previously characterized bacterially expressed VZV140k DNA binding domain (data not shown).

The 175R2DS hybrid protein has regulatory properties distinct from those of Vmw175 and VZV140k

The two main differences between Vmw175 and VZV140k in transfection assays are, firstly, that VZV140k fails to repress the HSV-1 IE3 promoter and, secondly, that VZV140k activates a wide variety of target promoters much more strongly than Vmw175 by itself. To determine the effects of the domain swap in these two assays, plasmids expressing Vmw175 (p175), VZV140k (p140SV) and the 175R2DS hybrid protein (p175R2DS) were transfected in increasing amounts into HeLa cells with either of two CAT indicator plasmids. Plasmid pgDCAT, which contains the HSV-1 gD promoter, was used as a representative target to monitor activation. Plasmid pIE3CAT was used to monitor the effects of the three proteins on the IE3 promoter. Plasmids p175, p140SV and p175R2DS all use the SV40 early promoter region to drive expression of the activator, and Western blot analysis verified that the three activators were expressed as intact proteins in the transfected cells (data not shown).

The results showed that p140SV gave high levels of activation of the gD promoter, while p175 is a much poorer activator by itself in this particular transfection system (Fig. 2).

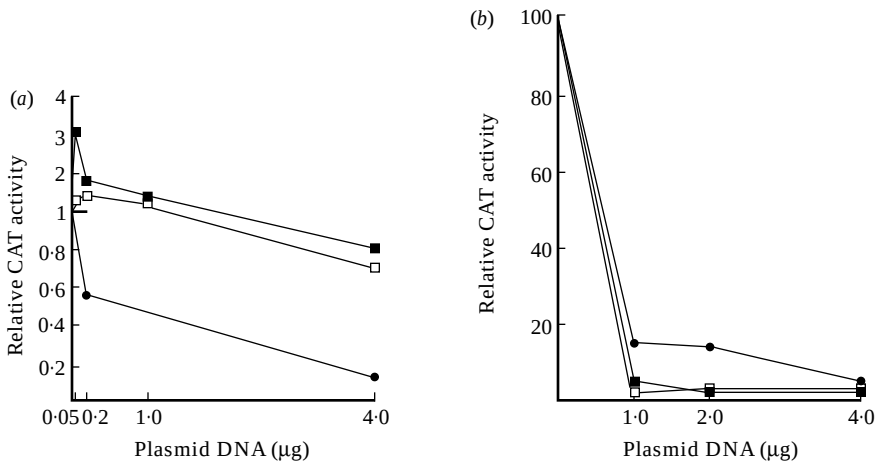

Fig. 3. Repression of the IE3 and VZV gene 62 promoters by Vmw175, VZV140k and the hybrid 175R2DS protein in transfection assays. (*a*) HeLa cells were transfected with 4 μg of pIE3CAT reporter and increasing amounts of p175 (●), p140SV (■) and p175R2DS (□). CAT activities were calculated as described in the legend to Fig. 2, and are plotted relative to the level obtained with pIE3CAT alone. (*b*) BHK cells were transfected with 4 μg p140CAT and effector plasmids, as above. Results of a single experiment, but typical of at least two independent repetitions, are shown. Note that it is only the coding sequences which differ between the three effector plasmids; the promoter, 5′ and 3′ bounding sequences and the vector are identical.

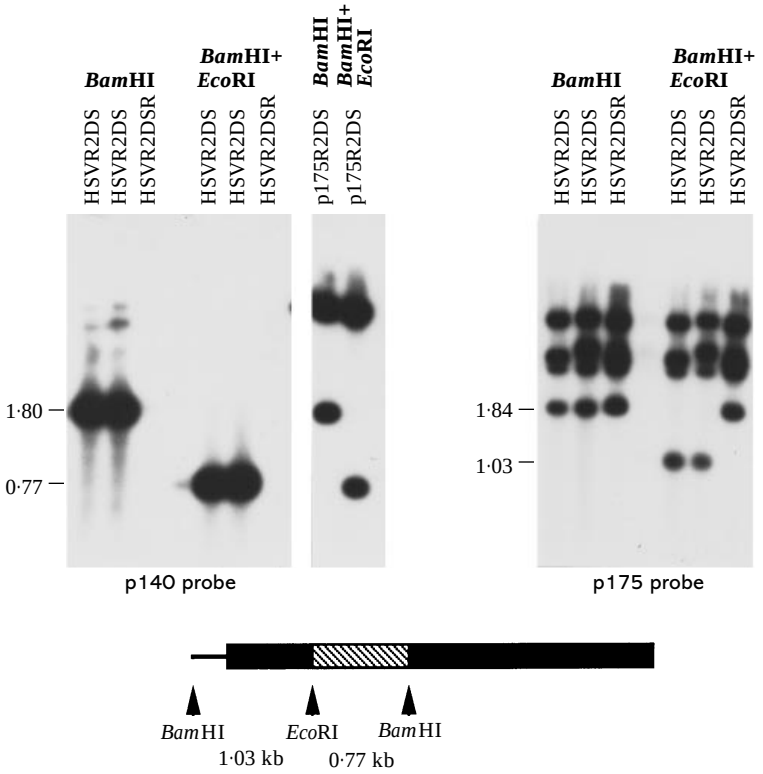

Fig. 4. Southern blot analysis of hybrid virus HSVR2DS. DNA from two independent final purified stocks of HSVR2DS was cut with restriction enzymes as indicated and compared to the rescuant virus HSVR2DSR and the parent plasmid p175R2DS. The tracks in the left-hand panel were probed for VZV sequences using p140 (which contains the whole of VZV gene 62), while the right-hand panel was probed for HSV sequences with p175. Below, a schematic of the hybrid gene is shown, with coding regions as boxes and non-coding DNA as a bar. The p140 probe detects only those fragments which include VZV sequences, namely the 1·8 kb *Bam*HI fragment and its 0·77 kb *Bam*HI–*Eco*RI sub-fragment which is composed entirely of VZV140k sequences (shown as hatched boxes in the schematic) (left-hand panel). The identities of these fragments were confirmed by comparison with fragments from their parental plasmid DNAs (centre panel). The p175 probe detects the 1·8 kb *Bam*HI band of HSVR2DS (which increases to 1·84 kb in the rescuant virus HSVR2DSR as the latter has 15 more codons) and its 1·03 kb *Bam*HI–*Eco*RI sub-fragment. The p175 probe also hybridizes to flanking IE3 fragments in the short repeat region of the genome which give rise to the multiple higher molecular mass bands in the right-hand panel.

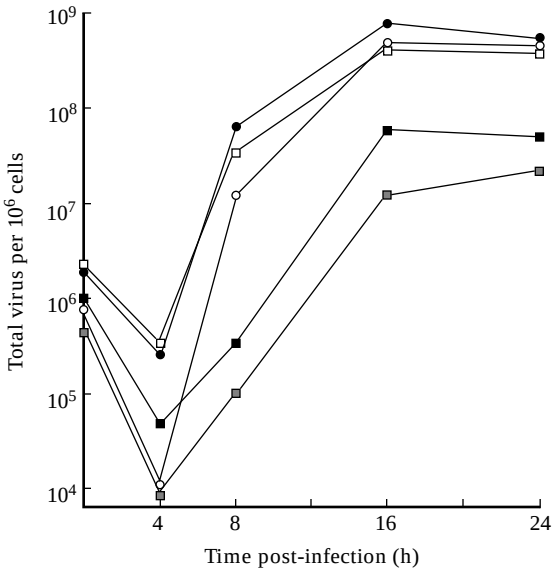

Fig. 5. Growth curve comparison of strain 17 (17^+; ●), HSVR2DS (■), HSV140 (⊠) and the rescuant viruses HSVR2DSR (○) and HSV140R (□). BHK cells on parallel plates were infected with the viruses as shown at an m.o.i. of 2 p.f.u. per cell. The cells and medium were harvested at the indicated times after infection and virus titres were determined using BHK cells. The results of a typical experiment are shown; the differences in growth potential were reproducible in repeated experiments.

Interestingly, p175R2DS gave an intermediate phenotype, with activation levels at about 25% of those seen with p140SV and significantly higher than those with p175 (Fig. 2). Importantly, this result confirms that the hybrid protein is folded into an active conformation.

It has been shown previously that Vmw175 represses the IE3 promoter in plasmid pIE3CAT, while VZV140k slightly activates it (Disney *et al.*, 1990). In this study, similar results were observed, with p140SV activating the IE3 promoter by a small, but reproducible degree at low input doses (Fig. 3*a*). In contrast to p175, plasmid p175R2DS failed to significantly repress the IE3 promoter even at high doses, while slight, but reproducible activation of the IE3 promoter by p175R2DS was observed at low input doses (Fig. 3*a*). The slight repression of the IE3 promoter observed at high doses of both p140SV and p175R2DS is probably due to promoter competition, since a plasmid carrying the SV40 promoter alone also shows similar repression at equivalent high doses (data not shown). These results suggest that exchanging the DNA binding domain modified the properties of the hybrid protein to resemble more closely those of VZV140k.

In contrast to the results with the IE3 promoter, the domain swap protein expressed by plasmid p175R2DS repressed the VZV gene 62 promoter in plasmid p140CAT to an extent similar to that achieved by both Vmw175 and VZV140k (Fig.

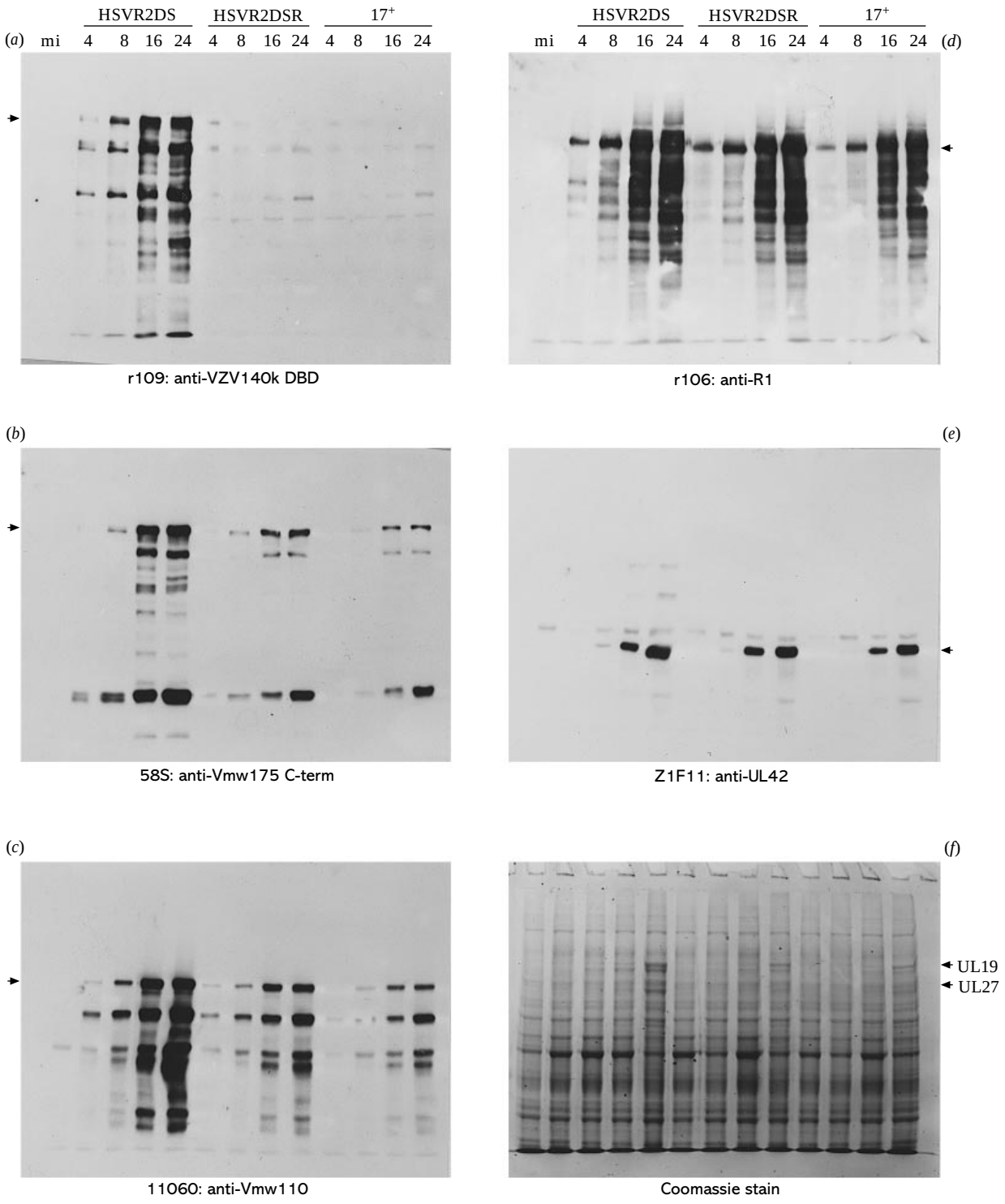

Fig. 6. Time-course of expression of selected viral proteins by HSVR2DS. Vero cells were infected at an m.o.i. of 5 p.f.u. per cell with HSVR2DS, HSVR2DSR and strain 17^+ and the cells were harvested 4, 8, 16 and 24 h later. Proteins were separated on 7·5% SDS gels and transferred to nitrocellulose by Western blotting. The nitrocellulose filter was incubated with antibodies as shown, and bound antibodies were detected by the ECL system (Amersham). The filter was stripped and re-probed with multiple antibodies. In (*f*), the same samples were analysed on a parallel gel and the proteins were stained with Coomassie blue. The arrows in panels (*a*–*e*) indicate the full-length protein, while smaller bands are breakdown products which are efficiently detected by the very sensitive ECL method. The major capsid protein (ICP5; UL19) and glycoprotein B (gB; UL27) bands are indicated in (*f*); other abundant viral proteins can also be seen, especially in the 24 h HSVR2DS track. mi, Mock infected.

3*b*). This experiment was an important control because it indicated that the hybrid protein was indeed competent for repression and that the results with the IE3 promoter were unlikely to be a consequence of a dysfunctional 175R2DS protein.

Construction of an HSV-1 virus which expresses the 175R2DS hybrid protein in place of Vmw175

The prediction from the above experiments is that a recombinant HSV-1 virus which expresses the hybrid 175R2DS protein in place of Vmw175 might activate viral gene expression more efficiently than wild-type HSV-1. In addition, due to the failure of the 175R2DS protein to repress the IE3 promoter, such a virus would be expected to express higher levels of the hybrid Vmw175 protein. In a manner analogous to the method of construction of HSV140 (Disney & Everett, 1990), co-transfection of the IE3 deletion mutant D30EBA viral DNA with linearized plasmid p175R2DS allowed the isolation of a virus which expressed the hybrid protein. The selected final isolate (virus HSVR2DS) was stable and had a restriction pattern in the IE3 region entirely consistent with the presence of the expected domain swap in both copies of the IE3 gene (Fig. 4). In turn, infectious DNA from virus HSVR2DS was co-transfected with linearized p175 DNA to construct a virus, named HSVR2DSR, in which the IE3 sequences had been repaired to re-establish a normal IE3 gene. Southern blots showed that, compared to the plasmid DNA controls, both HSVR2DS and HSVR2DSR had the expected restriction fragments which hybridized to the relevant HSV-1 and VZV sequence probes (Fig. 4).

Virus HSVR2DS is viable but exhibits impaired growth

To compare the growth efficiencies of different viruses, we monitored the production of progeny virus in a single-step growth curve in tissue culture. Parallel plates of BHK cells were infected with HSV-1 strain 17^+, HSVR2DS and HSVR2DSR, and the cells and medium harvested at intervals thereafter. Titration of the progeny on BHK cells indicated that HSVR2DS was about 10-fold less productive than strain 17^+, and that this defect was overcome in the HSVR2DSR repaired control (Fig. 5). The growth defect of HSVR2DS was also evident in its markedly smaller plaque size (data not shown). This result confirms that the hybrid protein is functional in the context of the virus but, despite its ability to achieve higher levels of activation than Vmw175 in transfection assays, HSVR2DS remains less growth efficient overall. Although we cannot exclude the possibility that HSVR2DS has accumulated other mutations within its genome during construction and isolation, the results with HSVR2DSR indicate that any such mutations have no measurable effect on growth efficiency in this assay.

In our earlier experiments, we found that it was difficult to see individual plaques after plating with high dilutions of HSV140, the virus which expresses VZV140k instead of Vmw175. The results with HSVR2DS are in apparent contrast to this, as small plaques were readily apparent. However, more recent experiments with faster growing cells have shown that HSV140 can produce visible plaques. Therefore, it was possible to compare the growth efficiencies of HSV140 and HSVR2DS with a repaired isolate of HSV140 (named HSV140R). The results (produced in parallel with the above experiment) showed that HSV140 was less productive than HSVR2DS, and the defect was, again, overcome by the repair (Fig. 5). The improved growth of HSVR2DS compared to HSV140 indicates that Vmw175 sequences outside the DNA binding domain make a specific contribution to virus growth since they are not fully interchangeable with the corresponding sequences of VZV140k.

Virus HSVR2DS expresses several viral proteins more abundantly than wild-type virus

To examine the properties of HSVR2DS in more detail, we monitored the efficiency of expression of a selection of IE and early gene products during the productive cycle. The results presented were obtained using Vero cells, and essentially similar results were obtained in BHK cells (not shown). Cells in Linbro wells were infected with viruses HSVR2DS, HSVR2DSR and HSV-1 strain 17^+ at an m.o.i. of 5 p.f.u. per cell and parallel wells were harvested after 4, 8, 16 and 24 h of infection. The viral proteins were separated by SDS–PAGE and detected by Western blotting. Initially, the efficiency of Vmw175 expression was monitored by probing with monoclonal antibody 58S, which recognizes an epitope near the C terminus of Vmw175 (Showalter *et al.*, 1981). The three viruses expressed a Vmw175 polypeptide of very similar gel mobility, but HSVR2DS reproducibly expressed significantly higher amounts, especially at late times of infection (Fig. 6*b*). The Vmw175-related polypeptide expressed by HSVR2DS was shown to include the VZV140k DNA binding domain by probing the same blot with r109 serum (Tyler *et al.*, 1994), which was raised against a bacterially expressed polypeptide equivalent to the VZV140k insert in virus HSVR2DS (Fig. 6*a*). The higher levels of expression of the hybrid Vmw175 protein by HSVR2DS are entirely consistent with its failure to repress the IE3 promoter in transfection assays (Fig. 3). This conclusion is also consistent with the observed higher levels of expression of VZV140k protein by virus HSV140 (Disney & Everett, 1990).

We were interested to see the effects of the hybrid protein on expression of other viral proteins. For this reason, similar blots were probed to detect the amounts of selected viral proteins: IE polypeptide Vmw110; ICP6 or R1, an early polypeptide with some IE characteristics; and UL42, an early polypeptide. The results showed that HSVR2DS expressed

higher amounts of Vmw110 than either HSVR2DSR or HSV-1 strain 17^+ (Fig. 6*c*), while there was little significant difference in the amounts of R1 expressed by the three viruses (Fig. 6*d*). The fact that the R1 promoter has been shown to be unresponsive to Vmw175, but instead appears to be activated by Vmw110 (Desai *et al.*, 1993) may be relevant. The levels of UL42 expressed by the three viruses were not significantly different (Fig. 6*e*), but Coomassie staining of the protein gels indicated that several abundant viral polypeptides were expressed by HSVR2DS at significantly higher levels, compared to the other two viruses (Fig. 6*f*). This was particularly clear in the 24 h samples, but the same phenomenon was also visible in the 16 h samples, and to a limited extent in the 8 h samples. The advantage of using Coomassie staining for this experiment is that, despite lower sensitivity, several viral proteins could be examined on the same gel and their amounts compared directly. Care was taken to ensure that the input multiplicities were equal in these experiments, and it was determined that the particle to p.f.u. ratios of the three virus stocks were also similar. Therefore, the hybrid protein appears to activate expression of several viral polypeptides more efficiently than normal Vmw175 during virus infection, which is consistent with the higher levels of activation that it achieved in transfection assays.

Discussion

The results presented in this paper re-emphasize the functional equivalence of Vmw175 and VZV140k, and illustrate that their properties are partially determined by the nature of their DNA binding domains. The VZV140k DNA binding domain has a more relaxed sequence specificity than that of Vmw175 (Tyler & Everett, 1993). This property might allow VZV140k to interact more efficiently with its target promoters and thus explain its apparently greater activation potential in transfection assays. The properties of the DNA binding domain swap hybrid protein are compatible with this suggestion. At the HSV-1 IE3 cap site, in contrast to its Vmw175 equivalent, the VZV140k domain does not bend the DNA and the two proteins give DNAse I footprints of different characteristics (Everett *et al.*, 1992; Tyler & Everett, 1993). Since the positioning of Vmw175 at a binding site in the vicinity of the IE3 transcriptional initiation site is crucial for the ability of Vmw175 to repress IE3 transcription (Roberts *et al.*, 1988; Michael & Roizman, 1993; Koop *et al.*, 1993), it is possible that the differences in detail of VZV140k binding at this site might also explain why VZV140k activates, rather than represses, the IE3 promoter. Indeed, a recent analysis of the Vmw175-induced DNA distortions that occur at the IE3 cap site suggested that Vmw175-induced DNA bending may play a role in repression of the IE3 promoter (Kuddus *et al.*, 1995). It is possible that the inability of VZV140k to introduce a DNA bend at this site is responsible for the failure of both VZV140k and the hybrid protein to down-regulate IE3 expression (Tyler & Everett, 1993).

However, the 175R2DS domain swap protein is not as efficient an activator as the VZV140k protein itself. This could be explained either by inappropriate folding of the hybrid protein, or by the absence of highly efficient activator sequences that are present in VZV140k but not in Vmw175. The former explanation seems unlikely, since the sequences immediately flanking the Vmw175 DNA binding domain are not crucial for function (Paterson & Everett, 1988*a*, *b*; Shepard *et al.*, 1989), and the available information indicates that this is probably also true for VZV140k (Baudoux *et al.*, 1995). The latter explanation is supported by the identification of a very strong activation sequence within residues 9–86 of the N terminus of VZV140k (Cohen *et al.*, 1993; Perera *et al.*, 1993) which is apparently not conserved in Vmw175. Therefore, it seems that the high activation potential of VZV140k might be explained by its possession of this activation sequence and also by the characteristics of its DNA binding domain.

The properties of the 175R2DS domain swap protein in transfection assays were reflected by the increased efficiency of expression of several viral polypeptides by the corresponding HSVR2DS virus. For example, the Vmw175 protein itself was detected at increased levels at later times during infection by HSVR2DS as compared to wild-type HSV-1. Obviously, there are many factors which influence the levels of viral polypeptide synthesis during infection, and the situation during HSVR2DS infection is further complicated by the presumably increased levels of polypeptides that would normally be down-regulated by Vmw175, but given the complexity of the controls that are operating, it is striking that substitution of the highly conserved DNA binding domain of Vmw175 can have such a significant effect upon gene expression during infection. It is interesting that the higher levels of viral gene expression achieved by HSVR2DS do not result in increased numbers of infectious virus particles. Instead, a reduced virus yield as compared to wild-type HSV-1 was observed, indicating that virus yield is not merely a function of the level of viral protein production, but is a carefully regulated process. This is exemplified here by the reduced virus production that results when gene expression is mis-regulated as a consequence of replacing the Vmw175 DNA binding domain with that of VZV140k.

In conclusion, the inference from these data is that the specific details of the DNA binding activities of Vmw175 and VZV140k play a significant part in regulating the specificity of their transactivation and repression properties that, in turn, determine the highly efficient production of infectious herpesvirus virions.

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References

Allen, K. E. (1993). *A mutational analysis of the DNA binding domain of the herpes simplex virus immediate early protein Vmw175*. PhD thesis, University of Glasgow, UK.

Baudoux, L., Defechereux, P., Schoonbroodt, S., Merville, M. P., Rentier, B. & Piette, J. (1995). Mutational analysis of varicella zoster virus major immediate-early protein IE62. *Nucleic Acids Research* **23**, 1341–1349.

Cabirac, G. F., Mahalingam, R., Wellish, M. & Gilden, D. H. (1990). Trans-activation of viral tk promoters by proteins encoded by varicella zoster virus open reading frames 61 and 62. *Virus Research* **15**, 57–68.

Cohen, J. I., Heffel, D. & Seidel, K. (1993). The transcriptional activation domain of varicella-zoster virus open reading frame 62 protein is not conserved with its herpes simplex virus homolog. *Journal of Virology* **67**, 4246–4251.

Conner, J., Furlong, J., Murray, J., Meighan, M., Cross, A., Marsden, H. & Clements, J. B. (1993). Herpes simplex virus type 1 ribonucleotide reductase large subunit: regions of the protein essential for subunit interaction and dimerisation. *Biochemistry* **32**, 13673–13680.

DeLuca, N. A. & Schaffer, P. A. (1985). Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. *Molecular and Cellular Biology* **5**, 1997–2008.

DeLuca, N. A. & Schaffer, P. A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. *Journal of Virology* **62**, 732–743.

Desai, P., Ramakrishnan, R., Lin, Z. W., Osak, B., Glorioso, J. & Levine, M. (1993). The RR1 gene of herpes simplex virus type 1 is uniquely transactivated by ICP0 during infection. *Journal of Virology* **67**, 6125–6135.

DiDonato, J. A., Spitzner, J. R. & Muller, M. T. (1991). A predictive model for DNA recognition by the herpes simplex virus protein ICP4. *Journal of Molecular Biology* **219**, 451–470.

Disney, G. H. & Everett, R. D. (1990). A herpes simplex virus type 1 recombinant with both copies of the Vmw175 coding sequences replaced by the homologous varicella-zoster virus open reading frame. *Journal of General Virology* **71**, 2681–2689.

Disney, G. H., McKee, T. A., Preston, C. M. & Everett, R. D. (1990). The product of varicella-zoster virus gene 62 autoregulates its own promoter. *Journal of General Virology* **71**, 2999–3003.

Everett, R. D. (1984). Transactivation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *EMBO Journal* **13**, 3135–3141.

Everett, R. D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2 and 3 can activate HSV-1 gene expression in *trans*. *Journal of General Virology* **67**, 2507–2513.

Everett, R. D., Paterson, T. & Elliott, M. (1990). The major transcriptional regulatory protein of herpes simplex virus type 1 includes a protease resistant DNA binding domain. *Nucleic Acids Research* **18**, 4579–4585.

Everett, R. D., Elliott, M., Hope, G. & Orr, A. (1991). Purification of the DNA binding domain of herpes simplex virus type 1 immediate-early protein Vmw175 as a homodimer and extensive mutagenesis of its DNA recognition site. *Nucleic Acids Research* **19**, 4901–4908.

Everett, R. D., DiDonato, J., Elliott, M. & Muller, M. (1992). Herpes simplex virus type 1 polypeptide ICP4 bends DNA. *Nucleic Acids Research* **20**, 1229–1233.

Everett, R. D., Cross, A. & Orr, A. (1993). A truncated form of herpes simplex virus type 1 immediate-early protein Vmw110 is expressed in a cell type dependent manner. *Virology* **197**, 751–756.

Faber, S. W. & Wilcox, K. W. (1986). Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. *Nucleic Acids Research* **14**, 6067–6083.

Felser, J. M., Kinchington, P. R., Inchauspe, G., Straus, S. E. & Ostrove, J. M. (1988). Cell lines containing varicella-zoster virus open reading frame 62 and expressing the 'IE' 175 protein complement ICP4 mutants of herpes simplex virus type 1. *Journal of Virology* **62**, 2076–2082.

Fields, B. N., Knipe, D. M. & Howley P. M. (editors) (1996). *Fields Virology*, 3rd edn. Philadelphia: Lippincott-Raven.

Flanagan, W. M., Papavassiliou, A. G., Rice, M., Hecht, L. B., Silverstein, S. & Wagner, E. K. (1991). Analysis of the herpes simplex virus type 1 promoter controlling the expression of UL38, a true late gene involved in capsid assembly. *Journal of Virology* **65**, 769–786.

Gu, B. & DeLuca, N. A. (1994). Requirements for activation of the herpes simplex virus glycoprotein C promoter *in vitro* by the viral regulatory protein, ICP4. *Journal of Virology* **68**, 7953–7965.

Gu, B., Kuddus, R. & DeLuca, N. A. (1995). Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. *Molecular and Cellular Biology* **15**, 3618–3626.

Imbalzano, A. N., Shepard, A. A. & DeLuca, N. A. (1990). Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. *Journal of Virology* **64**, 2620–2631.

Inchauspe, G. & Ostrove, J. M. (1989). Differential regulation by varicella-zoster virus (VZV) and herpes simplex virus type-1 trans-activating genes. *Virology* **173**, 710–714.

Koop, K. E., Duncan, J. & Smiley, J. R. (1993). Binding sites for the herpes simplex virus immediate-early protein ICP4 impose an increased dependence on viral DNA replication on simple model promoters located in the viral genome. *Journal of Virology* **67**, 7254–7263.

Kuddus, B., Gu, B. & DeLuca, N. A. (1995). Relationship between TATA-binding protein and herpes simplex virus type 1 ICP4 DNA-binding sites in complex formation and repression of transcription. *Journal of Virology* **69**, 5568–5575.

McGeoch, D. J., Dolan, A., Donald, S. & Brauer, D. H. K. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. *Nucleic Acids Research* **14**, 1727–1764.

Michael, N. & Roizman, B. (1989). Binding of the herpes simplex virus major regulatory protein to viral DNA. *Proceedings of the National Academy of Sciences, USA* **86**, 9808–9812.

Michael, N. & Roizman, B. (1993). Repression of the herpes simplex virus 1 alpha 4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. *Proceedings of the National Academy of Sciences, USA* **90**, 2286–2290.

Michael, N., Spector, D., Mavromara-Nazos, P., Kristie, T. M., Roizman, B. (1988). The DNA-binding properties of the major regulatory protein alpha 4 of herpes simplex viruses. *Science* **239**, 1531–1534.

Muller, M. T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. *Journal of Virology* **61**, 858–865.

O'Hare, P. & Hayward, G. S. (1985). Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene

expression in a pathway involving positive and negative feedback regulation. *Journal of Virology* **56**, 723–733.

Paterson, T. & Everett, R. D. (1988*a*). Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. *Virology* **166**, 186–196.

Paterson, T. & Everett, R. D. (1988*b*). The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. *Nucleic Acids Research* **16**, 11005–11025.

Paterson, T. & Everett, R. D. (1990). A prominent serine-rich region in Vmw175, the major transcriptional regulator protein of herpes simplex virus type 1, is not essential for virus growth in tissue culture. *Journal of General Virology* **71**, 1775–1783.

Perera, L. P., Mosca, J. D., Ruyechan, W. T., Hayward, G. S., Straus, S. E. & Hay, J. (1993). A major transactivator of varicella-zoster virus, the immediate-early protein IE62, contains a potent N-terminal activation domain. *Journal of Virology* **67**, 4474–4483.

Roberts, M. S., Boundy, A., O'Hare, P., Pizzorno, M. C., Ciufo, D. M. & Hayward, G. S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. *Journal of Virology* **62**, 4307–4320.

Schenk, P. & Ludwig, H. (1988). The 65 kd DNA binding protein appears early in HSV-1 infection. *Archives of Virology* **102**, 119–123.

Shepard, A. A., Imbalzano, A. N. & DeLuca, N. A. (1989). Separation of primary structural components conferring autoregulation, transactivation, and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. *Journal of Virology* **63**, 3714–3728.

Showalter, L. D., Zweig, M. & Hampar, B. (1981). Monoclonal antibodies to herpes simplex type 1 proteins including the immediate-early protein ICP4. *Infection and Immunity* **34**, 684–692.

Smiley, J. R., Johnson, D. C., Pizer, L. I. & Everett, R. D. (1992). The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein (gD) promoter are not essential for efficient gD transcription during virus infection. *Journal of Virology* **66**, 623–631.

Smith, C. A., Bates, P., Rivera-Gonzalez, R., Gu, B. & DeLuca, N. (1993). ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. *Journal of Virology* **67**, 4676–4687.

Tyler, J. K. & Everett, R. D. (1993). The DNA binding domain of the varicella-zoster virus gene 62 protein interacts with multiple sequences which are similar to the binding site of the related protein of herpes simplex virus type 1. *Nucleic Acids Research* **21**, 513–522.

Tyler, J. K., Allen, K. E. & Everett, R. D. (1994). Mutation of a single lysine residue severely impairs the DNA recognition and regulatory functions of the VZV gene 62 transactivator protein. *Nucleic Acids Research* **22**, 270–278.

Wu, C. L. & Wilcox, K. W. (1990). Codons 262 to 490 from the herpes simplex virus ICP4 gene are sufficient to encode a sequence-specific DNA binding protein. *Nucleic Acids Research* **18**, 531–538.

Wu, C. L. & Wilcox, K. W. (1991). The conserved DNA-binding domains encoded by the herpes simplex virus type 1 ICP4, pseudorabies virus IE180, and varicella-zoster virus ORF62 genes recognize similar sites in the corresponding promoters. *Journal of Virology* **65**, 1149–1159.

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