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Herpes simplex virus type 1 polypeptide ICP4 bends DNA

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ABSTRACT

ICP4, the major regulatory polypeptide of herpes simplex virus type 1, is expressed at the earliest stages of virus infection and is required for the activation of transcription from the majority of viral promoters. It is a DNA binding protein which specifically recognises bi-partite sites related to the sequence ATCGTnnnnnCGG. In this report we show that both partially purified ICP4, and its isolated DNA binding domain, bend DNA at occupied binding sites. The apparent angles of bend at two different binding sites were very similar and in both cases the centre of the bend was very close to the binding site sequence.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) encodes at least 70 distinct genes (1) which are expressed in a temporally regulated manner (for reviews, see references 2–4). The five immediate-early (IE) genes are expressed at the onset of infection, and the products of at least three of these are involved in the regulation of the later classes of genes. The product of IE gene 3, ICP4 (also known as Vmw175), is of crucial importance since its inactivation results in failure of the infection to progress beyond the IE phase (5,6). ICP4 is a sequence specific DNA binding protein which recognises sites throughout the viral genome (7, and references therein). Early comparisons of the sites bound by ICP4 suggested that there might be two sequence elements important for binding (8). Recent experimental analyses of binding site mutations which affect recognition by ICP4 have confirmed the theoretical deductions; the two important elements of the binding site are separated by five bases such that a prototype binding element can be written as ATCGTnnnnnCGG (9,10). This sequence is very close to that derived from a statistical comparison of a number of known ICP4 binding sites (7).

Many DNA binding proteins have been found to induce a bend at their DNA binding sites (11, and references therein). Some of these proteins, of which perhaps the best studied example is the E. coli CAP protein (12), bind as dimers to bi-partite symmetrical sites, and the bending is thought to have functional significance. We have investigated whether ICP4 also bends DNA. We have studied ICP4-induced bending using two different DNA binding sites with isolated, purified ICP4 DNA binding domain and partially purified preparations of the intact protein. The purified DNA binding domain caused a significant bend of similar magnitude at both binding sites, and intact ICP4 also bent the DNA at these sites. In all cases the center of the bend was at or very close to the binding sequence.

MATERIALS AND METHODS

Probes

The probes were derived either from the upstream region of the HSV-1 glycoprotein gD promoter (8), which contains a binding site known as gD site II (13), or from the cap site region of the IE-3 promoter (14). The experiments with the isolated ICP4 DNA binding domain used probe fragments which were cloned as dimers, while those using the partially purified whole protein used circularly permuted probes obtained after cloning of a single binding site into plasmid pBend2 (15).

(i) The gD site II binding site. Plasmid pRED21 (16) contains a XhoI linker in the HindIII site at position +11 of the gD promoter. M13 phage mpE21 contains a fragment of the gD promoter with a XhoI linker inserted at position −294 (17). The PvuII (−266) to XhoI gD promoter fragment of pRED21 was isolated and ligated with the XhoI-HindIII fragment of mpE21 into pUC9, which had been cut with SmaI and HindIII. This essentially creates a tandem duplication of the gD promoter region, which contains gD site II at position −107. Cutting of this fragment with BstNI, Sau3A, HinfI or XhoI plus HindIII creates a circularly permuted family of probes, each containing a single copy of gD site II, with the binding site at variable locations with respect to the ends of the fragments. These fragments were labelled with Klenow DNA polymerase and the appropriate alpha [^{32}P] dNTP. The reactions were completed with an excess of all four unlabelled dNTPs. Due to the precise locations of the restriction sites and the nature of their cleavages, the probes so generated have very slightly different lengths. The important details of these probes are shown in Figure 1A.

(ii) The IE-3 binding site. The region containing this site was dimerised by cloning the SalI-SphI IE-3 promoter region fragment (coordinates +77 to −200) between the SalI and SphI sites of a derivative of pUC19 which had been cut with EcoRI and XbaI, filled in and ligated (this removes the SacI, KpnI, SmaI and BamHI sites from the vector but recreates both the XbaI and EcoRI sites). The plasmid product of the first step was cut with HindIII (vector polylinker site) and partially with EcoRI (there

is an EcoRI site at the other end of the insert in the vector polylinker and another at position −108 of the IE-3 promoter). The larger partial EcoRI-HindIII complete promoter fragment and the smaller vector partial fragment (containing the SalI-EcoRI IE-3 promoter region) were isolated and ligated. The addition of the EcoRI-HindIII IE-3 promoter region fragment dimerises the IE-3 binding site region. Thus the final structure of the clone is EcoRI/XbaI/SalI(+77 to −108)EcoRI/XbaI/SalI(+77 to −200)SphI/HindIII. Cleavage of the resultant plasmid with XbaI (which is immediately 5′ of the SalI site), EagI, BamHI, AvaI or BstNI creates a family of circularly permuted probes with the IE-3 binding site at different distances from their ends. The important details of these probes are shown in Figure 1B.

(iii) Probes derived from plasmid pBend2. Plasmid pBEND2 contains a tandemly repeated sequence (which contains several restriction sites) with XbaI and SalI cloning sites between the two repeats (15). Plasmids p-108bend and pBA2bend were constructed by inserting the BamHI-EcoRI (+27 to −108) and BamHI-AvaI (+27 to −18) IE-3 promoter fragments into the XbaI site of pBEND2 by blunt-end ligation. These two plasmids have the IE-3 inserts in opposite orientations. Plasmid pJgDbend contains the HindIII-XmaIII (+11 to −121) gD promoter region cloned by blunt-end ligation into the XbaI site of pBEND2. The direct repeat in pBEND2 which flanks the HSV DNA inserts in the above plasmids contains, amongst others, BglII, XhoI, EcoRV, SmaI and RsaI restriction sites. Cutting with any of these enzymes produces fragments of 264bp from p-108bend, 189bp from pBA2bend and 274bp from pJgDbend. These fragments were 5′ end-labelled with polynucleotide kinase and isolated to produce three families of probes with the ICP4 binding site in circularly permuted positions with respect to their ends. These probes are shown in Figure 1C.

Proteins

(i) I10X. The DNA binding domain peptide of ICP4, here named I10X, was expressed in E.coli using a T7 expression system and purified to apparent homogeneity as described (10).

(ii) ICP4. Intact ICP4 was partially purified from BHK cells which had been infected for 11 hours with 10pfu per cell of HSV-1 strain KOS. The methods used were exactly as described (7).

Gel retardation experiments

Appropriate amounts of either ICP4 or I10X were incubated with end-labelled probes and the complexes resolved in 4% acrylamide gels in 0.5×TBE buffer as described (14). The dried gels were autoradiographed to detect free probe and the mobility of the bound complex. The relative mobility of each complex was measured as a proportion of the mobility of the relevent unbound probe. The results were plotted to illustrate the change in mobility of the complex with respect to the position of the binding site within the probes.

RESULTS

The isolated DNA binding domain of ICP4 causes a significant DNA bend at its binding site

The method by which DNA bending is detected is based on the principle that a bend near the end of a restriction fragment does not affect its electrophoretic mobility to the same degree as a bend in the middle. Thus, creation of a circularly permuted set of probes with a protein binding site at different distances from the ends of the fragments allows the detection and localisation of protein induced DNA bends (18). Accordingly, a fragment containing the IE-3 ICP4 binding site was cloned as a tandem duplication, as described in the methods section, to allow the preparation of a circularly permuted set of probes. The details of the relevant portion of the tandem duplication are shown in Figure 1. Fragments generated by cutting with XbaI (which cuts immediatly 5′ of both the SalI sites) EagI, BamHI, AvaI or BstNI contain a single ICP4 binding site which lies in the middle of the XbaI probe and very near the end of the AvaI probe. Purified ICP4 DNA binding domain protein I10X was incubated with each of these probes and the complexes separated on acrylamide gels. The results in Figure 2A show that there was a significant change in mobility of the primary DNA-protein complex depending on the probe used. Consistent with the formation of a DNA bend,

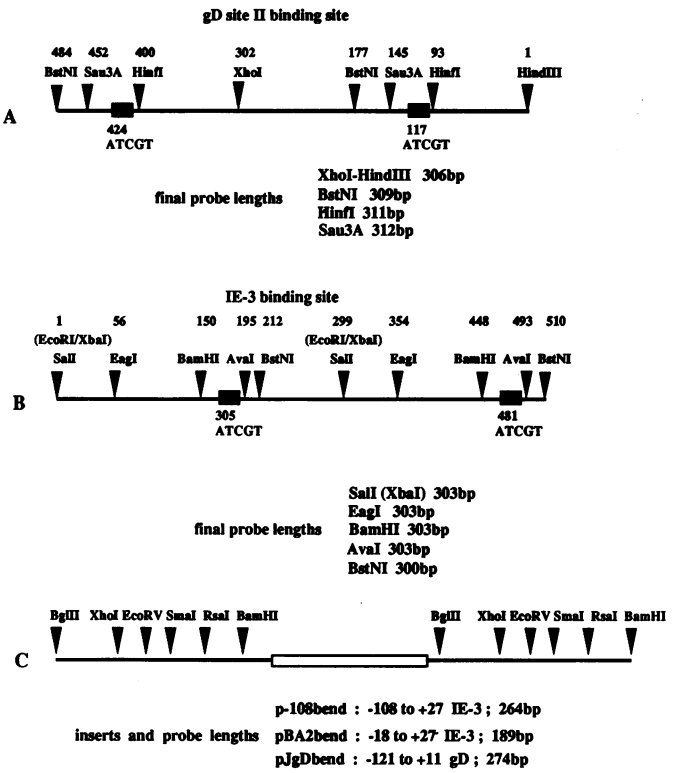

Figure 1. Probes used for gel retardation experiments with protein I10X. A. The important parts of the dimerised gD site II binding site. The construction of this fragment is described in the Methods. The XhoI-HindIII section on the right is repeated to the left of the XhoI site, with the addition of a few linker bases just 5′ of the central XhoI site. The positions of the first bases of the restriction sites (and of the ICP4 recognition sequence), relative to that of the HindIII site, are marked. The final all inclusive probe lengths have been calculated from these coordinates, taking into account the bases added during the labelling reaction. B. The dimerised IE-3 binding site region. At the left hand end are the contiguous sites EcoRI/XbaI/SalI; these are repeated at the middle. The coordinates are of the first bases of the marked recognition sequences relative to that of the SalI site at the left end. The fully inclusive probe lengths are given below. The probe which was generated by XbaI digestion has its ends shifted 6bp to the left of that which could be generated by digestion at the SalI sites marked. C. A schematic representation of the the IE-3 and gD binding site probes in plasmids p-108bend, pBA2bend and pJgDbend, which have been derived from vector pBEND2 (15). The inserted fragment is depicted by the open box, with the repeated sequences with multiple single restriction sites marked either side.

the mobility of the AvaI probe complex was the greatest, and that of the XbaI probe complex the slowest.

To ensure the generality of these results, the gD site II ICP4 binding site was also dimerised and probes generated by restriction with XhoI plus HindIII, HinfI, Sau3A and BstNI. In this case, as detailed in Figure 1 legend, the probes were of slightly different lengths because of the methods of their construction and labelling. However, a graphical representation of the mobility of the complexes relative to that of the cognate probe shows that I10X binding also results in alterations in the migration rates of the complexes depending on the location of the binding site (Figure 2B).

From the mobility differences illustrated in Figure 2 parts A and B, it is possible to deduce the location of the center of the DNA bend, and to obtain an estimate of the bend angle. The relative mobilities of the complexes were plotted with respect to the relative locations of the restriction sites. The center of the bend is then assumed to be at the extrapolated position of minimum mobility (18). In the case of the IE-3 probe, the data in Figure 2C strongly suggest that the center of the bend is very close to the AvaI site on its 5′ side, and nearer to the AvaI site than to the BamHI site. Note that the A of the ATCGT recognition sequence is 16bp from the end of the AvaI probe and 32bp from the end of the BamHI probe. In the case of the gD site II binding site, the center of the bend must be approximately midway between the Sau3A and HinfI sites (Figure 2D). The A of ATCGT is 28bp from the end of the Sau3A probe and 27bp from the end of the HinfI probe. Therefore the data are consistent with the location of the I10X induced bend being at or near the 5′ end of the ICP4 recognition sequence of both binding sites.

The actual bend angle at the two binding sites must be similar since the IE-3 and gD site II probe families are almost exactly the same size and the relative alterations in mobility are also very similar. Quantitation of the bend angle is more difficult since the actual data will be affected not only by the sizes of the probes and proteins, but also by the buffer conditions during electrophoresis (19). However, the differences between the mobilities of the fastest and slowest migrating complexes with I10X are similar to those observed with TFIIIA binding under similar conditions; in this latter case the bend angle was calculated as 60−65° (19).

Addition of larger quantities of protein I10X to the binding mixture results in the appearance of secondary complexes (labelled SC in Figures 2A and 2B) which are presumed to contain multiple dimeric I10X molecules. These additional dimers could be interacting by protein-protein contacts between I10X dimers alone, or they could interact with both the first bound dimer and adjacent DNA, or they could be binding at completely separate, secondary binding sites. It is intriguing that the apparent bend in both the gD and IE-3 binding site higher order I10X complexes is significantly reduced; therefore the conformation of the DNA must be altered by additional I10X dimers. This could be due to conformational changes of the the I10X protein itself (through multimerisation by protein-protein interactions), or neutalisation of the bending induced by the first bound dimer by subsequent dimer binding at adjacent or distant sites (so to produce an equivalent bend in the opposite orientation).

Partially purified intact ICP4 bends DNA

Full length ICP4 also induces a DNA bend. These experiments used probes which also contained the gD site II and IE-3 binding sites, which were obtained after cloning appropriate fragments into pBend2. This vector was constructed specifically to allow the preparation of probe families with a cloned binding site located at varying distances from the ends of the fragments (15). The probes are shown in Figure 1C, and the results summarised in Figure 3. Again, the results indicate that ICP4 induces a protein bend at both sites. The magnitude of the differences in complex mobility are not so marked as those with protein I10X, probably because intact ICP4 is five times the size of I10X. Therefore the

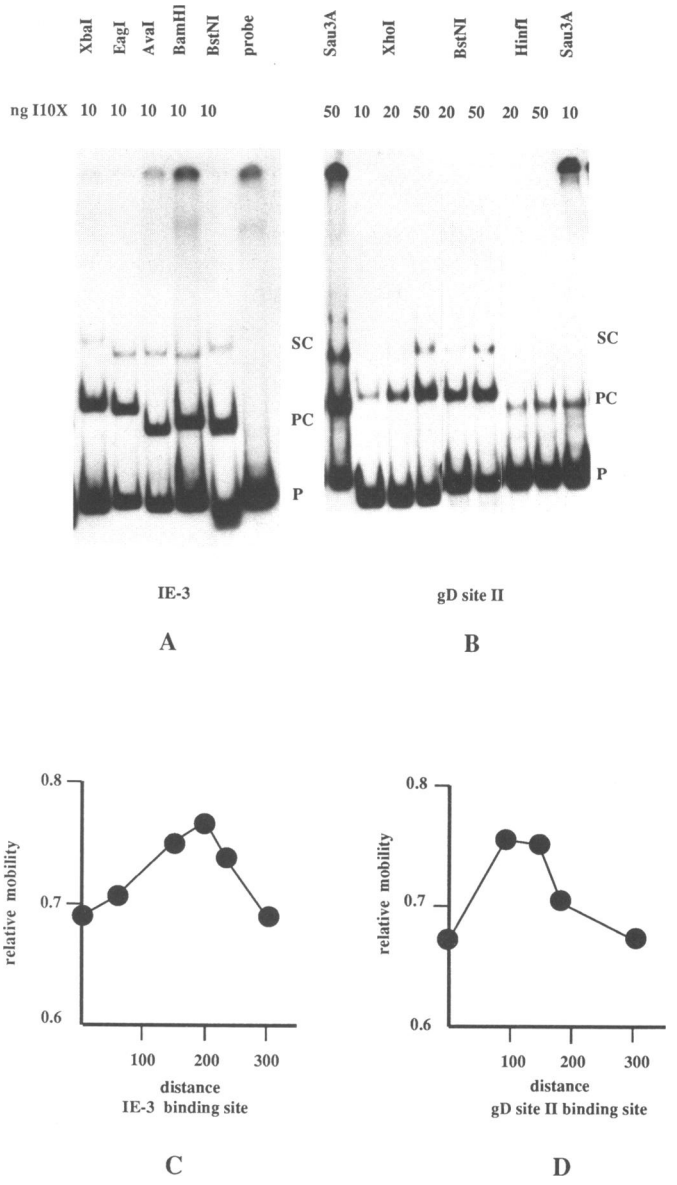

Figure 2. The circular permutation gel retardation assay with protein I10X. Panels A and B. Circular permutation gel retardation assays with the IE-3 and gD site II binding sites. P = free probe, PC = primary complex, SC = secondary complex(es). The probes used for each incubation and the amount of I10X protein used are marked above the panels. The slight differences in the mobilities of the probes are mainly due to their slightly different lengths, as explained in the legend to Figure 1. Panels C and D. The relative mobilities of the complexes compared to their cognate uncomplexed probes have been calculated and plotted in relation to the positions of the first bases of the restriction sites, as marked in Figure 1. The data with the slowest migrating complex has been shown at both extremities of the curves. The order of the probes in panels A and B is not the same as that plotted in panels C and D.

ICP4 complexes are themselves of much lower mobility, which partially masks the effect of DNA bending. Consequently, localisation of the bend centre was possible only with pBA2bend (Figure 3C, middle panel); however the slopes in the left and right panels of Figure 3C (p-108bend and pJgDbend) clearly indicate that bending is associated with ICP4 binding to these probes. In agreement with the results described above with I10X, the center of the ICP4-induced bend in the pBA2bend probe was close to the actual binding site sequence. Again, the bending effect was eliminated in the secondary complexes. Since the secondary complexes with intact ICP4 have been attributed to protein-protein (rather than protein-DNA) interactions (20), it seems likely that the loss of bending is due to conformational changes induced by ICP4 dimer multimerisation.

DISCUSSION

We have demonstrated that ICP4 induces a DNA bend at or very close to its binding site. This was confirmed using two different probes and with both the intact protein and its isolated DNA

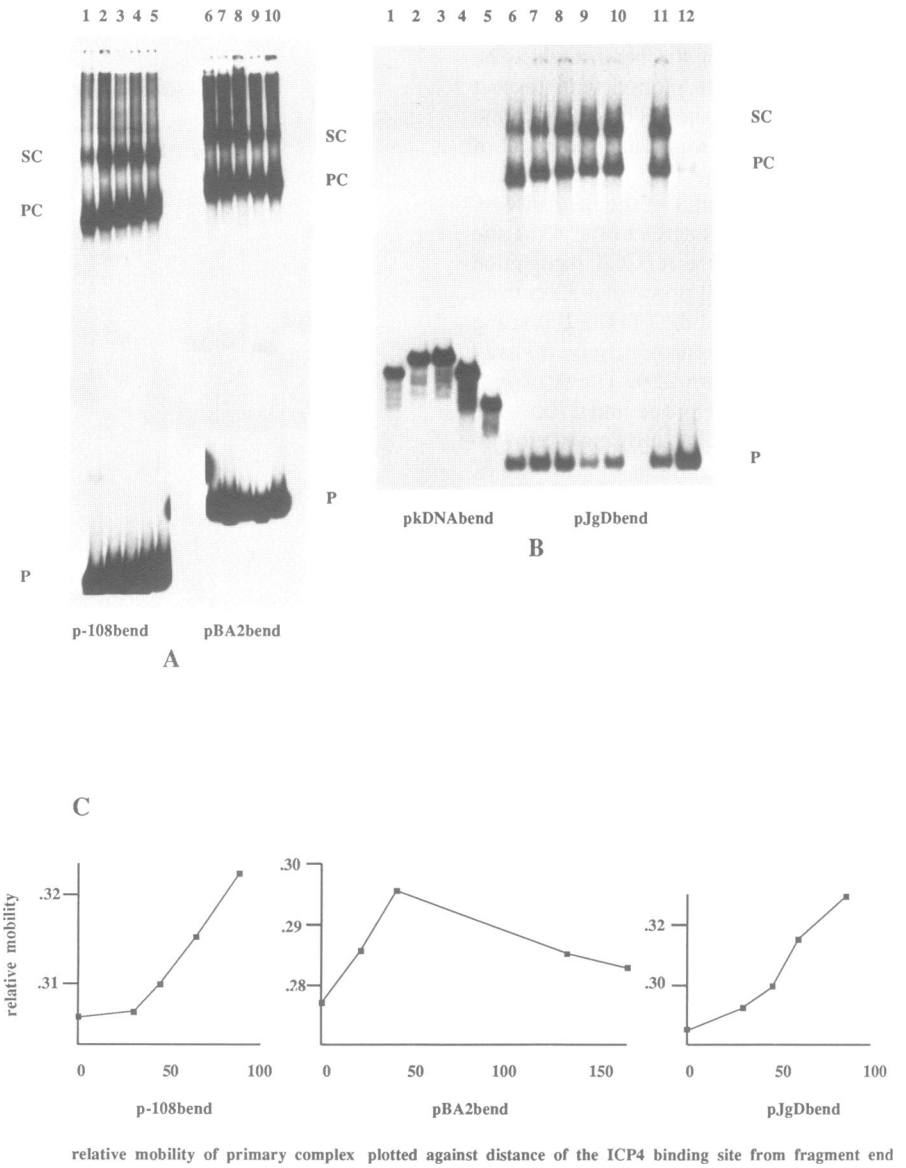

Figure 3. Intact ICP4 bends DNA. A. Bending of the IE-3 binding site. The probes in lanes 1 to 5 were derived from digestion of p-108bend with RsaI, SmaI, EcoRV, XhoI and BglII respectively. The probes in lanes 6 to 10 were derived from restriction of pBA2bend with BamHI, RsaI, SmaI, XhoI and BglII respectively. The probes were incubated with partially purified ICP4 and the complexes resolved on acrylamide gels. P = free probe, PC = primary complex, SC = secondary complex (marked both left and right of the panel). B. Bending of the gD binding site. Tracks 1 to 5 contain control fragments derived from digestion with different enzymes of pKDNAbend, which was constructed by insertion of an intrinsically bent kinetoplast DNA fragment into pBEND2. Tracks 6 to 10 contain probes from pJgDbend derived after digestion with RsaI, SmaI, EcoRV, XhoI and BglII. Track 11 is a control with the BglII probe with 2.5ng of non-specific competitor (poly $dGdC)_{17}$. The incubation loaded in track 12 contained 2.5ng of a competitor oligonucleotide encompassing the IE-3 binding site (−18 to +27); this illustrates the specificity of both primary and secondary complexes. C. Graphical representation of the results. The relative mobility (with respect to free probe) of the primary complexes from the different permutation probes of p-108bend, pBA2bend and pJgDbend have been plotted on the vertical axis, with the distance from the end of each probe to the A of the ATCGTC on the horizontal axis.

binding domain. The significance of DNA bending to the mechanisms of transcriptional regulation remain unclear, but this report shows that ICP4 can join the growing number of eukaryotic proteins which are involved in transcription and which bend DNA.

Detailed analyses of the E. coli CAP protein-DNA interaction have shown that the protein binds as a dimer to a bi-partite symmetrical recognition sequence such that the bend occurs in the middle of the binding site, between the monomer subunits (which each contact one half of the recognition palindrome) (12). It is interesting that both ICP4 and its isolated DNA binding domain can be isolated as dimers in solution (10, 21) and that ICP4 binds to DNA as a dimer (22) and that its recognition sequence is bi-partite (7,10). However, the ICP4 recognition sequence is not symmetrical. It is not easy to see how a functional ICP4 DNA contact interface could be formed by dimerisation of two identical subunits which each contribute (symmetrical) interactions with the asymmetrical parts of the recognition sequence. It may be that each monomer unit of the ICP4 dimer includes a complete DNA binding interface, in which case the bend may be caused by the DNA being wrapped around a protein monomer, rather than by the two subunits of the dimer bending it between them. This scenario is supported by the observation that heterodimers of wild type and DNA binding deficient mutant ICP4 molecules are able to bind to DNA (23).

This suggests that only one subunit is responsible for the initial binding event, which leaves open the possibility that the other subunit might also interact with DNA, perhaps to form a DNA loop.

It is clear that, whatever the biological significance of DNA bending by ICP4, more detailed understanding of how it interacts with DNA will be dependent on structural studies. The availability of large amounts of the purified DNA binding domain makes this an approachable goal.

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