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Nucleotides within both proximal and distal parts of the consensus sequence are important for specific DNA recognition by the herpes simplex virus regulatory protein ICP4

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ABSTRACT

The herpes simplex virus type 1 regulatory protein ICP4 is a sequence specific DNA binding protein which associates with a number of different sites, some of which include the consensus ATCGTCnnnnYCGRC. In order to investigate the involvement in DNA binding of conserved bases within the consensus, we have synthesised a family of mutant oligonucleotides and tested their ability to form a complex with ICP4. We have also compared the binding specificities of bacterially expressed fragments of ICP4 which include the DNA binding domain. Mutation of most (but not all) bases in the proximal part of the consensus greatly reduced binding by ICP4, as did a mutation affecting the distal part. Most (but not all) G residues identified in methylation interference assays were required for efficient binding. While a bacterially expressed ICP4 peptide encompassing amino acid residues 252–523 bound to DNA with a specificity similar to that of the whole protein, a shorter protein (residues 275–523) had a slightly relaxed DNA binding specificity.

INTRODUCTION

Analysis of the complete 150kb DNA sequence of herpes simplex virus type 1 (HSV-1) has predicted the presence of at least 70 distinct genes (1). These genes can be grouped into three principal temporally regulated groups depending on the kinetics and drug-sensitivity of their expression during virus infection in tissue culture (see references 2, 3 and 4 for reviews). The immediate-early (IE) genes can be transcribed in the absence of viral protein synthesis, while transcription of the later classes of genes requires IE polypeptide synthesis. Perhaps the most important of the IE polypeptides is ICP4 (also known as Vmw175) since it is absolutely required for progression of the lytic cycle beyond the IE phase (5,6).

ICP4 is a sequence specific DNA binding protein which recognises a number of sites throughout the HSV-1 genome (7–14). These sites appear to conform to either of two classes, firstly the so-called consensus sites which include the sequence ATCGTCnnnnYCGRC, first recognised by Faber and Wilcox (8). This consensus can be viewed as two conserved portions separated by a variable region. In addition, there are a number of other sites whose relationship to the consensus is not clear (13–15).

While there are several reports which suggest that the ability of ICP4 to bind to DNA is important for its functions (16–18), the role of individual binding sites is not so clear-cut. For example, while the binding site at the cap site of gene IE-3 is important for repression of the IE-3 promoter in transfection assays (19, 20) the role of other binding sites in the ability of ICP4 to activate early promoters is more controversial (13, 15, 21, 22). One problem in the interpretation of the role of ICP4 binding sites is that, given the variation in their sequence, it is not possible to predict from sequence data alone whether a site will be recognised or not. Therefore, in order to establish a greater understanding of the characteristics of an ICP4 binding site, we have undertaken a mutational analysis of the well- defined consensus site found upstream of the glycoprotein gD promoter region. We found that while mutation of several of the conserved residues in the proximal part of the site greatly reduced binding, not all such residues were essential. In addition, the distal part of the consensus sequence was found to be as important as the proximal.

As a first step towards determining the amino acid residues of ICP4 which are involved in sequence specific binding, we have expressed portions of the DNA binding domain in bacteria. Using the family of mutant oligonucleotide probes, it was found that one bacterially expressed peptide had a DNA binding specificity indistinguishable from that of the intact protein made in infected cells. However, a slightly smaller portion of the protein was somewhat less rigorous in its requirements for sequence specific binding.

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MATERIALS AND METHODS

Oligonucleotides and preparation of probes

A double stranded oligonucleotide corresponding to positions −89 to −110 of the gD promoter (with four nucleotide 5′ single-standed extensions identical to those produced by restriction enzymes SalI and XhoI) was synthesised to create a probe of wild type sequence:

5′ TCGACACTATCGTCCATACCGACCAC 3′

Mutant probes based on this sequence were also synthesised, as detailed in Figure 1. Further oligonucleotides containing the sequences between coordinates −109 to −92, and −96 to −79 (see Figure 1) were also synthesised, again with SalI and XhoI cohesive ends. After synthesis, the single-stranded oligonucleotides were annealed in appropriate pairs and purified by gel electrophoresis. Double-stranded oligonucleotides were labelled either with γ-^{32}P ATP and polynucleotide kinase or with α-^{32}P dATP and Klenow polymerase. Labelled probes were again purified by gel electrophoresis before use.

In some experiments the 47bp fragment between coordinates −121 and −82 was used either as a probe or as a competitor. The XmaIII-HinfI 47bp fragment from pJB3 (13) was isolated, mixed with XhoI linker oligonucleotides and ligated into the XhoI site of pΔgDCAT (22). Large scale plasmid preparations cut with XhoI yielded mg quantities of the 47bp fragment, which was purified by gel electrophoresis and quantitated by UV adsorbance. Labelled 47bp fragment probes were prepared using α-^{32}P dATP and Klenow DNA polymerase.

Gel retardation assays

A. Using intact ICP4 expressed during virus infection. Labelled oligonucleotide probes (0.5 to 1ng) were incubated for 60 min at 26°C with 3μl infected cell nuclear extract or partially purified ICP4 fraction VIII in 20μl reaction mixtures which also contained 10mM Tris-HCl (pH 7.6), 15mM KCl, 15μM EDTA, 0.1% NP40 and 250−400ng of *Escherichia coli lac* operator DNA as non-specific competitor. The procedures for separating the bound and unbound probe complexes on non-denaturing acrylamide gels have been described previously (13). The fraction of probe in the bound complex was determined by excision of the bands followed by liquid scintillation counting.

B. Using fragments of ICP4 expressed in bacteria. Suitable dilutions of bacterial extracts were mixed with approximately 0.1ng of Klenow-labelled oligonucleotide probes on ice in a buffer containing 10mM Tris-HCl pH 8.0, 1mM EDTA, 100mM NaCl, 1μg polydI/polydC and 0.1% NP40. After incubation for 20 minutes, the mixtures were applied to a non-denaturing 4% polyacrylamide gel with 0.5× TBE running buffer and run at 200V at 4°C for 2 to 3 hours. Complexed and unbound probe was detected by autoradiography of the dried gel.

Bacterial expression plasmids

The expression plasmid p585.4 (kindly provided by Dr. C. M. Preston) is a derivative of plasmid pET-8c (23) which has a NcoI and BamHI cloning sites for insertion of coding regions for expression. Plasmids pI9, pI10 and pI11 are derivatives of p175 (24) which contain EcoRI linker insertions into ICP4 coding sequences at codons 252, 275 and 292 respectively (25). The EcoRI-BamHI fragments of these plasmids, which include ICP4 sequences as far as the BamHI site at codon 523, were cloned into p585.4 between the NcoI and BamHI sites with the aid of adaptor oligonucleotides. The adaptors allowed the linkage of NcoI extensions to those generated by EcoRI and which maintained the ICP4 peptide reading frame (which is initiated at the ATG in the NcoI site). The sequences at the NcoI-EcoRI junctions were confirmed by direct sequencing of the resultant plasmids. The peptides expressed by these plasmids (pT7I9, pT7I10 and pT7I11) contain ICP4 coding sequences preceeded by five amino acids encoded in the N-terminal junction region (MARIR in pT7I9 and MAEFG in both pT7I10 and pT7I11). At the C-terminal end of these peptides there are twenty amino acids encoded by vector DNA 3′ of the BamHI site. Plasmids pT7I9X and pT7I10X are derivatives of pT7I9 and pT7I10 which have XbaI linker oligonucleotides containing stop codons in all three reading frames inserted into their BamHI sites so as to remove the vector-encoded 20 amino acid residues from the expressed proteins.

Expression of fragments of ICP4 in bacteria and their partial purification

The T7 expression plasmids were transformed into *E. coli* host BL21 (DE3) pLysS (23) and maintained as glycerol stocks. Bacterial colonies from a freshly streaked nutrient agar plate containing ampicillin at 100μg/ml were inoculated into YT-broth containing ampicillin at 100μg/ml and grown shaking at 37°C until the OD_{450} was about 0.5. IPTG was added to a final concentration of 150μg/ml and incubation continued for 1hr. The bacteria were harvested, resuspended in 1/100 volume of Tris-HCl pH 8.0, 1mM EDTA 1mM PMSF and 0.1mM DTT and frozen at −20°C. After thawing, sodium deoxycholate and lysozyme were added to final concentrations of 0.5% and 100μg/ml respectively. After lysis NaCl was added to 2M final concentration and the DNA pelleted by centrifugation at 20000 rpm for 1hr in the Sorvall SS34 rotor. The supernatants were made up to 25% saturation with ammonium sulphate and the precipitated proteins pelleted, dissolved in 50mM Tris-HCl pH 7.5, 1mM EDTA, 200mM NaCl, 0.1% CHAPS, 1mM PMSF and 0.1mM DTT. The extracts were dialysed against the same buffer (but with 0.01% CHAPS), mixed with an equal volume of glycerol and stored at −20°C.

Methylation interference assays

Either the lower or the upper strand oligonucleotide of the −109 to −92 probe was individually labelled with polynucleotide kinase, hybridised to the unlabelled complementary strand and then partially methylated with dimethyl sulphate. The probe was then incubated with infected cell extract and the ICP4 bound and unbound species separated by gel electrophoresis. The DNAs were electroeluted, treated with 1M piperidine at 90°C for 30 min and then loaded onto a 10% polyacrylamide 8M urea DNA sequencing gel.

Viruses, cells and nuclear extracts

The growth of HeLa cells and their infection with HSV-1 strain KOS has been described previously (26). Nuclear extracts were prepared by the method of Dignam et al. (27) 6 hours after infection. Partially purified ICP4 fraction VIII (a gift from Dr. K. Wilcox) was dialysed against Hepes-glycerol buffer D (27) prior to use.

RESULTS

The binding of ICP4 to site II in the gD promoter

ICP4 binding site II in the HSV-1 glycoprotein gD promoter was initially identified by an immuno binding assay, and the DNA binding region resolved by DNase footprinting (8) (Figure 1). Although there is no incontrovertible evidence that such ICP4 DNA binding sites play a specific and essential role in ICP4 function, there are situations both in vivo and in vitro in which the presence of gD site II contributes to the overall promoter activation induced by ICP4 (13, 22).

The aim of the work described here was to investigate the detailed DNA sequence requirements for binding at gD site II by ICP4. Initially, a gel mobility shift assay was used to confirm that ICP4 bound to site II. Using the 47bp XmaIII-HinfI fragment (see Methods) as probe, increasing amounts of infected cell nuclear extract produced increasing amounts of a slow moving complex (arrowed). This was shown to contain ICP4 by further retardation after incubation with the anti-ICP4 monoclonal antibody H944 (Figure 2). The specificity of ICP4 binding was indicated by the fact that the amount of complex formed could be reduced by adding unlabelled probe fragment but not by E. coli lac DNA (Figure 2, tracks I and J). Other retarded bands observed in this experiment were not further retarded by the antibody and were (at least to some extent) present in uninfected cell extracts and so they are not relevant to ICP4 binding.

Inspection of the ICP4 gD site II footprint from −115 to −75 reveals an imperfect axis of symmetry in which the core ATCGTC ICP4 binding consensus (8) is located on the 5′ side while an inverted sequence, GACGA, is located in the 3′ portion. This raises the question whether both of these sites bind ICP4 and thus contribute to the rather large region that is protected from the action of DNase. Accordingly, two oligonucleotide probes were synthesised, one containing the 5′ sequence from −109 to −92 and the other the 3′ region from −96 to −79 (Figure 3A). Gel retardation assays, using both infected cell nuclear extracts and partially purified ICP4 fraction VIII, showed that both probes formed a complex with ICP4. The 5′ oligo was bound about 3-fold more strongly than that containing the 3′ side. Formation of the ICP4 specific complexes with both oligonucleotides was efficiently inhibited by addition of the 47bp fragment (Figure 3B). We conclude that the 5′ and 3′ halves of the gD site II region can bind ICP4 independently, although a different approach would be necessary to determine exactly the relative ICP4 binding affinities of the two oligonucleotides. It should be noted that the 3′ probe also contains the distal core sequence (TGG in this instance; see below).

The fact that oligonucleotides including both the 5′ and 3′ sites bind ICP4 does not necessarily mean that both sites can be bound simultaneously. Since the sites are very close, binding to one may preclude binding to the other. Indeed, comparison of the mobility of the retarded complexes formed with site II and other

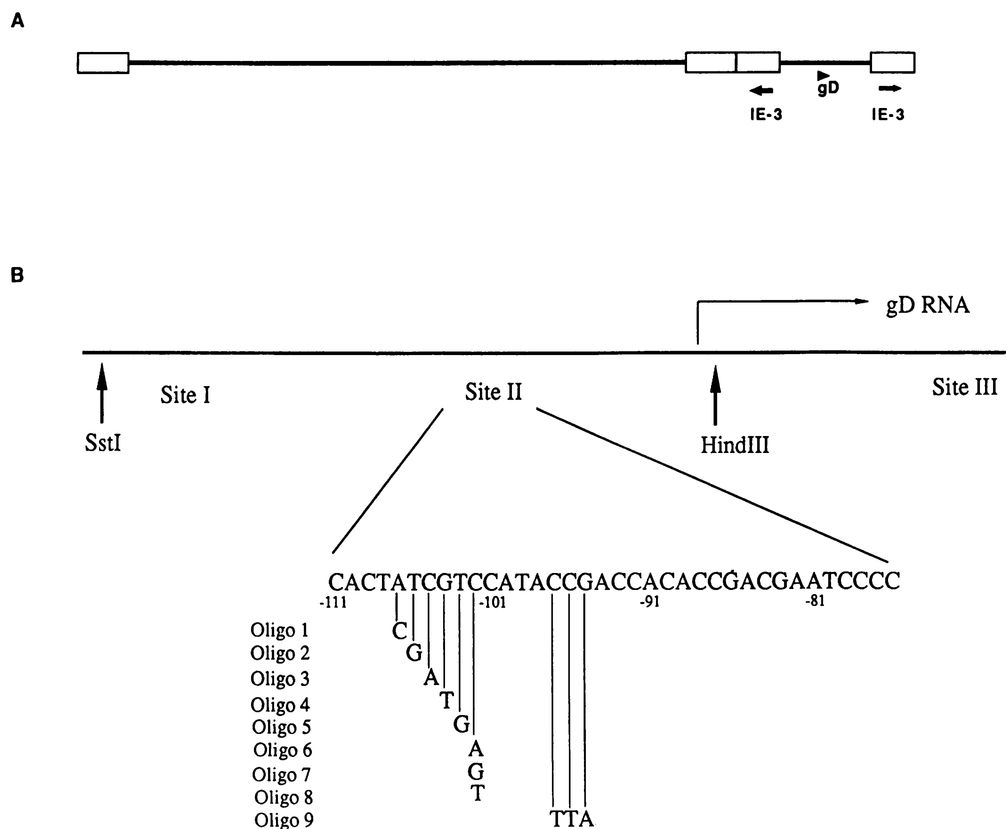

Figure 1. A. The HSV-1 genome with the location of the gD gene and the IE-3 gene encoding ICP4 is shown, with the boxed regions representing the repeats bounding the long and short unique regions of the genome. B. The HSV-1 gD promoter region with its three defined ICP4 specific binding sites between the HindIII site at +11 and the SstI site at −392. The sequence of the site II region is shown. The ATCGTC of the proximal part of the consensus starts at position −107. Mutant oligonucleotides 1 to 8 have the indicated point mutations in the ATCGTC consensus, with the complementary mutation on the other strand. Mutant oligonucleotide 9 has a triple mutation in the distal part of the consensus. Wild type and mutant oligonucleotides included bases −89 to −110 of the sequence shown here, with TCGA 5′ extensions so as to create SalI and XhoI restriction sites at their extremities.

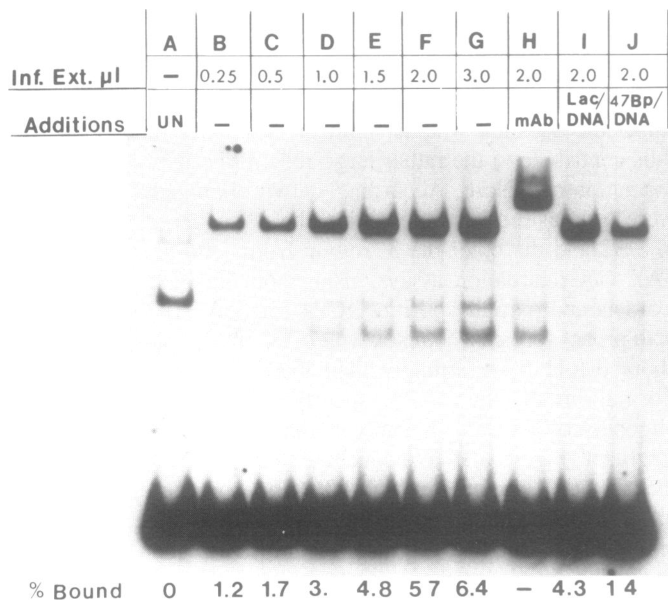

Figure 2. Gel mobility shift assay with the 47bp fragment gD site II probe. Purified XhoI-cut 47bp fragment was end-labelled and incubated with increasing amounts of infected cell extract in the presence of 3µg Salmon sperm DNA as non-specific competitor. The addition of monoclonal anti-ICP4 antibody, lac DNA (400ng) or unlabelled 47bp fragment (200ng) competitor is indicated above the appropriate lane. The percentage of DNA probe present in the ICP4 complex (which is indicated by the arrow) is given at the foot of each lane.

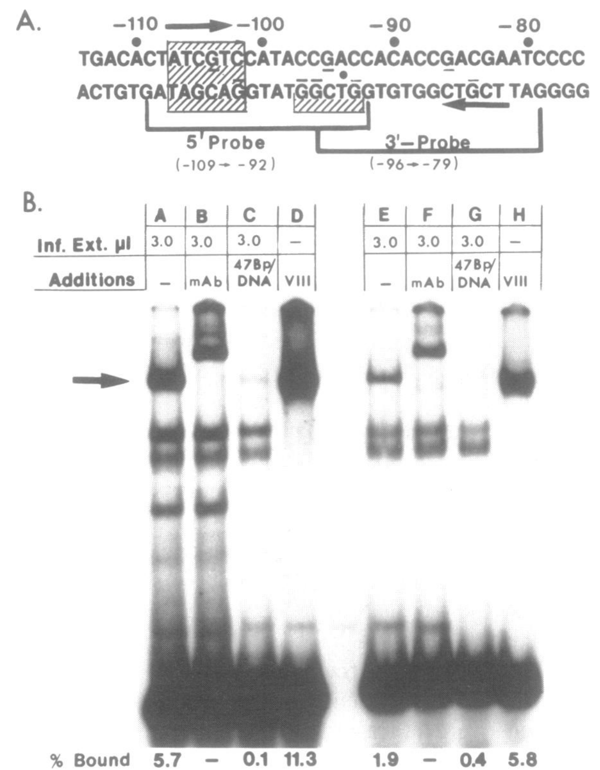

Figure 3. Gel mobility shift assay with 5′ and 3′ elements of gD site II. A. The complete sequence of the site II region is shown, with the 5′ and 3′ core elements of the consensus binding site marked by arrows. The extents of the 5′ and 3′ oligonucleotide probes are shown by horizontal lines. B. The upper strand of each synthetic oligonucleotide was labelled with γ-^{32}P ATP and polynucleotide kinase, annealed with the complementary lower strand and the double stranded probe incubated with infected cell nuclear extract or ICP4 fraction VIII. In this experiment 400ng lac DNA was used as non-specific competitor. The effects of adding anti-ICP4 monoclonal antibody (mAb) or 200ng of unlabelled 47bp fragment DNA were also determined. The percentage of oligonucleotide probe in the specific ICP4 complex (indicated by the arrow) is shown beneath each lane.

binding sites (13) suggests that these sites bind the same number of ICP4 molecules. Using a different set of binding sites, it has been shown that normally only one dimeric ICP4 molecule binds to each site (14). It would be difficult to prove whether there are situations in which both 5′ and 3′ sites in site II can be simultaneously occupied since increasing the amount of ICP4 in gel retardation assays generally results in novel lower mobility complexes (which probably contain additional ICP4 dimers), irrespective of the probe used (data not shown).

Methylation of guanosine residues in both proximal and distal parts of the consensus interfers with ICP4 binding

Methylation interference experiments with a DNA fragment from the IE-3 gene indicated that ICP4 binding involved G residues flanking the ATCGTC core (10). Figure 4 shows the result of a methylation interference assay using both top and bottom strands of a synthetic site II oligonucleotide containing sequences from −109 to −92. Decreased intensity of G residues in the tracks using DNA isolated from the ICP4 complex, compared to the free DNA, showed that methylation of some (but not all) G residues both within the ATCGTC core and in the distal part of the consensus sequence interfered with binding. The summary of the results in Figure 4c shows that the important G residues are localised on both faces of the DNA helix, illusitrating that in this instance bound ICP4 seems to wrap around the DNA.

Mutation of bases in both parts of the consensus inhibits binding by ICP4

A double stranded oligonucleotide (Figure 1) was synthesised which contains the 5′ portion of the footprinted region of the gD binding site II, which includes a single copy of the consensus (8; see above). After labelling, this probe was used in gel retardation experiments with nuclear extracts of HSV-1 infected cells. As with the 47bp fragment probe, complex formation between the oligonucleotide probe and a component of the nuclear extract was observed (Figure 5). This complex was shown to be ICP4-specific by the fact that it was not observed with extracts from mock infected cells and that it could be further retarded by the addition of an anti-ICP4 antibody (data not shown).

The sequence requirements for the binding of ICP4 to the oligonucleotide probe were investigated by synthesising a family of related probes with mutations at various positions. These mutations are shown in Figure 1. The results (Figure 5) show that mutation of the first five positions of the ATCGTC consensus resulted in large reductions in ICP4 complex formation (lanes B to F). However, mutation of the final C to an A had little effect (compare lanes A and G). The sequence requirement at this position was further investigated using oligonucleotides with changes to G or T. Again, the results indicate that these changes did not severely affect binding by ICP4 (lanes H and J). It is interesting that methylation of the G residue on the other strand at this position in the normal sequence interfered with ICP4 binding (Figure 4) whereas alteration of this base did not. Therefore the interference with binding by methylation at this position is more likely to be due to steric effects rather than a requirement for recognition of a specific base. An oligonucleotide with three changes in the distal part of the consensus sequence

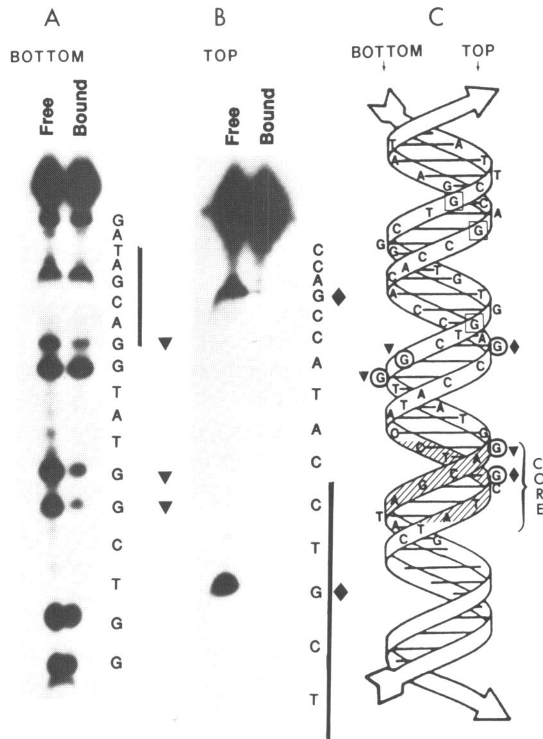

Figure 4. Methylation interference of ICP4 binding to gD site II. A synthetic double stranded oligonucleotide of the site II region was singly end- labelled on the lower strand (panel A) or upper strand (panel B) and partially methylated with dimethyl sulphate. The probe was then incubated with infected cell extract and the free and ICP4 bound species separated by gel retardation. After elution from the gel and treatment with piperidine, equalised counts of the free and unbound probes were analysed on a 10% acrylamide DNA sequencing gel. The sequence of the probe is shown next to the autoradiograph, with the ATCGTC core consensus marked by vertical lines. Part C shows the helical 3 dimensional structure of the site II region, with the important G residues marked in circles. Residues marked in boxes represent the Gs in the 3′ side of the site II sequence which are in positions corresponding to the critical Gs in the 5′ sequence. The proximal core consensus is shown as a shaded section of the helix. The G residues implicated in binding are marked with filled triangles or diamonds.

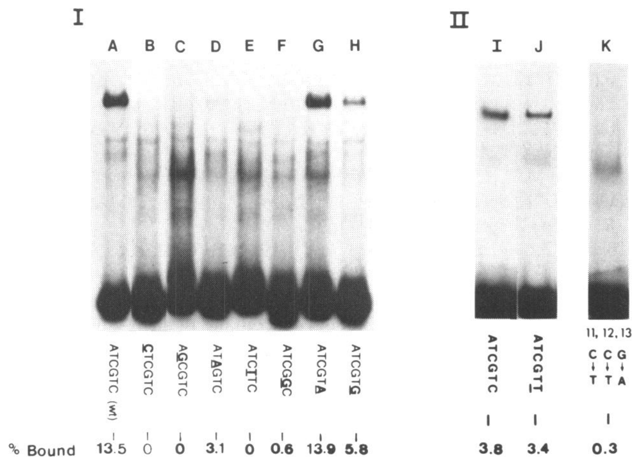

Figure 5. Effect of point mutations in the gD site II region on binding of ICP4. Mobility shift assays were conducted with oligonucleotide probes containing point mutations in the site II region, using 5μg of nuclear extract and 250ng 40bp lac non-specific competitor DNA. The ICP4 DNA complex is indicated by an arrow. The fraction of oligonucleotide probe in the ICP4 complex is indicated below each lane.

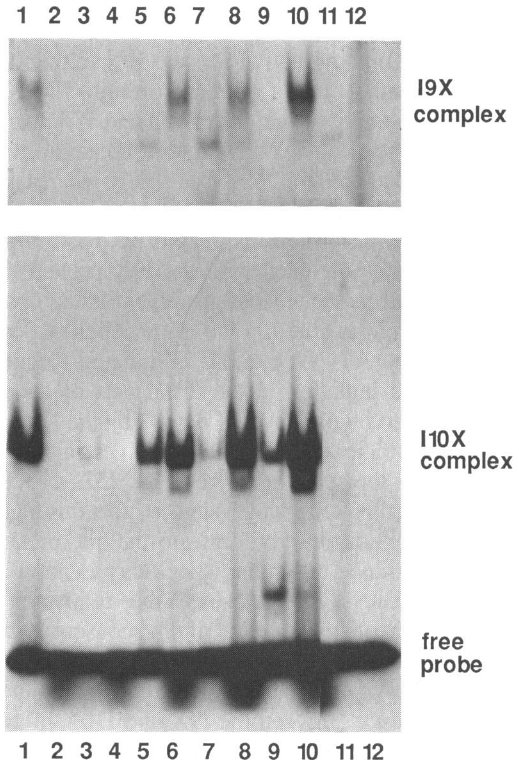

Figure 6. Binding of bacterial expression peptide I9X and I10X to gD site II oligonucleotide probes. The lower panel shows gel retardation experiments using I10X protein with (tracks 1 to 10 respectively) the wild type oligonucleotide probe and mutant oligos 2−6, 1, 7, 9 and 8 respectively. Track 11 contains the wild type probe with an extract containing the I11X protein and track 12 probe alone. The free probe and the specific retarded complex bands are marked. In the upper panel the same probes have been used with an extract containing the I9X polypeptide. Only the region of the gel with the specific retarded bands is shown.

did not form a complex with ICP4 (Figure 5, lane K). This illustrates the importance of both the proximal (ATCGT) and distal (YCG) parts of the consensus and indicates that not all sequences which include the ATCGTC motif will be bound by ICP4. This finding is consistent with a previous observation (20).

Alterations in the DNA binding domain of ICP4 can affect the specificity of its binding to DNA

A combination of mutational and functional studies have shown that the DNA binding domain of ICP4 lies within amino acid residues approximately 250−500 (16−18, 28, 29). As a first step towards identifying in more detail residues within this region which are directly involved in specific DNA recognition, fragments of this coding region were inserted into a bacterial expression vector and the resultant ICP4 peptides partially purified (as described in the Methods section). The ICP4 related proteins expressed by pT7I9X, pT7I10X and pT7I11 contain residues 252−523, 276−523 and 293−523 respectively; all were expressed in amounts sufficient to be visible on an acrylamide gel stained with Coomassie Blue (data not shown).

Gel retardation experiments using extracts containing the protein expressed by pT7I9X gave a similar spectrum of DNA binding specificity to that of intact ICP4 (Compare Figures 5 and 6). Mutations in the first five positions of the ATCGTC motif, and the mutation in the distal portion of the consensus, all greatly reduced binding. Extracts containing the protein expressed by

plasmid pT7I11, encompassing ICP4 residues 293–523, failed to form a complex with the probes (data not shown). This illustrates that the binding activity in the I9X extract is due to the I9X protein (rather than a bacterial protein). These results are consistent with the observations of Wu and Wilcox (28) who showed that a bacterial fusion protein containing residues 262–490 was able to bind to DNA specifically whereas a shorter fusion including residues 306–490 was not.

The results with the protein expressed by plasmid pT7I10X were similar to those obtained with the I9X protein, but with some intriguing differences. Most prominent is the observation that the I10X protein is able to bind quite efficiently when the fifth position of the ATCGTC motif is mutated (Figure 5). In addition, the triple mutation in the distal part of the complex apparently abolished binding by I9X but not by the I10X protein. Therefore the specificity of DNA sequence recognition has been slightly altered by the removal of residues 252- 275 from the bacterially expressed protein, which suggests that this highly basic part of the polypeptide may be close to the site of nucleotide contact. This difference in binding specificity is not a result of expression in bacteria *per se* since the relaxation of the requirement for the fifth position of the consensus was also observed using the fragment of virally expressed ICP4 which can be liberated (29) by proteinase k treatment (data not shown).

A further difference between the I9X and I10X proteins was that I9X was more difficult to isolate, with a greater percentage being lost in the insoluble pellet. Therefore I10X extracts contained significantly more expression protein than I9X extracts; this probably explains (at least in part) why the I10X retarded bands were more readily detectable (Figure 6).

DISCUSSION

The results presented in this paper show that both proximal and distal parts of the ICP4 consensus DNA binding site are important for specific binding but that strict conservation of the core ATCGTC motif is not. It is also apparent that not all guanosine residues which are identified as part of the ICP4 binding site by methylation interference are actually involved in sequence specific recognition. These results confirm and extend previous observations that the first two bases of the ATCGTC motif are important for binding (16, 20) and that the ATCGTC motif of itself is insufficient to allow binding by ICP4 (20). This information will allow more accurate prediction of the location of ICP4 binding sites within the HSV-1 genome, which will in turn be important in determining their role.

How the type of consensus site investigated here relate to other less obviously related binding sequences is unclear. Michael and Roizman (14) have identified two sites in the upstream portion of the IE-3 promoter which apparently do not conform to the ATCGTC consensus. However, examination of one of these sites (which can be written as ATGGGCnnnnCCGG) in the light of our data suggests that it may not be unusual. Both changes from consensus in the ATCGTC motif are at positions where mutations in gD site II did not totally eliminate binding (Figures 5 and 6). The other (which can be written as GCCGGCnnnnCGG) is less similar to the ATCGTC consensus. However, other such GC-rich ICP4 binding sites have also been detected in the gC promoter region (our unpublished results). A strict comparison of these apparently different classes of site requires careful determination of relative binding constants, and a more thorough mutagenic study will be necessary for further determination of the precise DNA sequence requirements for binding by ICP4.

The observation that the bacterial expression protein I9X has a sequence requirement for DNA binding essentially indistinguishable from that of authentic ICP4 reinforces a previous conclusion (28) that post translational modification has no apparent role in DNA binding by ICP4. Therefore, the bacterial expression system should prove ideal for the determination of the precise amino acid residues of ICP4 that are involved in sequence specific recognition. The results with the I10X protein suggest that these sequences may lie close (either in sequence or in three dimensional structure) to residues 253–272 (which affect specificity) and residues 273–291 (which are required for binding). It is intriguing that these segments of ICP4 are just outside a region which is very highly conserved in all members of the ICP4 family of proteins (30–32). Therefore differences in amino acid sequence in the less highly conserved part of the DNA binding domain could contribute to the differences in detail in the properties and functions of these proteins, through alteration of their DNA binding properties.

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