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Mutation of a single lysine residue severely impairs the DNA recognition and regulatory functions of the VZV gene 62 transactivator protein

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ABSTRACT

The product of varicella-zoster virus gene 62 (VZV 140k) is a potent transactivator protein. We have identified a region within the DNA binding domain of VZV 140k that shows a striking similarity to the DNA recognition helix of the homeodomain, with an especially highly conserved quartet of residues, WLQN. The 140k protein has functional counterparts within the other alphaherpesviruses, which include the major transcriptional regulatory protein of HSV-1, (ICP4), and the WLQN region is highly conserved among the members of this family of viral transactivators. Substitution of VZV 140k residue lysine 548, just adjacent to the WLQN region, drastically reduces the DNA binding activity of the 140k DNA binding domain and the intact 140k mutant protein fails to activate gene expression. Substitutions of two other VZV 140k residues in this conserved WLQN region result in alterations to the DNA binding interaction and reduced transactivation activities. All three mutations act at the level of DNA recognition, as they have no apparent effect on the dimerization state, solubility or efficiency of expression of the mutant peptides.

INTRODUCTION

Varicella-zoster virus (VZV) is a neurotropic alphaherpesvirus and is the causative agent of chickenpox and shingles in humans. The other members of the alphaherpesvirus family include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), pseudorabies virus (PRV) and equine herpesvirus type 1 (EHV-1). The complete genomic nucleotide sequences of HSV-1, VZV and EHV-1 have been derived (1–3) and they exhibit similar genomic organisation and many of the gene products have conserved amino acid sequences and similar locations within the genome. Much of our current understanding of alphaherpesvirus gene regulation has been derived from the intensively studied HSV-1 prototype system. The 75 HSV-1 genes are regulated in a complex temporal cascade and the genes are classified as immediate-early (IE), early (E) or late (L) (for reviews, see 4,5). The transcription of the five IE genes occurs without prior viral protein synthesis and is induced by VP16, a component of the infecting virus particle (6). At least three of the HSV-1 IE gene products encode important transactivators that are essential for progression to the E and L phases of the transcriptional cascade (7). The product of the IE3 gene, ICP4, is considered to be the major transcriptional regulatory protein of HSV-1 because ICP4 is absolutely required for transactivation of E and L gene expression (8,9) and also for autoregulation of its own HSV-1 IE3 promoter (10–12).

Protein homologues of HSV-1 ICP4 have been identified in the other alphaherpesviruses on the basis of predicted amino acid sequence homology. These include the 140k protein of VZV (2), the IE1 protein of EHV-1 (13) and the IE180 protein of PRV (14). The product of VZV gene 62, 140k, is a large protein containing 1,310 amino acid residues, having a predicted molecular mass of 140,000D. VZV 140k can functionally substitute for HSV-1 ICP4 (15,16), indicating that 140k is likely to play an essential role in the VZV life cycle, equivalent to that played by HSV-1 ICP4. In transient transfection experiments, VZV 140k is a potent transactivator of VZV putative early and late genes and some heterologous genes (17–20). 140k has been reported to either autoregulate or transactivate its own gene 62 promoter in transient transfection assays, depending on the cell type used (21,22).

The primary sequence of the VZV 140k protein and its homologues in the other alphaherpesviruses have been divided conceptually into five colinear regions on the basis of the degree of amino acid similarity between them (23,13,14). The HSV-1 ICP4 protein binds directly to DNA (24) and its DNA binding activity is abolished by small (2–4 amino acid) insertion mutations at positions throughout the highly conserved region 2 and the C-terminal end of the less well conserved region 1 of the protein (25,26). Subsequently, it was found that peptides spanning these regions of HSV-1 ICP4, and the equivalent regions of the VZV 140k and PRV IE180 proteins, yield stable, physically separable (27) DNA binding domains, when expressed in isolation in bacteria (28–31). The ICP4 protein, and its isolated DNA binding domain, recognizes various DNA binding sites that

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conform (to a greater or lesser extent) (32) to the bipartite ICP4 consensus sequence ATCGTCnnnnYCGRC (33,28,34). In addition, the VZV 140k DNA binding domain can also bind to ICP4 consensus binding sites and many of the 140k binding sites recognised within its own VZV gene 62 promoter show some similarity to the ICP4 consensus, although the 140k domain is less sequence specific than that of ICP4 (31). The 140k DNA binding domain interacts with its DNA binding sites as a stable homodimer (35) and DNA binding is likely to be a requirement for the transactivation and repression functions of 140k, as an insertion of four amino acids into region 2 renders the protein functionally inactive (21).

Structural studies and sequence comparisons have found that many DNA binding proteins can be grouped into classes that use related structural motifs for DNA recognition (for reviews, see 36,37). Like several other important viral transactivator proteins, for instance SV40 large T antigen (38), the VZV 140k DNA binding domain does not contain sequences that fit neatly into any of the recognised classes of DNA binding motif. However, we have identified a short region of sequence showing significant homology to the DNA recognition helix (helix 3) of the homeodomain DNA binding motif. Homeodomains have been found in proteins of virtually all eukaryotes examined, ranging from yeast to man (39,40) where they play central roles in gene regulation. The homeodomain is structurally related to the prokaryotic helix-turn-helix (HTH) motif, and typically comprises 60 residues arranged in three α-helical segments, helices 2 and 3 comprise the HTH, and the third helix is the principal determinant for DNA recognition. Recently, numerous highly divergent classes of homeodomains / eukaryotic HTH proteins have been discovered (for reviews, see 40,41). Studies on non-classical homeodomains, such as the dimeric hepatocyte transcription factor HNF1 (or LFB1) (42) reveal their almost identical spatial arrangement of helices to those found in the corresponding HTHs of the more typical *Drosophila engrailed(en)* (43) and yeast MATα*2* (44) homeodomains, despite their very low level of sequence identity outside of the recognition helix.

The WLQN region of homology to the homeodomain recognition helix that we describe in this paper lies towards the centre of the 140k DNA binding domain (residues 537–553) and is highly conserved among the other alphaherpesvirus transactivator homologues. Residues both within and flanking the WLQN region, corresponding to those that make critical homeodomain-DNA contacts, are also well conserved. Although the presence of proline 'helix-breaking' residues in the WLQN region of 140k suggest that this sequence is unlikely to adopt a structure precisely equivalent to that of the homeodomain, we decided to analyse the possible functional implications of the conservation of the WLQN region by mutagenesis of the VZV 140k sequence. The results presented here provide evidence that residues within this WLQN homology region are directly involved in DNA recognition and are required for the transactivation function of the 140k protein.

MATERIALS AND METHODS

Bacteria and cells

Plasmids were constructed and maintained in *E. coli* strain DH5a using standard methods (45). For the bacterial expression experiments, plasmids were transformed into *E. coli* strain BL21 (DE3) pLysS (46). Bacteriophage M13 derivatives were transfected into *E. coli* strain TG1. The TG1 host bacterium was used for all subsequent growth and maintenance of M13 bacteriophage. HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100units/ml penicillin, 100μg/ml streptomycin, 2.5% (v/v) newborn calf serum and 2.5% (v/v) foetal calf serum. Baby hamster kidney cells (BHK-21 clone 13) (47) were grown in Glasgow MEM (Gibco) supplemented with 100units/ml penicillin, 100μg/ml streptomycin, 10% (v/v) tryptose phosphate and 10% (v/v) newborn calf serum.

Oligonucleotide directed mutagenesis

The oligonucleotides given in Figure 1B (incorporating novel restriction enzyme sites, *Hin*dIII, *Pvu*II and *Sac*I respectively) were created using a Cruachem synthesiser to enable substitution of single VZV 140k residues; Q545L, N546L and K548E respectively. Oligonucleotides were purified by gel electrophoresis, phosphorylated and used in mutagenesis reactions using the Amersham M13 mutagenesis system (Version 2). The template for mutagenesis was single stranded bacteriophage M13mp18EK, which contains the VZV gene 62 *Eco*RI-*Kpn*I fragment isolated from plasmid p585T7aVT2X (see below) cloned into the *Eco*RI-*Kpn*I sites of the multiple cloning region of the M13mp18 vector. After mutagenesis, the reactions were transfected into competent TG1 bacteria and plaques picked for screening. Positive bacteriophage M13Q545L, M13N546L and M13K548E were identified by restriction enzyme digestion at the introduced restriction sites and confirmed by direct plasmid sequencing of the mutated region.

Construction of plasmids expressing mutants of the 140k DNA binding domain and intact 140k peptides

Plasmid p585T7a was adapted from the pET-8c T7 expression vector (46) to facilitate insertion of DNA fragments and their subsequent in-frame expression as described (31). Plasmid p585T7aVT2X was a derivative of p585T7a and included VZV gene 62 nucleotide sequences encompassing codons 417 to 647 (31). Plasmid p585T7aVT2X expressed the 140k DNA binding domain peptide, referred to as VT2X (Fig. 2A), which had a predicted molecular mass of 26.7kD. Plasmids pVT2XQ545L, pVT2XN546L and pVT2XK548E were derived from plasmid p585T7aVT2X as described below and express versions of the VT2X peptide containing single amino acid substitutions as described by the name of each plasmid. *Eco*RI-*Kpn*I fragments carrying gene 62 sequences containing specific nucleotide changes incorporated by oligonucleotide-directed mutagenesis were isolated from replicative forms of the M13 bacteriophage M13Q545L, M13N546L and M13K548E. Each mutated *Eco*RI-*Kpn*I fragment was ligated into the *Eco*RI-*Kpn*I digested p585T7aVT2X plasmid; the digested vector fragment had previously been isolated away from the wild type *Eco*RI-*Kpn*I fragment using agarose gel electrophoresis and GENECLEANII (BIO 101 Inc., La Jolla) procedures. This cloning procedure produced plasmids pVT2XQ545L, pVT2XN546L and pVT2XK548E; direct plasmid sequencing was used to confirm the incorporation of the intended nucleotides and also the sequences spanning the *Eco*RI and *Kpn*I junctions.

Plasmid p140 contained the entire VZV gene 62 open reading frame and promoter region on the *Cla*I-*Pst*I fragment of plasmid pVZVSstf (17) in a pUC9 background as described (48). Nucleotide changes introduced by oligonucleotide-directed mutagenesis were incorporated into the intact gene 62 open

reading frame as follows: *Nar*I-*Kpn*I fragments isolated from plasmids pVT2XQ545L, pVT2XN546L and pVT2XK548E were each co-ligated with three fragments (*Kpn*I-*Pst*I fragment, 2414bp; *Pst*I-*Sph*I fragment, 4572bp; *Sph*I-*Nar*I fragment, 632bp) isolated from plasmid p140. The resultant positive plasmids p140Q545L, p140N546L and p140K548E were identified by extensive restriction enzyme digest analysis using the restriction sites introduced by the mutagenesis procedure.

Expression and partial purification of the VZV 140k DNA binding domain

E. coli strain BL21 (DE3) pLysS transformed with the T7 expression vectors were grown up, induced, harvested and crude 35% ammonium sulphate cut extracts prepared from 100ml cultures exactly as described (31). Samples of the lysed bacteria after induction and the final crude extract were analysed by 12.5% SDS PAGE followed by Coomassie blue staining. The relative amounts of the mutant and wild type VT2X peptides in the partially purified extracts were determined by scanning densitometry for each set of protein preparations; equivalent amounts of the peptides were included in the DNA binding analyses (below).

DNA fragment probes

The following DNA restriction fragment probes, radiolabelled at a single fragment end, were used for DNA binding analyses: The VZV gene 62 promoter probe fragment (31) encompassed sequences −130 to +57 of gene 62. The HSV-1 IE3 promoter probe (35) encompassed nucleotides −18 to +27 of the IE3 gene and includes the strong consensus ICP4 binding site spanning the mRNA start site (49). The HSV-1 gD promoter probe encompassed nucleotides −262 to +11 and was derived from a *Pvu*II-*Hin*dIII fragment of the HSV-1 glycoprotein D (gD) promoter as follows: plasmid pgDCAT (50) was cut with *Hin*dIII and the overhang filled in by Klenow enzyme in the presence of α^{32}P-dATP, followed by digestion with *Pvu*II. The labelled *Pvu*II-*Hin*dIII fragment which includes the ICP4 consensus binding site upstream of the gD mRNA start site (33) was eluted from a 5% polyacrylamide gel and purified by ethanol precipitation.

DNA binding analyses

Appropriate amounts of partially purified bacterial extracts (see legend to Fig. 3)were incubated on ice with 1ng of radiolabelled DNA fragment probes for 20min and the products resolved by native gel electrophoresis essentially as described (31). In these experiments 0.5μg of pUC9 was included in each incubation instead of the polydI.polydC non-specific competitor.

Determination of site specific protection of 4ng radiolabelled DNA fragments from partial DNase I digestion, by the partially purified bacterial extracts of wild type and mutant VT2X peptides (amounts as given in legend to Fig. 4), was exactly as described previously (31).

DNA transfection and CAT assay

HeLa cells were seeded at 10^6 cells per 50mm petri dish and were transfected by the calcium phosphate precipitation method 24hr later (51). The reporter plasmid pgDCAT contains the HSV-1 gD promoter / leader sequences fused to the chloramphenicol acetyl-transferase (CAT) open reading frame. Transfections used 4μg of the pgDCAT plasmid and variable amounts of the p140 derived effector plasmids; pUC9 was used to equalise the amount of DNA within experiments. After a further 24hr the cells were washed and re-fed with fresh medium; 24hr later total cell extracts were prepared and CAT activities were assayed as described by Gorman (52). The radioactivity in the substrate and product was quantitated using a PhosphorImager scanner with Image-Quant software (Molecular Dynamics). The protein concentration of HeLa cell extracts was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions, in order to calculate the percentage conversion from substrate to product per microgram protein.

Immunofluorescence

BHK cells were seeded at 5×10^5 cells per 50mm petri dish and 24hr later were transfected with 6μg of plasmid p140 or its derivatives using a liposome-mediated DNA transfection procedure (53) as described (54). 24hr after transfection, the cells were washed and re-fed. After a further 24hr the cells were fixed, permeabilised and stained for immunofluorescence essentially as described (55). The rabbit polyclonal antiserum 109 was used at a dilution of 1:500 in PBS as the primary antibody, then fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin was used to detect bound antibody before examination by UV fluorescent microscopy. Polyclonal 109 was raised against the bacterially expressed 140k DNA binding domain peptide VT2, which had been highly purified by column chromatography (35).

Computer analysis

Protein sequences were aligned using programmes from the Sequence Analysis Software Package (Genetics Computer Group, Inc., Madison, Wis.).

RESULTS

Identification of a region of sequence homology to the homeodomain recognition motif

The minimum defined DNA binding domains of the VZV 140k (31) and HSV-1 ICP4 (28,29) proteins are fairly large (162 and 216 residues respectively) compared to the sizes of the recognised classes of DNA binding domain structures so far determined (36,37). We have been interested in defining specific sequences within these large protein domains that mediate the DNA recognition function of the alphaherpesvirus regulatory proteins. A comparative analysis of the sequences of the VZV 140k homologue proteins with the various established classes of DNA binding motifs has identified a sequence (referred to as the WLQN region) showing significant similarity to the homeodomain DNA recognition helix. The WLQN region of homology is roughly in the centre of the defined DNA binding domain of the VZV 140k and HSV-1 ICP4 proteins and is highly conserved within the alphaherpesvirus family.

The numerous homeodomains that have been described fall into distinct groups, residues at several positions are significantly conserved throughout all the homeodomain groups, but the remaining residues show considerable divergence (39,40). In essence, the homeodomain is a structural fold carrying the DNA recognition helix. Figure 1A shows the recognition helix of the prototype Group 7 *Drosophila Antennapedia* (*Antp*) homeodomain aligned with the homology region identified within the VZV 140k alphaherpesvirus homologues. The asterisks in Figure 1A indicate positions that are invariant or nearly invariant within the homeodomains. These residues are also conserved or semi-

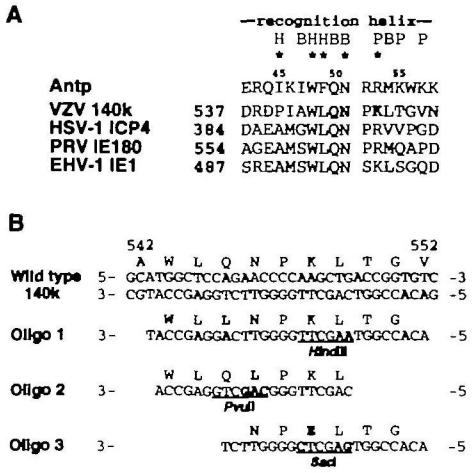

Figure 1. The WLQN region and its mutational analysis in the VZV 140k protein **A.** Alignment of the recognition helix of the *Drosophila Antp* homeodomain to the alphaherpesvirus homologues of VZV 140k. The top sequence shows the *Antp* recognition helix (homeodomain helix 3). *Antp* amino acids are numbered according to convention to indicate their position in the 60-residue homeodomain (40). Positions corresponding to the hydrophobic core (H), base contact (B) and phosphate contact (P) amino acids of the *Antp* (56) MATα2 (44) and *en* (43) homeodomain structures are indicated above the *Antp* sequence. Positions that are invariant or nearly invariant within all known homeodomains are indicated with asterisks. Alphaherpesvirus region 2 sequences are aligned beneath. The VZV 140k residues that were subject to mutation are shown in bold. **B.** Oligonucleotides used for oligonucleotide-directed mutagenesis of VZV 140k. At the top is shown the wild type protein sequence and the sequences of both gene 62 DNA strands which encompass the region of mutagenesis. Below are given the three oligonucleotides (corresponding to the gene 62 non-coding strand) and the relevant coding potentials. The nucleotides that differ from the wild type sequence and the resultant amino acid residue alterations are shown in bold. Restriction enzyme sites introduced by taking advantage of the degenerate nature of the genetic code are also indicated.

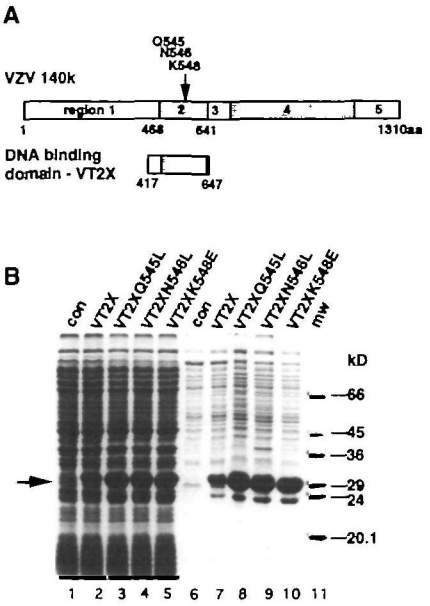

Figure 2. The location of the VZV 140k DNA binding domain and its expression in bacteria. **A.** Diagrammatic representations of VZV 140k and its DNA binding domain peptide, VT2X. The sequences of regions 2 and 4 are highly conserved (46% and 56% identity respectively) between VZV 140k and HSV-1 ICP4 (23) and are shown shaded, amino acid coordinates of the boundaries of the conserved regions are given (inclusively). Regions 1, 3 & 5 are less highly conserved between the two proteins (15–25% identity). The VT2X peptide contains VZV 140k residues 417 to 647 (31), encompassing the highly conserved region 2. The arrow indicates the position of the three residues whose substitution is discussed in the text. **B.** Coomassie-stained 12.5% SDS PAGE analysis of the partial purification of bacterially expressed wild type and mutant VT2X peptides. Expression of 140k DNA binding domain peptides was induced in *E. coli* from the appropriate T7 expression plasmids (Materials & Methods). Tracks 1–5 contain 2μl of the total soluble proteins from 100ml of each induced bacterial culture after resuspension in 1ml buffer, bacterial lysis and centrifugation (31). Tracks 6–10 contain 2μl of partially purified extracts after 35% ammonium sulphate precipitates were resuspended in 300μl buffer. 'con' is an extract prepared from induced bacteria carrying the p585T7a plasmid. The arrow shows the position of the 26.7kD VT2X peptide; a smaller proteolytic degradation product is apparent beneath. Track 11 contains molecular weight standards: BSA, 66kD; albumin, 45kD; glyceraldehyde-3-phosphate dehydrogenase, 36kD; carbonic anhydrase, 29kD; trypsinogen, 24kD; trypsin inhibitor, 20.1kD.

conserved within the alphaherpesvirus proteins. The structures determined for three homeodomain-DNA complexes (43,44,56) demonstrate that the invariant residues either mediate specific DNA contacts or maintain the hydrophobic core contacts that stabilise the homeodomain protein fold, as indicated in Figure 1A. However, unlike the homeodomain recognition helix, the WLQN region of 140k is unlikely to be folded into a single uninterrupted α-helix due to the presence of proline residues at amino acids 540 and 547, but could quite possibly adopt two helical turns between residues 540 and 547 and resume a helical conformation after residue 547.

Mutagenesis, expression and purification of the 140k DNA binding domain

We were interested to investigate whether this potentially interesting WLQN region might mediate DNA recognition by the VZV 140k family of proteins. We used *in vitro* mutagenesis to alter the charges on specific VZV 140k residues, while maintaining the size of the amino acid side chains to minimise conformational alterations in the protein. We mutated two single conserved residues within the WLQN motif itself: The 140k residue Gln 545 was replaced with Leu (Q545L) and residue Asn 546 was replaced with Leu (N546L). The corresponding residues of the homeodomain, residues 50 and invariant Asn 51 (Fig. 1A), both point into the major DNA groove and hydrogen bond with DNA bases, in the known homeodomain-DNA structures. In addition, genetic and biochemical analyses have shown the residue at homeodomain position 50 determines the differential binding specificity of the homeodomain-DNA interaction (57–59). For the third mutation, we chose to replace VZV 140k residue Lys 548 with Glu (K548E), due to the conservation of a basic residue at this position in the alphaherpesvirus proteins and also the homeodomains. The corresponding homeodomain residue Arg 53 forms critical hydrogen bonds with two phosphate groups of the DNA backbone to stabilise the protein-DNA interaction.

The substitutions were initially introduced into a peptide encompassing the VZV 140k DNA binding domain. This DNA binding domain peptide, referred to as VT2X, encompasses 140k residues 417 to 647 (Fig. 2A) and includes the highly conserved protein region 2 (codons 468 to 641). These 140k sequences yield a stable domain when expressed in isolation of other viral sequences in *E. coli* using the T7 expression system, and is capable of binding to DNA in a sequence specific manner (31). Partially purified extracts of the wild type and mutant VT2X peptides were produced as described (31). Figure 2B shows for each peptide, samples of initial soluble (tracks 1–5) and partially

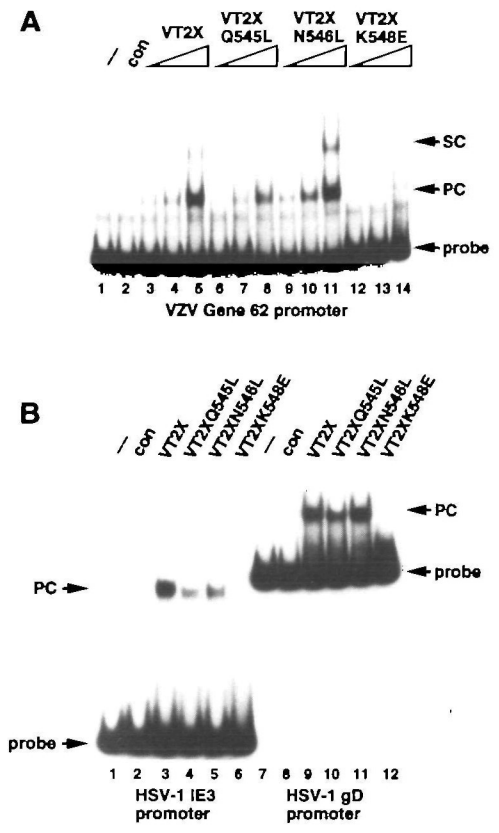

Figure 3. Gel retardation analyses of the DNA binding activities of the mutant 140k DNA binding domain peptides **A.** A radiolabelled DNA fragment derived from the VZV gene 62 promoter (Materials & Methods) was incubated with 25ng, 50ng or 100ng of the partially purified bacterial extracts of wild type or mutant VT2X peptide indicated above tracks 3–14. The incubation reaction for track 1 contained no extract and track 2 contained a control extract prepared from bacteria carrying the p585T7a expression vector. After incubation, the reactions were electrophoretically separated on a native 4% polyacrylamide gel and prepared for autoradiography (31). The positions of the free DNA probe, primary (PC) and secondary (SC) protein–DNA complexes are indicated on the right-hand side. **B.** Radiolabelled DNA fragments from the HSV-1 IE3 gene promoter (tracks 1–6) and the HSV-1 gD early gene promoter (tracks 7–12) were incubated with 40ng and 30ng (respectively) of the partially purified bacterial extracts of the VT2X derived peptides. The components of the reactions were resolved by electrophoresis as above.

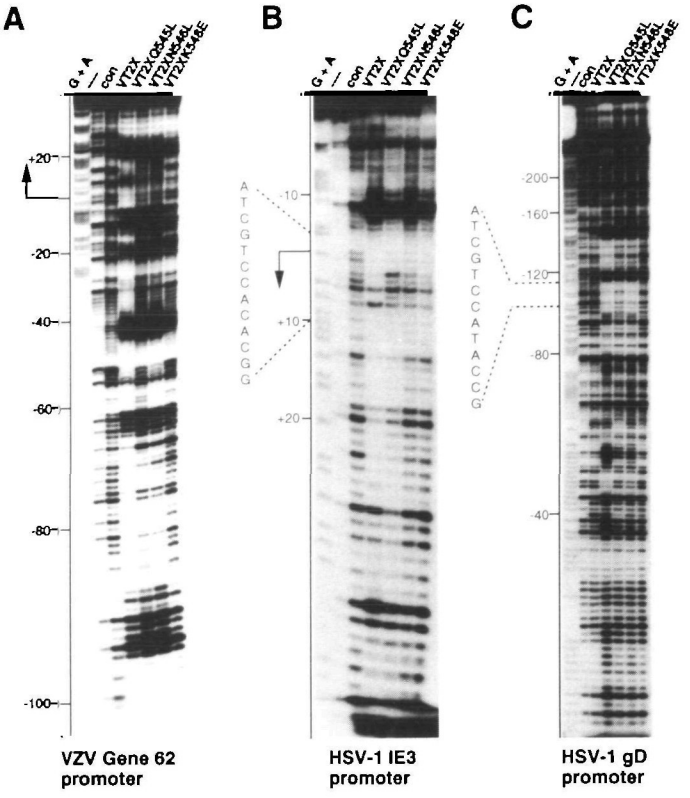

Figure 4. DNase I footprinting analyses of the DNA binding activities of the mutant VZV 140k DNA binding domain peptides. DNase I footprinting assays were performed with end-labelled gene promoter DNA fragments and partially purified bacterial extracts as described (31). The wild type and mutant VT2X tracks contained 1.6μg of the DNA binding domain peptide indicated above the tracks. Tracks labelled 'G+A', '----' and 'con' contain Maxam and Gilbert sequencing reactions, no extract and induced bacterial extract carrying the p585T7a vector respectively. The identity of the DNA probe fragment used for each experiment is given beneath the panel (details given in the Materials and Methods section). Radiolabelling of the VZV gene 62 promoter fragment was on the bottom DNA strand, the HSV-1 IE3 and gD promoter fragments were labelled on the top strand. The numbered scale on the left of each panel identifies the positions of nucleotides included within each DNA fragment; numbered with respect to the mRNA start site (+1). The bent arrows indicate proposed mRNA start sites. The positions and sequences of the ICP4 consensus binding site over the HSV-1 IE3 gene mRNA start site (49) and also the ICP4 consensus site 5′ of the HSV-1 gD mRNA start site (33) are indicated.

purified (tracks 6–10) bacterial extracts, following SDS PAGE analysis and Coomassie-staining. The mutations did not effect the efficiency of expression in bacteria, as all the peptides were induced to high levels (Fig. 2B, tracks 2–5). In addition, all the peptides were highly soluble indicating that the mutations did not induce conformational disruptions leading to protein precipitation.

Mutation of a lysine residue within the WLQN region severely impairs sequence specific DNA binding by the 140k DNA binding domain

The VZV 140k DNA binding domain binds various sequences within its own VZV gene 62 promoter (31) including a site over the mRNA start site that is thought to be involved in the autoregulation of the gene 62 promoter at later times during infection (30). Initially the mutant VT2X peptides were analyzed for their abilities to recognise the gene 62 promoter in gel retardation analyses (Fig. 3A). Incubation of an equivalent range of amounts of each peptide with the gene 62 promoter probe yielded primary protein–DNA complexes for wild type VT2X, VT2XQ545L and VT2XN546L (Fig. 3A, tracks 3–5, 6–8, 9–11 respectively) and secondary complexes were produced with higher amounts of these peptides. Other experiments using *in vitro* translated heterodimer polypeptides strongly suggest that the simplest interpretation is that the primary complex corresponds to a peptide dimer bound to DNA; additional experiments have found that the 140k DNA binding domain peptides exist totally in the dimeric form in solution and therefore the secondary complexes are assumed to contain a pair of dimers (35). The VT2X, VT2XQ545L and VT2XN546L peptides had similar DNA binding affinities as determined by competition

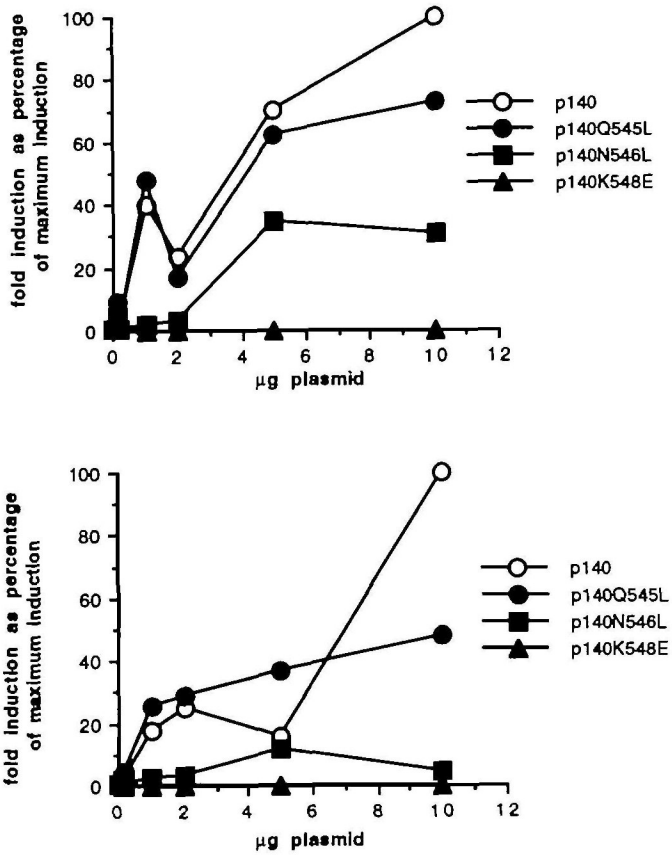

Figure 5. Transactivation efficiencies of the mutant VZV 140k proteins. The graphs show data obtained from independent experiments measuring activation of expression from HSV-1 gD promoter-leader sequences by the VZV 140k protein and its mutant derivatives. Increasing amounts of each effector plasmid were co-transfected with the reporter construct pgDCAT into HeLa cells, the symbols corresponding to the effector plasmids expressing the gene 62 sequences are as explained in the key. Fold induction promoter activity induced by each effector plasmid was calculated by dividing the percentage conversion from substrate to product per microgram protein obtained at a particular plasmid amount, by the control value obtained when no effector plasmid was present (the control value represented an average of three individual transfections). The fold induction for each transfection is expressed as a percentage of the maximum induction induced by 10µg of plasmid p140 (100%).

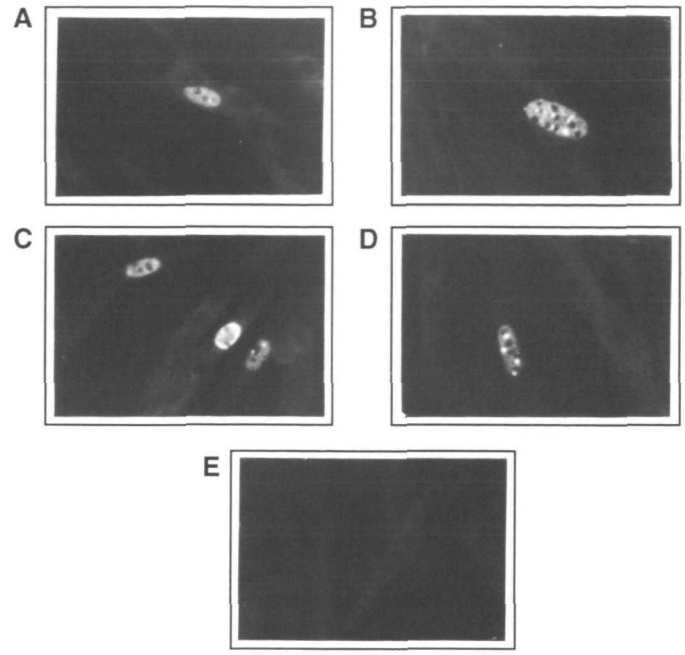

Figure 6. Expression of VZV 140k mutants in BHK cells. Plasmids expressing **A.** 140k (p140), **B.** 140Q545L (p140Q545L), **C.** 140N546L (p140N546L), **D.** 140K548E (p140K548E) and **E.** no plasmid were transfected into BHK cells and stained with polyclonal antiserum to the VZV 140k DNA binding domain. Granular and diffuse nuclear staining characteristic of the VZV 140k protein, is present in A–D.

experiments (data not shown). The VT2XK548E peptide produced a very faint, smeary protein–DNA complex with the gene 62 promoter fragment (Fig. 3A, track 14) when included at an amount which yielded secondary complexes with the other three peptides. The greatly reduced DNA binding activity of VT2XK548E could be accounted for by a lower DNA binding affinity or unstable interactions with DNA, as a result of the substitution.

The VZV 140k DNA binding domain peptide, VT2X, can also recognise binding sites within HSV-1 gene promoters, including sites fitting the ICP4 binding consensus (31,60). To analyze whether the effects of the three mutations on DNA binding were specific to sites within the gene 62 promoter, we investigated whether the mutant VT2X peptides could recognise DNA fragments from the HSV-1 IE3 and HSV-1 glycoprotein D (gD) promoters (Fig. 3B). Both of these probes contained ICP4 consensus binding sites (Fig. 4B & C) that are recognised by wild type VT2X (31). This experiment used an equivalent amount of each peptide that gave only the primary protein-DNA complex. As seen above with the VZV gene 62 promoter fragment, mutations Q545L and N546L had little effect on the DNA binding affinity of VT2X to either the IE3 or the gD probe (Fig. 3B), although slight differences in the relative intensities of complexes produced by these two mutant peptides, as compared to wild type VT2X were seen on the three probe fragments. No detectable binding of peptide VT2XK548E to the HSV-1 IE3 promoter fragment was observed (Fig. 3B, track 6) and a barely detectable complex was seen on the gD promoter fragment (Fig. 3B, track 12). These consequences of mutation K548E are consistent with the result on the VZV gene 62 promoter fragment (see above) and indicate that the effects of this substitution are not specific to the gene 62 promoter, but reflect a generalised disruption of the VZV 140k DNA binding interaction.

The 140k DNA binding domain is a stable dimer and dimerization appears to be a prerequisite for DNA binding (35). Therefore, it was necessary to eliminate the possibility that mutation K548E might act by disrupting dimerization rather than affecting DNA binding directly. Aliquots of each mutant VT2X peptide were analyzed by gel filtration chromatography as described previously (35) and were all found to elute as single discrete species with retention volumes identical to that of the VT2X homodimer (data not shown). Therefore mutation of residue K548 in the 140k WLQN region does not affect subunit interaction, and is likely to interfere with DNA binding directly.

Mutations in the WLQN region alter DNA recognition by the 140k DNA binding domain

DNase I footprinting analysis was used to provide further information about the effects of the mutations in the 140k WLQN

region on the specificity of DNA binding. Particular care was taken to ensure equal input of DNA binding domain peptides in these experiments. Footprinting on the VZV gene 62 promoter probe showed that VT2X provided characteristic protection of multiple sequences from DNase I digestion, flanked by marked sites of DNase I hypersensitivity (31); the most apparent sequences protected by VT2X from DNase I digestion were centred over the gene 62 mRNA start site, and around nucleotides −30, −50 and −80 (Fig. 4A). Peptides VT2XQ545L and VT2XN546L both provided protection for sequences similar to those recognised by VT2X, albeit more faintly. This weaker DNase I protection was not merely a consequence of reduced DNA binding affinity (see above). These two mutant peptides also induced sites of DNase I hypersensitivity in similar positions to those produced with VT2X, although closer examination of the VT2X, VT2XQ545L and VT2XN546L tracks reveals slight differences in the details of these hypersensitivity sites. The VT2XK548E peptide failed to produce any footprinting pattern, although on comparison with the control track, it is apparent that a degree of DNase I hypersensitivity was induced by this peptide at similar positions to those produced by the other three peptides. Perhaps peptide VT2XK548E interacts transiently with DNA, but not so as to provide sequence specific protection or yield stable protein-DNA complexes detectable by gel retardation assays.

Further DNase I footprinting experiments were performed upon the same HSV-1 IE3 and gD promoter probes used in the gel retardation analyses above (Fig. 3B). On the HSV-1 IE3 probe, VT2X protected both halves of the bipartite consensus site and faint protection extended beyond the 3′ end of the consensus site to nucleotide +25 (Fig. 4B). VT2XQ545L weakly protected some similar sequences to those recognised by VT2X, although on close comparison with the VT2X track, slight differences in hypersensitivity and also in the DNase I protection pattern were apparent. Interactions between the VT2XN546L and VT2XK548E peptides and DNA was illustrated by the DNase I hypersensitivity induced to the 5′ of the consensus binding site but there was little specific protection from DNase I digestion. The HSV-1 gD promoter fragment contained multiple sequences weakly recognised by VT2X, flanked by sites of DNase I hypersensitivity (Fig. 4C). The sequences protected by VT2X were centred over nucleotides −130, −100 (the consensus ICP4 binding site), −45 and also to a lesser degree around nucleotide −85. The gD ICP4 consensus sequence (33) was weakly recognised by VT2XQ545L and VT2XN546L, but not by VT2XK548E. Examination of the sites of DNase I hypersensitivity induced by VT2X and its mutant derivatives demonstrates that the three mutant peptides had differing effects on the details of the DNase I footprint on the HSV-1 gD promoter fragment.

The lysine mutation that disrupts sequence specific DNA recognition also destroys the transactivation function of 140k

Having found that amino acid substitutions within the WLQN region affected, to varying degrees, the interaction of the isolated VZV 140k DNA binding domain with DNA, we wanted to analyze the effects of these mutations, if any, on the transactivation function of the 140k protein. Each mutation was incorporated into the intact 140k protein (Materials and Methods), and the transactivation efficiency of each mutant 140k protein, as determined by CAT assay, was compared to that of wild type 140k. Transactivation of the HSV-1 glycoprotein D (gD) promoter was measured in these experiments because VZV 140k is an extremely potent activator of this particular promoter (61). Additionally, the effects of the mutations on transactivation and DNA binding can be compared directly, as the gD promoter region was included in the DNA binding analyses above. For each mutation, consistent alterations of the DNA binding interaction was observed on all three promoters tested above; therefore it is likely that the consequence of each mutation on the regulatory functions of 140k, as measured on the HSV-1 gD promoter, would apply to all target promoters. A range of concentrations of each effector plasmid and a fixed amount of the gD promoter-CAT reporter construct were transiently co-transfected into HeLa cells. Graphical representations of the results of two repeats of such an experiment are shown in Figure 5, and indicate the reproducibility of the effects of each mutation. As anticipated, wild type VZV 140k very strongly transactivated (up to 118 fold and 191 fold induction over plasmid pgDCAT alone, for each graph in Fig. 5 respectively) the HSV-1 gD promoter in a dose dependent manner. The 140Q545L protein had a slightly impaired transactivation efficiency, whereas the reduction as a result of the N546L mutation was more pronounced (Fig. 5). The 140K548E protein completely failed to activate gene expression from the gD promoter. The lack of function of the 140K548E protein was not specific to the HSV-1 gD promoter but represented a general phenomenon; 140K548E failed to transactivate the HSV-1 IE1 and HSV-1 tk promoters, and also the SV40 early promoter-enhancer regions (data not shown).

The fact that the isolated 140k DNA binding domain peptides containing mutations within the WLQN region were stable, soluble dimers (see above), suggests that conformational alterations are not responsible for the altered DNA binding and regulatory functions. To check that the mutations had not unfavourably affected expression or stability of the full length 140k proteins, we compared the immunofluorescence profiles obtained for wild type and mutant 140k proteins. All four 140k derivative proteins localised to the nucleus with the characteristic distribution of nuclear staining (Fig. 6A−D). For each set of transfections, similar intensity fluorescence was observed in comparable numbers of cells for the wild type and mutant proteins. Additionally, a polypeptide band with the same mobility as wild type 140k was expressed to equivalent levels for each mutant 140k peptide, as determined by western blot analysis of extracts of transfected BHK cells (not shown). We conclude that the mutations in the WLQN region were not deleterious to the expression or stability of the 140k protein. Therefore, the differential efficiencies of transactivation induced by the mutant 140k proteins were likely to be a consequence of their altered DNA binding activities, as identified in the DNA binding analyses above.

DISCUSSION

From the available functional data and by analogy with HSV-1 ICP4, the VZV 140k transactivator protein is likely to be particularly important for the regulation of gene expression of all proposed temporal classes of VZV genes (18,20−22). Again by analogy with ICP4, VZV 140k is likely to act at the level of transcriptional initiation (12). It is a reasonable assumption that the mechanisms underlying these processes involve VZV 140k binding directly to DNA in a sequence specific manner. The effects of mutating three single residues in a conserved sequence within the 140k DNA binding domain, including the residues WLQN, are consistent with this WLQN region being

directly involved in the DNA binding and transactivation functions of VZV 140k.

The bacterially expressed isolated 140k DNA binding domain provided a well characterised and convenient tool for the analysis of the effects on DNA binding caused by mutations in the WLQN region. Due to the poor growth characteristics of VZV in tissue culture, the study of the intact 140k protein has been hampered by the inability to obtain large enough amounts of 140k from VZV infected cells for DNA binding analyses (61). However, the 140k DNA binding domain has been closely defined and is stably expressed in bacteria as a separable domain and further post-translational modifications are not required for its sequence specific DNA binding activity (31). At least for the homologous HSV-1 ICP4 protein, the isolated DNA binding domain peptide has a very similar specificity to that of the intact protein (34). By analogy, the consequence of point mutations in the WLQN region on the binding activity of the 140k DNA binding domain peptide are likely to reflect their effects upon the intact 140k protein.

Replacement of VZV 140k residue lysine 548 with glutamic acid (K548E) drastically reduced the sequence specific DNA binding activity of the mutant 140k DNA binding domain, and resulted in a non-functional intact 140k protein, as determined by transient transfection assays. Given that no gross changes in dimerization state, stability, solubility or efficiency of expression were detected as a result of the K548E mutation, these findings suggest that DNA binding is essential for the transactivation function of the VZV 140k protein. The relationship between the DNA binding and regulatory functions of the equivalent protein of HSV-1, ICP4, is slightly controversial. Detailed mutational and functional analyses have shown that mutations which disrupt the DNA binding activity of ICP4 abrogate its regulatory functions (25,26,62), with one exception. This solitary case comprised a pseudorevertant of an inactive ICP4 protein which had restored regulatory activities (in the context of viral infection) and impaired DNA binding properties (63). However, the isolated DNA binding domain of the ICP4 pseudorevertant protein was able to bind to DNA, at least at room temperature (K.E. Allen, N. DeLuca, R. Everett, unpublished data).

Although substitution K548E had pronounced effects on the DNA binding and regulatory functions of VZV 140k, the consequences of two further single mutations in the WLQN region were more subtle. Replacement of 140k residue glutamine 545 with leucine (Q545L) and asparagine 546 with leucine (N546L) had no apparent effect on DNA binding affinity as measured by gel retardation analysis. However, the specific details of their DNA binding interactions, as determined by DNase I footprinting analysis, significantly differed from those of either wild type VT2X or VT2XK548E. Mutation Q545L caused a minor reduction in the transactivation efficiency of the 140k protein, and the N546L mutation resulted in a yet more pronounced reduction in gD promoter induction. The effects of these two mutations on the transactivation function are most easily explained as a consequence of their slightly altered interactions with DNA, although they may be caused by other factors not addressed in this study, such as subtle conformational changes within the domain resulting in defects in other macromolecular interactions. The effects of these three 140k mutations on DNA binding support the idea that this WLQN homology region is directly involved in the DNA binding interaction. Verification of the importance of the WLQN region will be provided by the analysis of the effects of these, and further amino acid substitutions, on the life cycle of the alphaherpesviruses.

The homeodomain forms a stable folded structure that is capable of binding to DNA by itself (64–66) therefore this 60 residue domain is much smaller than the defined 140k DNA binding domain (162 residues) (31). However, the structures of larger variant homeodomains have recently been determined, for example, the 'winged-helix' DNA binding motif of proteins such as HNF-γ3 (67) is well over 100 amino acids in size. The DNA binding activity of the HSV-1 ICP4 protein is highly sensitive to insertion mutations throughout its entire DNA binding domain (25,26), and it is possible that other additional sequences within these large domains are directly involved in DNA binding. Indeed, a HTH motif has been proposed on the basis of secondary structure predictions further C-terminal to the WLQN region of the HSV-1 ICP4 protein (26). Several substitutions within this helical region result in a temperature sensitive DNA binding phenotype, while two other single mutations in the proposed 'turn' disrupt DNA binding (K.E. Allen, unpublished data). Although it is a possibility that this predicted HTH sequence may encode a structural scaffold which holds the WLQN region in such a way that it can be stably presented to DNA, it is not uncommon for proteins to couple additional DNA binding motifs or domains to increase their affinity or specificity of binding. For example, prokaryotic HTH proteins such as λ repressor, use an extended region of peptide chain to wrap around the DNA (68). Similarly, the POU proteins have a conserved 65–75 residue segment, the POU-specific domain, on the N-terminal side of the homeodomain (69) which contacts the DNA sequence ATGC adjacent to the homeodomain target sequence (70). Thus each of the two DNA binding motifs of the POU protein contacts one half of a non-palindromic bipartite target sequence; the bipartite nature of the non-palindromic ICP4 consensus binding site (33) is suggestive of an analogous DNA binding mechanism for the alphaherpesvirus proteins. Other DNA binding domains found in association with homeodomains include the paired domain (71), C_2H_2 zinc fingers and even additional homeodomains. The *Drosophila Zfh-2* protein, containing three homeodomains and 16 zinc fingers, is an extreme example of this domain coupling (72). The involvement of several independent structural elements in the interaction with DNA would be consistent with the large size of the DNA binding domains of the VZV 140k and HSV-1 ICP4 proteins.

It may transpire that many DNA recognition motifs are variations on the same theme (73) and DNA binding proteins have evolved different structural scaffolds to present their DNA recognition motif to probe the DNA. The 140k alphaherpesvirus DNA recognition region may be an example of a divergent motif that has retained a minimum set of residues necessary for its interaction with DNA. The differences between the potential WLQN recognition sequence of the 140k protein and the known types of recognition motifs may facilitate its particular DNA binding characteristics such as its low DNA binding specificity, which allows it to bind multiple recognition sequences (31) within a presumably very wide range of VZV promoters. The details of the involvement of the WLQN region in the DNA binding interaction awaits future structural analyses.

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