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The major transcriptional regulatory protein of herpes simplex virus type 1 includes a protease resistant DNA binding domain

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ABSTRACT
Herpes simplex virus type 1 expresses five immediate-early (IE) polypeptides. In the absence of functional Vmw175 (the product of IE gene 3) activation of transcription of later classes of viral genes and repression of IE gene expression does not occur. The recognition of specific DNA sequences by Vmw175 requires, as determined by sensitivity to mutation, a part of the protein highly conserved in the corresponding proteins of related herpes viruses. However, mutations in other parts of the protein can also disrupt specific DNA binding. This paper shows that the DNA binding domain of Vmw175 can be liberated as a functional unit by digestion with proteinase K. Analysis of mutant Vmw175 proteins showed that the proteinase K resistant domain has an amino terminus between amino acid residues 229 and 292, while its carboxy terminus is between residues 495 and 518. Mutations outside this region which affect DNA binding by the intact protein do not eliminate binding of the proteinase K resistant domain. This implies that direct DNA binding by Vmw175 involves a linear subsection of the polypeptide, and that mutations in other parts of the polypeptide which affect DNA binding of the whole protein do so by indirect means.

INTRODUCTION
Herpes simplex virus type 1 (HSV-1) is a common human pathogen with a double stranded DNA genome of 150kb, the complete sequence of which is known (1). The genome includes at least 70 distinct open reading frames, which are expressed from transcripts that can be divided into three main temporal classes, immediate-early (IE), early and late (for reviews see references 2 and 3). Four of the five IE polypeptides have some role in subsequent viral gene regulation, but perhaps the most important is the product of IE gene 3, Vmw175 (or ICP4). Viruses with temperature sensitive (ts) lesions in Vmw175 are generally inefficient or completely unable to activate transcription of later classes of genes, and are unable to repress IE gene expression at non permissive temperature (npt) (4—8). Viruses with ts and deletion mutations in Vmw175 fail to form plaques on normal tissue culture cells at npt. Thus Vmw175 plays a central and crucial role in the regulation of HSV-1 gene expression and as such has been the focus of extensive study.

Vmw175 is a phosphorylated nuclear protein (9) which can be poly(ADP)ribosylated in vitro (10) and which migrates as multiple species on SDS polyacrylamide gels with an approximate molecular weight of 175kd (11). It is translated from an unspliced IE-3 transcription unit in the short repeat regions of the viral genome (Figure 1) and the DNA sequence predicts a translation product of 1298 amino acid residues (12). The predicted primary sequence can be divided into five regions on the basis of similarity to the corresponding proteins expressed by other alphaherpesviruses (12—14). Regions 2 and 4, composed of residues 315 to 484 and residues 797 to 1224 respectively, are the most highly conserved and include the locations of several ts lesions (6,8,15). Mutagenesis of Vmw175 has revealed that regions 2 and 4 are more sensitive to disruption of Vmw175 function by mutation than other regions (16—22).

Vmw175 is a sequence specific DNA binding protein which recognises sites which include the consensus ATCGTC and also other sites that have been less well defined (23-26). The relationship of the DNA binding activity of Vmw175 to its functions is not completely clear. There is a strong binding site at the capsite of the IE-3 promoter which is necessary for the repression of this promoter by Vmw175 both in transfection assays (27) and in the viral genome (16). Few of the promoters that are activated by Vmw175 contain ATCGTC sequences in their vicinity (although the presence of other non-consensus sites can not be excluded). Where Vmw175 binding sites do occur, they can contribute to the activation of in vitro transcription by Vmw175 (24, 28, 29), but they are not necessary for activation by Vmw175 in transfection experiments (28), especially in the presence of the other HSV IE gene products (30,31). However, mutational analysis of Vmw175 clearly shows that mutations in region 2 which affect transcriptional activation and repression also affect the ability of Vmw175 to bind to its recognition

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sequences (8, 16, 32, 33). The implication is that DNA binding is, in some way as yet poorly understood, important for transcriptional activation by Vmw175.

In this study we show that a domain of Vmw175 capable of specific DNA binding can be separated from the whole protein by prior proteinase K digestion. We have defined the part of the protein involved by analysis of insertion, deletion and ts mutations; the results show that the distal end of region 1 and a substantial part or the whole of region 2 are involved in the formation of the DNA protein complex. In addition, we have shown that mutations in other parts of the polypeptide which affect binding of the whole protein do not affect the production or activity of the proteinase K resistant domain. This raises the interesting possibility that the properties of the DNA binding domain of Vmw175 might be influenced by other sequences in the whole protein.

MATERIALS AND METHODS
Plasmids and Bacteria
Plasmid p175 contains the HSV-1 IE-3 transcription unit linked to the SV40 early promoter and enhancer in a pBR322 vector. All insertion and deletion mutant plasmids were derived from p175 as described (17). Plasmids were propagated in Escherichia coli strain HB101 using standard methods.

Viruses and Cells
HSV-1 Glasgow strain 17 syn + was the wild type virus from which all ts mutants were isolated. Mutant tsK, with a single base substitution in IE gene 3, has been previously characterised (4, 7, 15, 34). Mutant viruses ts1211, ts1219, ts1221, ts1223 and ts1225 have been described (8). All viruses were propagated in BHK C13 cells grown in Glasgow modified Eagles medium (GMEM) supplemented with 10% newborn calf serum and 10% tryptose phosphate broth. HeLa cells, supplied by Flow Laboratories from the ATCC collection and grown in GMEM supplemented with 10% foetal calf serum, were infected with 5pfu per cell of wild type and mutant viruses at either permissive temperature or npt. Five hours after a 1 hour absorption period the cells were harvested for extract preparation.

Transfection of tissue culture cells and preparation of cell extracts
HeLa cells were seeded at 3 x 10^6 cells in 15ml medium into 90mm culture dishes. The following day 20ug of the relevant plasmid DNA was transfected into the cells by the method of Corsalvo and Pearson (36). Whole cell extracts were prepared two days later exactly as described before (32, 37).

Analysis of the DNA binding capability of Vmw175 by gel retardation
The ability of Vmw175 to bind to the IE gene 3 capsite region was assayed using the conditions and probe described by Muller (25) as described in detail before (32).

Treatment of cell extracts with proteinase K
Incubations of cell extracts for gel retardation assays were set up as described above and the appropriate amount of proteinase K added at times and temperatures as indicated in the text. When proteinase K treatment was carried out prior to the binding reaction, PMSF was added to 0.2mM to inhibit the protease before addition of the probe.

Estimation of the amount of Vmw175 in cell extracts
The amount of Vmw175 in cell extracts was estimated by an ELISA technique using the monoclonal antibody 58S, which recognises an epitope near the carboxy terminus of Vmw175, as described before (17, 38).

RESULTS
A proteinase K resistant domain of Vmw175 binds to the IE-3 capsite
Vmw175 in nuclear extracts binds specifically to DNA sequences which contain the consensus ATCGTC (23, 39), including that at the capsite of the IE-3 promoter (25, 32). Mutational analyses of the portions of Vmw175 that are important for this binding have strongly suggested that conserved region 2 (12) is part of the DNA binding domain (16, 32, 33) but the involvement of other portions of the protein could not be excluded (8, 32). Work in other fields has demonstrated the usefulness of proteinase K in trimming DNA-bound proteins to leave a resistant core (40, 41). We have used this method to define the region of Vmw175 which is closely in association with DNA.

Extracts from mock infected or cells infected with HSV-1 were prepared for use in gel retardation assays. In a normal binding experiment the IE-3 capsite probe gives two complexes with mock infected extracts (b and c) and a further strong complex (a') with extracts containing Vmw175. The presence of Vmw175 can be shown by the further retardation of complex a' by subsequent addition of monoclonal antibody 58S (Figure 2a). To investigate the effect of proteinase K on these protein-DNA complexes increasing amounts of protease were added after complex formation and the results analysed on retardation gels. Figure 2b shows that both cell complexes b and c are susceptible to digestion, giving bands of increased mobility. The infected cell extracts gave a further strong complex, p, which was present only when extracts which contained Vmw175 were used. The minor band appearing just above p in Figure 2b tracks 5-8 is probably an intermediate digestion product of Vmw175. Rather surprisingly, complex p could also be formed by incubation of
the cell extract with proteinase K before addition of the probe (Figure 2c). Prior treatment with proteinase K eliminated the bands derived from the cellular proteins, indicating that the proteins that form them are partially protected from protease only when bound to DNA. In most subsequent experiments prior treatment with 20ng of proteinase K was used since this gave the highest yield of complex p in this experiment (Figure 2c).

Attempts to identify which parts of Vmw175 might remain in complex p by incubation (after inhibiting the proteinase K with PMSF) with anti-Vmw175 antisera were not successful (42; results not shown). However, using a panel of point mutations in the Vmw175 binding sequence located upstream of the glycoprotein gD promoter, it was shown that mutations which eliminated binding of intact Vmw175 also severely reduced binding by the proteolytic fragment (D. Tedder, R.D. Everett and L.I. Pizer, manuscript in preparation). This confirms that complex p includes the DNA binding domain of Vmw175.

Identification of region 2 of Vmw175 as the DNA binding domain

Cells which have been transfected with plasmids which express Vmw175 contain sufficient amounts of the protein for use in gel retardation assays (32). The availability of a large number of insertion and deletion mutants in the Vmw175 coding region in such plasmids allows an investigation of which parts of the polypeptide are present in complex p. The insertion, deletion and ts mutants that were used in this study are illustrated in Figure 3 and Table 1.

Previous studies have indicated that region 2 of Vmw175 is most likely to be the DNA binding domain (16,32,33) and therefore it was considered most likely that this part of the protein was contained in complex p. When insertion mutants p18 through to p20 were used in binding assays without proteinase K (Figure 4a), normal amounts of the full size complex ‘a’ was obtained with p18, p19, p13, p17, p19 and p20. Plasmids p11 and p18 gave reduced amounts of the normal complex while p10 gave a smear. As previously described (32) plasmids p14, p15 and p16 were negative. In the previous study plasmid p12 was weakly positive (in the presence of antibody which may stabilise the complex) whereas here, despite ELISA detectable Vmw175 in the extract, p12 was negative.

After prior proteinase K treatment, complex p was obtained with some but not all of the insertion mutants. Plasmids p18, p19, p10 and p20 gave normal amounts of complex p while plasmids p11, p13 and p19 gave reduced amounts. The simplest interpretation of the failure to form complex p by p112, p14, p15 and p16 is that the insertions are in critical parts of the DNA binding domain. The protein expressed by plasmid p17 is unable to form large amounts of complex p even though the intact protein binds to DNA quite efficiently (compare Figure 4a and 4b tracks 11). This suggests that the insertion at residue 398 is in the complex p region and either it disrupts the binding of the proteolytic fragment directly or changes the protein structure such that the protease is able to remove residues essential for DNA binding. On the basis of its poor binding, it is likely that the insertion in p18 is also in the complex p region.

Note that protease K treatment frequently (but not always) resulted in apparently more complex p than the complex ‘a’ formed by the intact protein. Because of its smaller size, complex p is probably more stable in the separation gel than the full size complex (which often forms a smear, particularly with some of the mutants, as the protein dissociates during the run). Alternatively, partially degraded Vmw175 in the extract (which might bind but would form a smear) might be reduced to the normal complex p size by proteinase K and therefore yield a unique sized complex.

In order to confirm and extend the above results the analysis was continued using a selection of deletion mutants in the Vmw175 coding region (see Figure 3 and Table 1). Deletions pD1 and pD13 gave normal amounts of complex p, thus

Figure 2. A proteolytic fragment of Vmw175 binds specifically to the IE gene 3 capsid region. A. Extracts from mock infected (MI) and HSV-1 infected (17+) cells were prepared and incubated with the IE gene 3 probe. Free probe, host complexes b and c and the Vmw175 specific complex a' are marked. Complex a' is formed by the further retardation of complex a by the Vmw175 specific monoclonal antibody 58S. B. Binding reactions containing the IE-3 probe and mock infected (tracks 1-4) and HSV-1 infected (tracks 5-8) extracts were further incubated at room temperature for 10 minutes with proteinase K after the initial DNA binding reaction. Tracks 1-4 and 5-8 were incubated with 0, 1, 10, and 20 ng of proteinase respectively. Complexes b, c and a' are the products of protease digestion of host complexes b and c, while complex p is derived from digestion of the Vmw175 specific complex a’. C. An HSV-1 infected extract was incubated with tracks 1-4) 20, 10, 1 and 0 ng proteinase K and then incubated with the IE-3 probe. In track 5, proteinase K (20ng) was present during the binding reaction. The complexes are labelled as in B.

Figure 3. Insertion and deletion mutations in Vmw175. The 1298 codon coding region of Vmw175 is represented as a horizontal line with the positions of the insertion mutations used in this study shown as short vertical lines. A number of the insertion mutations are identified. The extents of the deletion mutations are also indicated. The precise details of these mutations are given in Table 1. Conserved regions 2 (codons 315-484) and 4 (codons 797-1224) are also indicated.
TABLE 1. The locations of the insertion deletion and temperature sensitive mutations in Vmwl175 used in this study.

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<th>MUTATION</th>
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<th>LOCATION</th>
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<th>BINDING</th>
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The mutations in Vmwl175 used in this study are shown. The location of the insertion mutants indicates the residue affected or the site of insertion between two residues. The original sequence at these locations, and those of the ts mutations, is shown to the left of a slash with the new sequence to the right. The co-ordinates of the deletions indicate the residues that have been deleted; those replaced are given in the last column. The details of the construction and characterisation of these mutations are given in references 8 and 17. The column labelled binding summarises the DNA binding results obtained previously with intact insertion or deletion polypeptides (32). Binding was estimated by visual inspection. For the insertions, - indicates no binding, +/- barely detectable, + to +++ increasing binding activity to wild type levels. For the deletions, binding is indicated simply a positive, negative or weak.

The amount of Vmwl175 in all the extracts used in these experiments was determined using an ELISA assay with monoclonal antibody 58S. The results confirmed that all the extracts contained Vmwl175, but in variable quantities. Extracts which gave neither complex p nor a complex with the full size protein contained at least as much ELISA-detectable Vmwl175 as others which were positive in gel retardation assays (data not shown). Unfortunately, due to the intrinsic variability of
transfections and uncertainty of the precise consequences of the mutations on Vmw175 stability or solubility, it is not possible to obtain exactly equivalent extracts using all the mutant proteins presented in this study.

**Mutations in regions of Vmw175 outside the DNA binding domain can affect the ability of Vmw175 to bind to DNA**

In a previous analysis of the effects of mutations in Vmw175 on its ability to bind to the IE-3 capsite probe, we found that certain mutations, distant from the DNA binding region defined above, could also affect the efficiency of Vmw175 DNA binding. Many of these mutations are in conserved region 4 (8,32). Some of these mutants gave poor, smeared complex formation. We have reanalysed these mutants by performing the DNA binding reactions after proteinase K digestion. The results (Figure 6) show that all the insertion mutants which bound poorly as intact proteins (pI22, pI24, pI27, pI28, pI29, pI32 and pI33; Table 1) gave amounts of complex p frequently comparable with that of the wild type protein. The simplest interpretation of these results is that mutations in regions 3 and 4 can affect the structure of the whole protein so as to compromise the function of the DNA binding domain. The alternative explanation is that the DNA binding domain involves two or more regions of the polypeptide that are distant from one another in the primary sequence. This is very unlikely given the results defining the limits of complex p.

The effect of changes in region 4 on the function of region 2 is also graphically illustrated by the DNA binding properties of Vmw175 proteins with ts lesions that map in region 4. The mutations in viruses ts1211, ts1219, ts1221, ts1223 and ts1225 (Table 1) map in the carboxy terminal third of Vmw175, and the lesions in ts1219, ts1221 and ts1225 have been localised in region 4 by DNA sequence analysis (8). Mutant tsK, with a single amino acid substitution at residue 475 in region 2 (15), was
included in these experiments for comparison. Extracts were prepared from cells five hours after infection at npt with HSV-1 strain 17 and the six ts viruses. A preliminary experiment confirmed previous results that all the Vmw175 proteins expressed from these viruses bound to the IE-3 capsite probe at room temperature (Figure 7a), but that only those expressed by strain 17 and ts1225 were able to bind at 39.5°C (8). After prior proteinase K treatment, all the extracts formed complex p after incubation at room temperature (Figure 7a), and all except tsK also gave complex p after incubation at npt (Figure 7b). These results show that the position of the lesion in tsK must be in that part of Vmw175 which is included in complex p and that the mutations in region 4, despite their effects on the DNA binding potential of the intact protein, are not in the minimal DNA binding domain. Thus single amino acid changes in a part of the protein distinct from the DNA binding domain can affect DNA binding.

**DISCUSSION**

The results presented in this paper clearly demonstrate that a portion of the Vmw175 polypeptide comprising the distal end of region I and all of conserved region 2 forms a domain of the protein that is separable from the whole by proteinase K digestion and which binds to a specific DNA sequence at the capsite of IE gene 3. The observation that deletions in the Vmw175 coding sequence which remove codons 12–229, 518–1133 and 1196–1269 do not affect the size of complex p shows that none of these regions form part of protease resistant domain. The affects of insertions pl12, pl14, pl15, pl16, pl17, pl18 and mutation tsK indicate (given the other data on the limits of complex p) that these mutations affect sequences important for DNA binding. These results are consistent with previous studies on the location of the DNA binding domain of Vmw175 (16,32,33). However, because it is not possible to estimate accurately the size of the protein retained in complex p, it is not clear if all of the amino acid residues between the estimated limits of complex are present. Furthermore, these data do not imply that the whole of the proteolytic fragment is important for DNA binding since its size is dependent on the location of the protease sensitive sites and is not affected by the presence of bound DNA. Indeed, insertion pl13 is within complex p but does not greatly affect its ability to bind to DNA. Further definition of the DNA binding domain of Vmw175 will require more extensive mutagenesis of the complex p region.

The basic conclusions of this study have recently been strongly supported by a study using fragments of Vmw175 that have been expressed in bacteria (43). Wu and Wilcox showed that a peptide including residues 262–490 was sufficient for DNA binding, that peptides from 262–464 and 306–490 did not bind to DNA and that the bacterially expressed polypeptide gave the same DNA binding footprint as the intact protein (43). Their mapping is entirely consistent with the data presented here. It should be noted that our results do not simply duplicate those using only a fragment of the protein since our results do not simply duplicate those using only a fragment of the protein.

**Figure 7.** The effect of ts mutations in Vmw175 on the formation of complex p at permissive and non-permissive temperature. A. Extracts from cells infected with HSV-1 strain 17 (tracks 1 and 9), tsK (tracks 2 and 10), ts1211 (tracks 3 and 11), ts1219 (tracks 4 and 12), ts1221 (tracks 5 and 13), ts1223 (tracks 6 and 14), ts1225 (tracks 7 and 15) and mock infected (track 8) were incubated with the IE-3 probe either in the absence (tracks 1–8) or the presence (tracks 9–15) of proteinase K. All incubations were performed at room temperature. B. Extracts from cells infected with strain 17, tsK, ts1211, ts1219, ts1221, ts1223, ts1225 or mock infected were treated with proteinase K at room temperature and then incubated (after addition of PMSF) with the IE-3 probe at 39.5°C. The lesion in tsK is the only one to affect complex formation. In this experiment the gel was run at 37°C, which might explain the rather broader than usual mobility of complex p. Such increased mobility occasionally occurred in other experiments but was not apparently caused by more extensive protease digestion.

binding data using mutants of Vmw175. Mutations in the intact protein may affect DNA binding by indirect conformational or stability effects, while studies using only a fragment of the protein may not be entirely relevant to the complete protein during virus infection. Although a detailed analysis of the complex p region may allow the definition of the amino acid residues that are involved in sequence specific DNA recognition, it will be necessary to study the effects of mutations in these sequences in the whole protein in order to determine their relevance during virus infection.

Perhaps the most potentially interesting aspect of this study is the concept that small changes in conserved region 4 can affect the function of conserved region 2. Although there may be several trivial or technical reasons for this (involving protein stability, solubility or gross conformational change), it is possible that protein-protein interactions between sequences in region 4 and other viral or cellular proteins could affect the conformation of region 4, which might then (in a similar way to the structural alterations induced by the insertion and ts mutations) affect region
2. Therefore DNA binding by Vmwl75 could be modulated by interactions with other transcription factors. Such interactions might affect the efficiency of promoter activation by Vmwl75, or even whether a promoter was activated or repressed.

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REFERENCES
