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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

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ABSTRACT

The immediate-early (IE) protein Vmw175 (ICP4) of HSV-1 is required for the transcription of later classes of viral genes and the repression of IE gene expression. We have previously constructed a panel of plasmid-borne insertion and deletion mutants of the gene encoding Vmw175 and assayed their ability to regulate transcription in transient transfection assays. By this approach we have mapped the regions of the Vmw175 amino acid sequence that are required for transcriptional activation and repression of herpes virus promoters. This paper describes the use of nuclear extracts, made from cells transfected with these mutant plasmids, in gel retardation DNA binding assays in order to define the regions of Vmw175 involved in binding to a specific Vmw175 DNA binding site. The results show that amino acid residues 275-495 (a region which is highly conserved between Vmw175 and the varicella-zoster virus "IE" 140K protein) include structures which are critically required for specific DNA binding, transactivation and repression. This raises the interesting paradox that although the specific DNA sequence recognized by Vmw175 is not commonly found in its target promoters, the protein domain required for recognition of this sequence is required for promoter activation.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large complex virus containing a double stranded DNA genome of around 150kb. Upon infection of tissue culture cells the genes of HSV-1 are expressed in a sequential cascade of three broad temporal classes, defined as immediate-early (IE), early and late (1). Expression of IE proteins is required for transcription of later classes of genes (2,3,4) and there is evidence that at least four of the five IE gene products are regulators of viral gene expression (reviewed, 5). However, characterization of temperature-sensitive (ts) and deletion mutations in these five genes has revealed that only Vmw175 (ICP4) and Vmw63 (ICP27)

(the products of IE genes 3 and 2) are essential for virus growth in cell culture (6-12). Functional Vmw175 is required for the transcriptional activation of early and late genes and also the repression of IE genes (13). Both of these activities can be studied using cloned copies of IE gene 3 in transient transfection assays to transactivate viral early gene promoters or to repress expression from its own promoter (5). Transactivation and repression have also been reproduced in vitro using partially purified Vmw175 (14,15).

Vmw175 is a large, phosphorylated, nuclear protein (16) which can be isolated as a homodimer (17). Whilst most of the IE proteins bind to DNA to some extent in vitro (18) Vmw175 is the only one known to interact with specific target sequences which include a proposed consensus binding sequence ATCGTC (15,19). Vmw175 has been shown to bind to this sequence in the promoter regions of the IE1, IE3 and glycoprotein gD genes (19-22) but has also been reported to bind to several viral DNA fragments which lack the consensus (20,23). Evidence from a variety of laboratories suggests a correlation between the ability of Vmw175 to bind ATCGTC at the transcriptional start site of IE gene 3, and autoregulation of this promoter. However, less is known about the mechanism by which Vmw175 transactivates promoters. Most such promoters lack sequences closely related to the consensus binding site, and indeed there is no evidence for any transactivation specific promoter sequences being necessary for the action of Vmw175 (24-26). Recent evidence suggests that Vmw175 can also bind to alternative sites not related to the consensus binding-site, both in promoter regions and the 5' transcribed, non-coding regions of late genes (23); the significance of this is not yet known.

IE gene 3 is present in two copies in HSV-1, being carried on the repeat sequences bounding the short unique region of the genome (27). The sequence of the gene reveals an uninterrupted open reading frame encoding a protein of 1298 amino acids having a predicted unmodified molecular weight of 133kDa (28). The protein is homologous to a predicted 140kDa protein of a related alphaherpesvirus, varicella-zoster virus (VZV) (28,29). The VZV 140K product is also a potent activator of transcription (24,30,

unpublished results) and can complement HSV-1 mutants with ts lesions in Vmw175 (31,32). On the basis of the sequence homology between the two proteins, and various other criteria, the Vmw175 sequence has been divided into five structural regions, with regions 2 and 4 containing the main regions of homology with the VZV protein (28,29) (see Figure 3).

We have previously described the construction and functional analysis of a large number of small, in-frame, insertion and deletion mutants of a plasmid-borne copy of the gene encoding Vmw175 (33). By studying the activity of the resultant mutant polypeptides in transient transfection assays we have defined the regions of the protein which are important for repression of its own promoter, and those important for transactivation of the promoter of the HSV early gene encoding glycoprotein gD, in the presence of another HSV-1 IE transactivator, Vmw110. Independent analyses performed by DeLuca and Schaffer (34,35) using chain-termination mutants of the gene encoding Vmw175 in both plasmids and recombinant HSV-1 virus have defined similar functional domains.

This paper presents the results from further analyses of plasmid-borne insertion and deletion mutants in Vmw175. Extracts were made from transfected cells and used in gel retardation assays to measure the ability of each mutant polypeptide to bind to a DNA probe containing the consensus Vmw175-binding site at the transcription start site of IE gene 3. The results show that the regions of Vmw175 required for DNA binding closely correspond to those involved in transcriptional regulation. In particular, a region crucial for site specific DNA binding lies within amino acids 275-495.

MATERIALS AND METHODS

Plasmids. Plasmid p175 expresses Vmw175 under the control of the SV40 early promoter and enhancer (36). Plasmids p111 (36) and p63 (37) express other HSV-1 IE gene products, Vmw110 and Vmw63. pGX38 carries the gene encoding the major DNA binding protein, ICP8. The insertion mutant series pI1-39 was derived from p175 by the insertion of 12bp EcoRI linkers at random restriction sites and the deletion mutants were derived from these (33). Deletions pD13, pD14 and pD15 were created by recombining pairs of insertion mutants at the inserted EcoRI linker to generate inframe deletions (see Table 2).

Cell culture, infection and transfection. HeLa cells were obtained from the ATCC through Flow Laboratories and grown in Glasgow Modified Eagle's Medium containing 10% foetal calf serum. The viruses used were derived from HSV-1 strain 17 syn^+ (38). Cells were infected at a multiplicity of infection of 5pfu per cell and nuclear extracts made 5h after a 1h adsorption period. Cells on 90 or 135mm plates (3 or $7x10^6$ cells per plate) were transfected by the procedure of Corsalo and Pearson (39). In some recent transfections HBS was replaced by the more efficient BBS (40). 30 or 60ug of pl75 derived plasmids were used plus 15 or 30ug of pUC9 as carrier. Nuclear extracts were made 45-50h post transfection.

Preparation of nuclear extracts. Nuclear extracts of infected and transfected cells were prepared by a procedure modified from that of Dignam et al.,(41). Cells scraped from 135 or 90mm culture dishes were washed in phosphate buffered saline (PBS) and resuspended in 2 volumes buffer A plus 0.5% NP40 (10mM Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT). After 10 minutes lysis on ice nuclei were pelleted by successive 2krpm/10 minute and 12krpm/20 minute spins in the Sorvall SS34 rotor. Proteins were eluted from the nuclei by incubating for 30 minutes on ice in 2 volumes buffer C (20mM Hepes pH7.9, 25% glycerol, 0.42mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT) before clearing with a 30 minute 15krpm spin. The supernatants were flash frozen and stored at -170^oC.

Some whole cell extracts were produced by lysing cells with buffer C plus 0.5% NP40; the results using extracts made by the two methods were generally indistinguishable.

Determination of the relative quantities of Vmw175 in nuclear extracts. Nuclear extracts were assayed by the ELISA technique in order to determine the relative amount of Vmw175 present. Microtitre wells were coated overnight with extract (5ul) plus 100ul of a solution containing 0.05% sodium azide, 1% ovalbumin, 1% BSA, 0.1% Tween 20 in PBS. 100ul of a 1/5000 dilution of the monoclonal antibody 58S (kindly supplied by M.Zweig, 42) was adsorbed to the coated wells by shaking for 1h at 37^oC then 100ul of a 1/3000 dilution of BIO-RAD horseradish peroxidase labelled goat anti-mouse IgG was bound to this at 20^oC for 1h. Extensive washes with PBS/0.1% Tween 20 were carried out between all steps. The absorbance at 405nm was measured 10 minutes after addition of substrate.

Gel retardation assay. The binding of Vmw175 to the IE gene 3 cap site was assayed as described by Muller (22) using the gel retardation DNA binding assay (43). The 45bp Ava I/BamHI DNA fragment spanning the IE3 cap site (-17 to +27) was end labelled by filling in using T4 DNA polymerase, separated on an 8% polyacrylamide gel, eluted and purified through a Sephadex G-50 column. Binding reactions were generally carried out at 20^oC for 20 minutes in a 20-30ul mix containing lug poly(dI).poly(dC), about 0.1ng probe (2000cpm) and 1-4ul nuclear extract (4-20ug protein) in 10mM Tris.HCl pH7.6, 1mM EDTA, 0.1% NP40. The variation in final salt concentration (20-80mM NaCl) did not affect the formation of the specific Vmw175 complex, which is stable up to at least 300mM NaCl (results not shown). The complexes were resolved on 4% polyacrylamide gels, run in 0.5xTBE buffer, which were dried before autoradiography.

Antibody shifts were carried out by incubating the binding reaction for an additional 10-15 minutes after adding 1ul of a 1/100 dilution of monoclonal antibody 58S.

RESULTS

Vmw175 in nuclear extracts made from transfected cells forms an authentic complex with the IE gene 3 cap site.

We have used a gel retardation assay similar to that of Muller (22) to show that nuclear extracts made from HeLa cells transfected with a plasmid expressing HSV-1 Vmw175 form a Vmw175 specific complex with the IE3 cap site which is essentially identical to that formed using extracts of virus infected cells. Using a short end-labelled DNA probe spanning the cap site of this gene a single major, viral specific, retarded complex is detected on non-denaturing polyacrylamide gels after incubation of the probe with infected cell nuclear extract for 20 minutes at 20^{o}C (Figure 1A, tracks 3 and 4). This complex is further retarded after additional incubation with the Vmw175 specific monoclonal antibody 58S (42) which recognizes an epitope near to the C-terminus of Vmw175 (33). These complexes were not formed using extracts made from mock infected cells nor from cells infected with an HSV-1 mutant which produces no Vmw175, in1411 (44) (Figure 1A). The complex was also formed by extracts made from cells infected with dl1403, a mutant lacking both copies of IE gene 1 which encodes Vmw110 (8).

An apparently viral specific complex, labelled 'z', of much greater mobility was occasionally formed (Figure 1B), especially if the binding reaction was carried out at elevated temperatures. This complex was not further retarded by the antibody 58S, indicating that it might be formed by a protein unrelated to Vmw175, or, by a proteolytic fragment of Vmw175 lacking the C-terminal 58S epitope.

Extracts made from cells transfected with plasmid p175, which expresses HSV-1 Vmw175, also formed a complex which was usually indistinguishable from the viral specified complex and which also contained Vmw175 (Figure 2A). However, transfected cell extracts occasionally formed two specific complexes which were both recognized by 58S (Figure 2B). The smaller complex could be formed by an abnormally processed form of Vmw175 or it

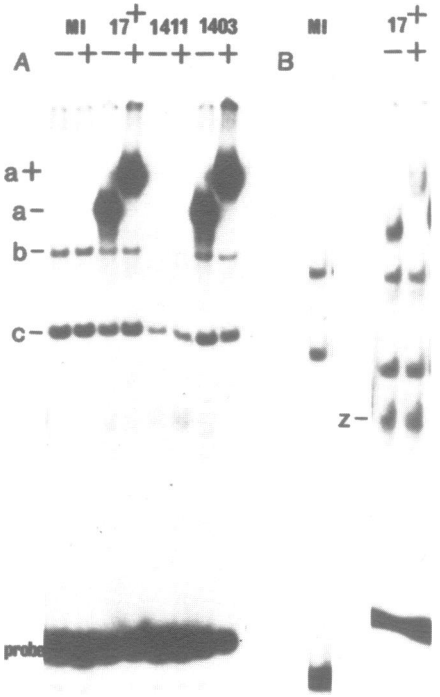

Figure 1: Autoradiograph of a gel retardation experiment showing the protein-DNA complexes formed using nuclear extracts of HSV-1 infected cells bound to a DNA probe spanning bases -17 to +27 of the HSV-1 IE gene 3. Assays were performed as described in the materials and methods, with (+) or without (-) addition of monoclonal antibody 58S. Assays perfomed at (A) 20°C or (B) 40°C. MI= mock infected, 17^+= HSV-1 strain 17^+, 1403= HSV-1 mutant dl1403, 1411= HSV-1 mutant in1411, a= retarded complex containing Vmw175, a^+= complex a further retarded with antibody, b and c are complexes containing host factors, z= novel retarded band, probe= unbound DNA probe.

might lack an unknown factor. It usually appeared when the overall efficiency of the transfections was poor and could reflect a lower level of Vmw175 in the transfected cells.

That cells transfected with p175 formed a complex identical to that formed with infected cell extracts indicates that Vmw175 is the only viral protein present in the complex. No other HSV proteins could be detected in the complex using monoclonal antibodies (against the major DNA binding protein) or antipeptide antisera (against Vmw110 and Vmw63, results not shown). The

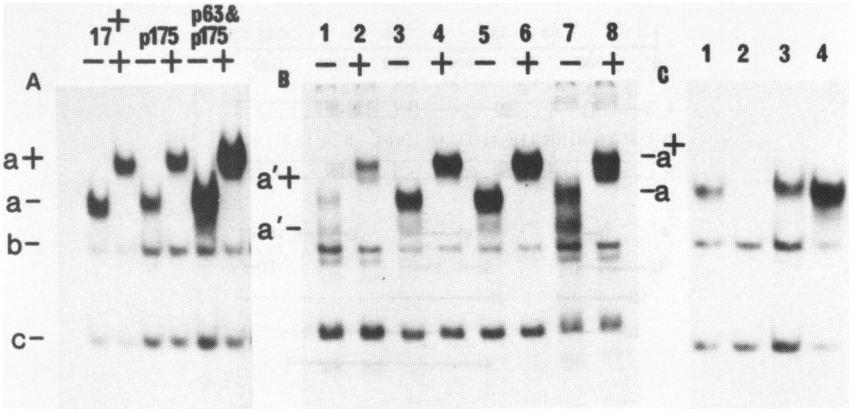

Figure 2: Nuclear extracts containing Vmw175 expressed in cells transfected with plasmid p175 form an apparently identical DNA complex to that formed by Vmw175 present in infected cell extracts. (A) Binding assays were carried out as described in Figure 1 using extracts of cells infected or transfected as shown. Unbound probe has been cut off the bottom of the portion of the gel shown. Plasmids used express Vmw175 (p175), Vmw110 (p111), Vmw63 (p63) and ICP8 (pGX38). (B) cells were transfected with p175 and the following plasmids: lanes 1 & 2, p111; 3 & 4, p111 and p63; 5 & 6, p111, p63 and pGX38; 7 & 8, p175 alone. a'= secondary complex, shifted to a'+ with antibody. (C) Addition of extracts containing Vmw63 does not enhance Vmw175 DNA binding. Lane 1, p175; lane 2, p63; lane 3 mix of extracts used in 1 & 2, lane 4, cotransfection of p175 and p63.

presence of other HSV-1 genes in the transfection had no effect on the nature of complex formed, but addition of the gene encoding Vmw63 did increase its amount (Figures 2A,B). However, Vmw63 itself did not form a complex with this DNA probe, and the addition of an extract containing Vmw63 to one containing Vmw175 did not enhance binding (Figure 2C). Rather the effect of Vmw63 seemed to be on expression of Vmw175, as the extract made from Vmw63 and 175 encoding genes transfected together had 2.5 fold more Vmw175 than the extract from cells transfected with p175 alone, as determined by ELISA.

The regions of Vmw175 important for DNA binding closely correspond to those important for transcriptional regulation.

1. Analysis of insertion mutants. We have previously constructed a series of derivatives of p175 with twelve base pair insertions

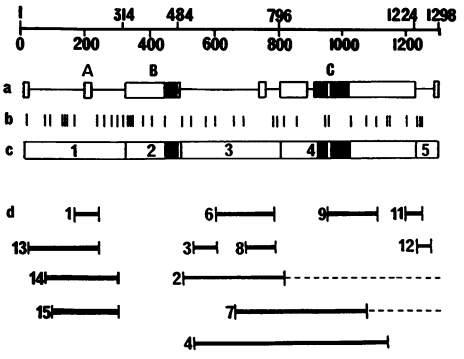

Figure 3: Map of the Vmw175 coding sequence showing the position of the insertion and deletion mutants used in this study. (a) Homology map of Vmw175 in relation to VZV 140K. The three main homology regions A, B and C are indicated. Clearly homologous regions are shown as boxes, with the most homologous sections filled (at least 80% identity). (b) Position of each linker insertion (I1-39) is represented by a vertical line. (c) Colinear map showing the 5 regions of Vmw175. (d) Extent of deletions used in this study, dotted lines represent out of frame sequences. The scale is given in amino acid residues.

at random sites in the Vmw175 coding sequence (Figure 3) and used these to define the regions of the protein that are important for transactivation of an HSV-1 early promoter and repression of the viral IE3 promoter (33). We have now investigated the DNA binding activity of these mutants by using nuclear extracts made from HeLa cells transfected with each of these 39 plasmids in gel retardation assays.

Nuclear extracts were prepared from $7x10^6$ transfected cells and one twentieth (4ul) of each was incubated with the IE3 cap site probe in a 30ul reaction volume at 20^oC for 20 minutes, then further incubated with 1ul 1/100 dilution of monoclonal 58S prior to separation on 4% polyacrylamide gels. The resulting autoradiographs are shown in Figure 4. The antibody shift was carried out to clearly resolve the Vmw175 complex from the nonspecific complexes (b) and (c) although in some cases the addition of antibody seemed to stabilize the degree of complex formation (results not shown). In addition to the major (a^+) complex, the minor (z) complex (Figure 1B) was also occasionally

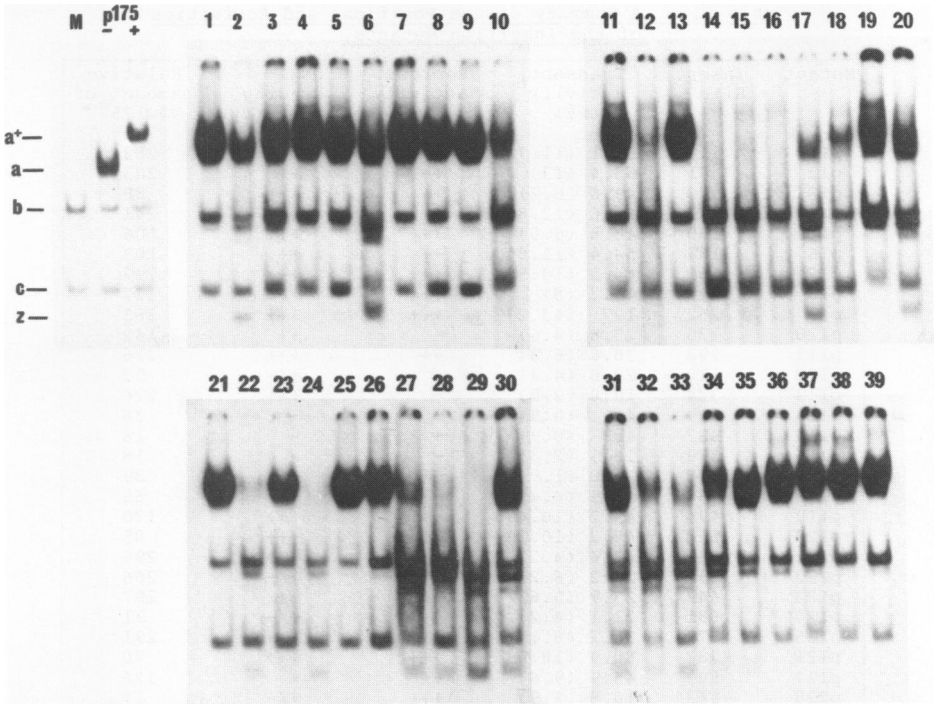

Figure 4: Binding assays using extracts of cells transfected independantly with each of the 39 insertion mutants pI1-pI39. All assays were incubated with antibody 58S except M (mock transfected) and p175/- (positive control). Complexes a, a^+, b, c and z are as described in Figure 1.

present. The binding reactions were carried out at 20°C; at higher temperatures the binding of wild type Vmw175 was significantly reduced (results not shown) although temperature sensitive mutants can be studied in this manner (unpublished results).

The amount of Vmw175 in each extract was measured by ELISA and compared to the p175 control transfection to ensure that the transfections were working with comparable efficiency and that the Vmw175 polypeptide was present in the extract. These results, together with a summary of the binding results in Figure 4, are presented in Table 1, which also summarizes the map

Table 1. A Summary of the Positions and Activities of the Insertion Mutants.

Mutant	Insert Site[a]	Transact. activity[b] x (SE)	Repress. activity[c]	DNA binding activity[d]	Relative amount of Vmw175[e]
pI1	12	35.1 (11.7)	+++	+++	288
pI2	71	53.4 (13)	++	++	203
pI3	83	49.6 (6.7)	++	+++	88
pI4	123	65.6 (12.8)	++	+++	241
pI5	130	43.8 (6.5)	+++	+++	106
pI6	137	54.4 (11.8)	++	+++	103
pI7	161	44.5 (10.5)	+++	+++	297
pI8	229	45.7 (8)	+++	+++	127
pI9	252	127 (43.2)	+++	+++	263
pI10	275	25.8 (4.6)	-	++	142
pI11	292	30.8 (6.3)	++	+++	276
pI12	310	20.0 (4.4)	+	+/-	97
pI13	324	10.0 (2.9)	-	+++	226
pI14	329	4.9 (0.9)	-	-	38
pI15	337	4.5 (0.9)	+	-	18
pI16	373	16.5 (2.8)	-	-	18
pI17	398	11.8 (1.9)	-	+	39
pI18	438	15.5 (4.4)	-	+	65
pI19	494	49.2 (10.4)	+++	+++	170
pI20	518	47.2 (10.2)	++	+++	85
pI21	561	43.4 (4.2)	+++	+++	294
pI22	591	26.2 (6.2)	++	+/-	206
pI23	649	23.9 (5.6)	++	++	297
pI24	681	21.1 (4.2)	++	+/-	91
pI25	774	38.2 (6.1)	+++	+++	291
pI26	787	80.9 (18.5)	+++	+++	70
pI27	803	45.9 (9.6)	++	+	126
pI28	843	10.8 (3.9)	+++	+/-[f]	15[f]
pI29	934	3.4 (0.5)	+	-[f]	0[f]
pI30	943	48.2 (12.3)	++	+++	58
pI31	1021	22.4 (3.7)	+++	+++	100
pI32	1066	10.3 (3.0)	+++	+	91
pI33	1098	30.0 (8.9)	++	+	15
pI34	1133	20.8 (2.3)	++	++	27
pI35	1139	48.2 (13.6)	++	+++	121
pI36	1195	86.9 (18)	+++	+++	218
pI37	1231	47.5 (10.3)	+++	+++	221
pI38	1236	63.1 (11.2)	+++	+++	245
pI39	1239	51.1 (8.6)	+++	+++	248

a The site (amino acid) of linker insertion.

b The activation of the gD promoter (using pgDCAT in conjunction with Vmw110 provided by p111) is given as a percentage of that obtained in parallel experiments with p175. The mean of at least four independent determinations is given, with the standard error of the mean. Wild type p175 activated pgDCAT expression 20-fold over uninduced levels (33).

c The qualitative ability to repress the IE3 promoter of pIE3CAT compared to p175 in cotransfection titration experiments. Repression activity is scored from - (essentially no activity) to +++ (wild type activity) (33).

d The ability of nuclear extracts made from HeLa cells transfected with each of the mutants to bind to the IE3 cap site. Binding activity is scored from visual inspection of Figure 4: - no binding, +/- barely detectable, + to +++ increasing binding activity.

e Relative amount of Vmw175 in each nuclear extract determined by ELISA. (p175=100)

f Further experiments with this mutant did produce detectable amounts of pI29 Vmw175 as described in the text and Figure 5.

positions and earlier transcriptional regulation results published for these plasmids (33).

Table 1 shows that mutants impaired in their ability to bind to the IE3 cap site in vitro broadly correspond to those impaired in their ability to transactivate and to repress transcription in transfection assays. For example insertion mutants pIl2 to pIl8 (with the exception of pIl3) are all significantly reduced in the transactivation and repression assays and also in their ability to bind to the IE3 cap site. This is especially true of mutants pIl4-pIl8. Indeed it is clear that this portion of the protein, (region 2; amino acids 315-484) is of critical importance for both transcriptional control and DNA binding. Only one mutant in region 2, pIl3, can efficiently bind to this DNA probe, but fails to express either repression or transactivation activity.

Insertion mutants throughout the majority of region 1 are virtually unimpaired in DNA binding activity or transcriptional regulation. However, two mutants, pIl0 and pIl2, situated at the end of region 1 close to the junction with region 2, are significantly reduced in their abilty to repress the IE3 promoter, and both are impaired in their ability to bind to the IE3 cap site, pIl2 markedly so.

However, DNA binding does not show an absolute correlation with either transactivation or repression. For example two insertion mutants in region 3, pI22 and pI24, are moderately efficient transcriptional regulators, but bind to the IE3 promoter very poorly in vitro. Similarly several mutants in region 4 have markedly reduced DNA binding activity, and whilst some of these have deficient transactivation activity (pI28, pI29 and pI32) all except pI29 are fairly efficient repressors of IE3 expression. These individual descrepancies in the correlation between DNA binding and transcriptional activity may reflect differences between binding in vitro and in the nucleus of the transfected cell.

One mutant, pI29, which failed to bind DNA in Figure 4 failed to produce detectable Vmw175 in these initial nuclear extracts. However, previous studies had detected low numbers of cells expressing nuclear Vmw175 by immunofluorescence of cells transfected with pI29. When the transfections were repeated

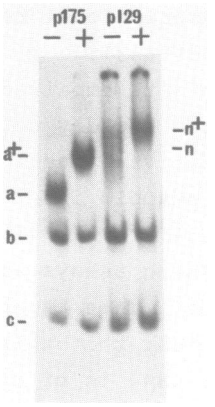

Figure 5: An abnormal complex is formed by total cell extracts of cells transfected with pI29. Binding is performed at 0°C with (+) or without (-) addition of antibody 58S using whole cell extracts of cells transfected with pl75 or pI29. Complexes a, a^+, b and c are as described in Figure 1, the novel complex, n, formed by pI29 transfected extracts is shifted to n^+ by addition of antibody.

using BBS instead of HBS, detectable amounts of Vmwl75 could be recovered and shown to bind to the IE3 cap site probe when incubated at 0°C (Figure 5). However, the complex formed was of reduced mobility both in the presence and absence of antibody. This may indicate that the I29 mutation induces incorrect processing or folding of Vmwl75. Total cell extracts of cells transfected with pI29 had greater amounts of Vmwl75 binding activity than nuclear extracts which may indicate that the polypeptide encoded by pI29 is deficient in nuclear localization.

It is also possible that the protein expressed by pI29 is unstable either in vivo or in vitro. The clustering of insertion sites which result in plasmids producing reduced amounts of Vmwl75 in nuclear extracts (Table 1) may indicate that certain regions are important for protein stablity, for example the regions of the protein defined by insertions I14-18, I28-30 and I33-34. Interestingly, mutations in the main DNA binding domain (I14-18) may result in a less stable protein. Apparently low levels of protein expression do not of themselves explain poor DNA binding since some extracts containing low levels of ELISA-detectable protein are capable of normal DNA binding, for

Table 2. A Summary of the Extent and Activities of the Deletion Mutants.

Mutant	Deleted amino acids	Frame[a]	IIF[b]	Transact.[c] act. x (SE)	Repress. act.[d]	DNA binding act.[e]	Relative amount of Vmw175[f]
pD13	12-229	IN	n.d.[g]	n.d.	n.d.	weak	25
pD14	72-292	IN	n.d.	n.d.	n.d.	-	14
pD15	84-292	IN	n.d.	n.d.	n.d.	-	15
pD1	162-229	IN	nuc.	30 (4.8)	+++	+	216
pD2	495-803	OUT	-	23 (4.0)	-	-	n.d.
pD2F	496-803	IN	cyt.	5 (0.5)	+	weak	52
pD3	518-591	IN	nuc.	4 (0.5)	+++	-	107
pD4	518-1133	IN	cyt.	18 (3.3)	++	-	27
pD5	562	OUT	-	3 (1.3)	++	-	n.d.
pD6	592-774	IN	cyt.	29 (2.8)	+	weak	25
pD7	650-1066	OUT	-	27 (5.4)	-	-	n.d.
pD7F	650-1066	IN	cyt.	10 (1.5)	+	-	88
pD8	682-774	IN	cyt.	57 (5.6)	-	weak	95
pD9	934-1098	IN	nuc.	4 (0.6)	+++	+	13
pD11	1196-1239	IN	nuc.	6 (2.2)	+++	+	59
pD12	1232-1269	IN	nuc.	7 (2.2)	++	+	52

a Phase of reading frame 3' to deletion
b Cellular localization of protein as determined by immunofluorescence (33). nuc. nuclear, cyt. cytoplasmic, - not detectable by immunofluorecence
c The activation of the gD promoter as described in Table 1 (and ref. 33).
d The qualitative ability to repress the IE3 promoter as described for Table 1 (and ref. 33).
e The ability of whole cell extracts made from cells transfected with each of the mutants to bind to the IE3 cap site probe in gel retardation assays (at 0°C). Binding is scored simply as positive (+), negative (-) or weak.
f Relative amount of Vmw175 in each nuclear extract determined by ELISA. (p175=100, the results are given for the extracts used in Figure 6, or, in the case of the non-binding mutants, for extracts containing the most Vmw175; out of frame proteins could not be detected by ELISA.)
g n.d. not done

example pI34 and the deletion mutant pD9 (Table 2). It is also possible that the low ELISA measurements could be explained by failure to efficiently extract or detect mutant polypeptides if, for example, insertions effect protein solubility or expression of the 58S epitope.

2. Analysis of deletion mutants. We have previously described the construction of a number of in frame deletion mutants within the Vmw175 coding region using the inserted EcoRI sites (33).

These mutants were assayed for their ability to bind to the IE3 DNA probe at 0°C in order to increase the sensitivity of the assay. These results are presented in Table 2 with the previously published results from transcription and immunofluorescence assays and the relative amount of ELISA detectable protein in each extract. Transfections were repeated at least three times for those extracts which failed to bind DNA and the highest ELISA measurement recorded.

DeLuca and Schaffer (35) have previously reported that a truncated polypeptide, encoding only the first 590 amino acids of Vmw175, is able to bind to DNA under some conditions. The results presented in Table 2 and Figure 6 confirm their results and allow us to map more finely the regions of Vmw175 unimportant for DNA binding.

Plasmids which expressed variants of Vmw175 with some DNA binding activity all have deletions which do not include amino acids 229-495, (Table 2, Figure 6). Whilst not all plasmids with deletions lying totally outwith this region express binding competent proteins (for example pD3, pD5 and pD7F) individual deletions which do not completely abolish DNA binding cover most of the rest of the gene (deletions 1, 2F, 6, 8, 9, 11, 12 and 13; Table 2). Because large deletions of the protein may cause many unpredictable changes in protein stability and conformation, perhaps only those deletions which do not totally abolish DNA binding activity should be considered significant results. The results with the deletion mutants, therefore, confirm the conclusion from the insertion mutant assays that the main DNA binding domain of Vmw175 centres around region 2.

The correlation between the DNA binding, transactivation and repression activities of the deletion mutants is less clear than with the insertion mutants (Table 1). However, the results with the deletion mutants do not separate any protein regions uniquely involved in each activity. It is perhaps suprizing that there are mutants which, although unable to bind to DNA in our assay, are able to activate repression. It is possible that such mutants retain some DNA binding capability in the cell. These mutants are severely impaired in their ability to transactivate, and therefore demonstrate overlap between the protein domains

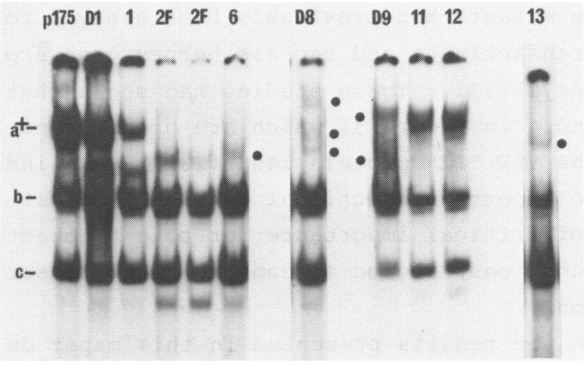

Figure 6: Binding assays performed at 0°C with antibody shift, using total cell extracts of cells transfected with deletion mutation plasmids pDl-15 (see Table 2). Only mutants with some detectable binding activity are shown. The wild type (pl75) shifted complex (a^+) is labelled, as are the host derived complexes b and c; weak complexes are highlighted.

required for at least two of the three functions assayed.

Interestingly, deletions in regions 4 and 5 (pD9, Dll and Dl2) give rise to somewhat larger DNA complexes than that formed by wild type Vmwl75, although these deletion plasmids are predicted to express proteins of a lower molecular weight. As suggested above for the insertion mutant pI29 this might be caused by a partial denaturation of these mutant polypeptides *in vivo* or *in vitro*. Some proteins deficient for nuclear localization (pD2F, D6 and D8) were able to bind to the IE3 probe, albeit very weakly, when extracts were prepared from whole cells (Figure 6); nuclear extracts of these deletion mutants did not contain significant amounts of Vmwl75 (results not shown). It is also noteworthy that deletions pDl and pDl3 which remove a serine rich phosphorylation site in region 1 are also able to bind DNA.

DISCUSSION

In order to investigate the relationship between the functional activities of Vmwl75 and its site-specific DNA binding property a panel of 39 in-frame insertion and sixteen deletion mutants were assayed for their ability to bind a DNA probe *in*

vitro. These mutants had previously been assayed for their ability to transactivate and repress herpes gene expression in transient assays (33). These studies had shown that two large regions, 2 and 4 in Figure 3, which are highly conserved between Vmw175 and the VZV "IE" protein 140K (28,29), do indeed correspond to important functional regions. Whilst region 2 seems to be of critical importance for both transactivation and repression functions, region 4 seems to be of lesser importance for repression.

Although the results presented in this paper do not demonstrate an absolute correlation between in vitro DNA binding activity and either transactivation or repression, it is clear that the general regions of the protein involved in all three phenotypes are similar. In particular the integrity of region 2 is of critical importance for each property investigated, excepting nuclear localization.

Previous studies have attempted to map the regions of Vmw175 involved in DNA binding by a variety of approaches. Kristie and Roizman (21) have reported that binding of monoclonal antibody H950 to Vmw175 abolishes DNA-binding activity. The epitope for this antibody has been mapped to within amino acids 21-36 (45) and therefore lies within a section of region 1 shown not to be required for DNA binding both by our results (Tables 1 and 2) and also by DeLuca and Schaffer (35). Therefore it is likely that the antibody is blocking DNA binding through longer range steric affects.

Hubenthal-Voss et al., (45) have also attempted to map the DNA binding domain of Vmw175 by using short synthetic oligopeptides to interfere or compete with functional sites for binding the recognition sites in the IE1 (α0) promoter. Their results, which mapped regions important for DNA binding activity near to the amino terminus, are at variance with our results. Indeed we show that the region they investigated seems to be of little importance, at least in binding the IE3 (α4) recognition site.

DeLuca and Schaffer (35) have used a set of viral mutants expressing truncated forms of Vmw175 to map the DNA binding

domain. The results obtained with their panel of deletion and nonsense mutants allowed them to present a map of the functional domains of the protein which is in good agreement with our own results presented here and previously (33). Mutants deleting the first 90 amino acids of the protein and those removing residues downstream from 773 were essentially wild type in their ability to bind an IE3 promoter DNA probe, and in their capacity to regulate IE3 and early gene transcription, although viruses encoding the downstream deletions were somewhat impaired in late gene expression. A mutant deleted from amino acid 590 was able to form a novel DNA complex at elevated salt concentrations, but possesed no regulatory actvity in the virus.

However, this approach lacks sensitivity unless applied from both the amino and carboxyl termini, and truncated proteins may exhibit conformational abnormalities making interpretation of results more complicated. Indeed from the results of our assays it is apparent that the insertion mutants give a more consistent pattern of results, and perhaps with the larger deletions only positive results should be considered significant. Our deletions pD13, pD1 and pD2F show that amino acids outside residues 229-496 are probably not directly involved in site-specific DNA binding. Those mutant polypeptides with insertions and deletions outwith this region which fail to bind DNA may be inactivated by longer range steric effects, for example disruption of protein folding.

Under the conditions of binding used by DeLuca and Schaffer (35) the Vmw175 DNA complexes were salt labile when the concentration was raised from 15 to 65mM NaCl. Furthermore they observed that some of their mutants formed novel complexes at elevated salt concentrations. We did not observe this salt lability under our conditions of binding and indeed the wild type Vmw175 DNA complex was stable up to 300mM NaCl (results not shown). Nor did we see any novel complexes formed at higher salt concentrations although these experiments were not performed on each mutant. We confirm their observations that deletion of the nuclear localization site, centred around amino acid 728 (33,35), did not prevent DNA binding of mutant protein recovered from the cytoplasm.

Michael et al (23) have recently reported that different post-translationally modified forms of Vmw175 possess different binding affinities for DNA probes which do not include an ATCGTC consensus. DeLuca and Schaffer (35) have shown that the serine rich tract in homology region A, which is conserved between VZV and HSV, is probably the major site of phosphorylation for this protein. However, we have shown that deletion of this region does not abolish affinity for the IE3 probe which includes the ATCGTC consensus, nor is this region essential for transactivation or repression (33). Interestingly, it has recently been reported that the serine phosphorylations of the adenovirus Ela protein do not affect transactivation or repression (46).

Because the regions of Vmw175 required for transcriptional activation and repression overlap with each other and with the region required for site-specific DNA binding we have not been able to directly associate site-specific binding uniquely with either regulatory activity. The weight of experimental evidence supports a role for the Vmw175 binding site at the cap site of IE gene 3 in autoregulation but it is still an enigma as to why the same protein sequences should apparently mediate this function and trancriptional activation which is not sequence specific. It is possible that Vmw175 possesses a second less specific DNA binding activity, perhaps encoded by the same protein sequences or alternatively elsewhere in the protein. Indeed Michael et al (23) have observed Vmw175 binding to the 5' transcribed non-coding regions of late genes which do not possess the consensus site-specific binding site. It will be interesting to investigate which regions of the protein are required for this activity, and whether these coincide with the regions (4 and 5) identified by DeLuca and Schaffer as being involved in late gene activation (35,47).

The precise functional role of regions 4 and 5 of Vmw175 is still obscure. We have previously shown that insertions and deletions in these regions interfere with transactivation and repression in a complicated manner (33) although clearly some mutants with large deletions in this region still encode proteins functional in transfection assays (34,48) and in recombinant

virus (35). Whether this portion of the coding sequence is simply important for conformational stability of the protein, or whether it directs a novel, uncharacterized activity is unknown. However, the strong conservation of region 4 between VZV and HSV, and the occurence of many temperature sensitive mutations in this region (13,47,49, unpublished results) suggests that this region does perform an essential role in the virus life-cycle.

Taking into consideration the known properties of Vmw175 it is possible to propose a simple functional model that could account for its dual roles of transcriptional activator and repressor. The model proposes that Vmw175 interacts simultaneously both with DNA and with host trancription factors, such as the TATA box binding factor TFIID (50,51), in order to produce a functional stabilized transcription complex. If the DNA binding region of Vmw175 encounters a very strong binding site sequence, such as that at the IE3 cap site, then the high affinity constant of the DNA/Vmw175 complex could effectively immobilize the transcriptional machinery. If the DNA sequence is less strongly bound by Vmw175, then the stabilized transcription complex would be free to proceed. Note that the proposed site of interaction with the host proteins may also map in region 2 which, at over 200 amino acids, is of itself almost as large as the adenovirus E1a protein. Thus the same region of the protein could be important for transactivation, repression and DNA binding. This model is consistent with the importance of the TATA/cap region for activation of the tk promoter (26) and the binding of Vmw175 to (non-consensus and possibly weak) sites in the 5' untranslated regions of other activated HSV genes (23). This model can be tested experimentally.

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