We demonstrate that the immediate early 175K protein (IE175K) of herpes simplex virus type 1 binds to the cap site of the latency-associated promoter (LAP) in an unusual manner. The complex formed on the LAP cap site was significantly larger than that formed on the IE175K cap site and the requirements for binding were qualitatively distinct with respect to both the primary sequence determinants at the site, and the regions of IE175K protein required for binding compared to those for the IE175K cap site. Although purified IE175K was sufficient for this larger complex formed on the LAP cap site, the DNA-binding domain was unable to bind efficiently. This contrasted strikingly with the IE175K cap site where, using precisely analogous probes, the DNA-binding domain exhibited a strong interaction. Surprisingly, from dissociation kinetics we show that binding of the intact protein to the LAP cap site is considerably more stable than the binding of IE175K to its own cap site (half-lives of the complexes 15 min and < 1 min respectively), and this was reflected in more efficient repression of LAP-driven expression than IE175K promoter-driven expression by IE175K. Moreover, primary sequence requirements for IE175K binding to the LAP cap site region differed from previously identified IE175K recognition sequences in that in addition to a partially conserved consensus sequence, neighbouring bases were necessary for binding. Although the LAP cap site exhibits a pseudopalindromic arrangement of core consensus sites, we show that this is not the basis for the higher order, more stable binding to this region. Together these results indicate that IE175K forms an unusual complex at the LAP cap site, broadening the range of previously defined sequences recognized by IE175K.

Introduction

Herpes simplex virus (HSV) establishes a latent infection within the neuronal cell population of sensory ganglia which innervate primary sites of HSV infection (reviewed by Wildy et al., 1982; Hill, 1985; Roizman & Sears, 1987). During the latent phase of infection there is a dramatic qualitative change in virus gene expression from that operating in productively infected cells, and transcription is restricted to a single unit which initiates within the long repeat region of the genome. The most abundantly expressed transcripts, the latency-associated transcripts (LAT), are transcribed from a short region partially overlapping the 3' end of the immediate early 110K gene in an antisense direction (Rock et al., 1986, 1987; Croen et al., 1987; Deatly et al., 1987; Spivack & Fraser, 1987; Stevens et al., 1987, 1988; Gordon et al., 1988; Wagner et al., 1988; Wechsler et al., 1988). There is, however, evidence to indicate that the LAT species are the processed products of a large unstable primary transcript which initiates 660 bases upstream from the LAT region (Dobson et al., 1989; Mitchell et al., 1990a, b, c; Zwaagstra et al., 1990; Devi-Rao et al., 1991). Indeed, it has been proposed that the LAT species are stable introns derived from the primary transcript (Devi-Rao et al., 1991; Farrell et al., 1991). Consistent with the location of the 5' end of the primary transcript, we and others, using transient expression assays, have demonstrated relatively strong promoter activity in this upstream region (Zwaagstra et al., 1989, 1990; Batchelor & O'Hare, 1990, 1992; Devi-Rao et al., 1991). In addition, we have identified fragments within this promoter (termed the latency-associated promoter, LAP) which selectively functioned in a human neuroblastoma cell line, IMR-32 (Batchelor & O'Hare, 1992).

One striking feature of our earlier results was the finding that, in cotransfection experiments, LAP was very efficiently repressed by the HSV-1 immediate early 175K protein (IE175K, also termed ICP4 or Vmw175). IE175K is an essential regulatory protein, which plays a pivotal role in the activation of early and late genes, and is also involved in the negative regulation of its own expression (Preston, 1979; Dixon & Schaffer, 1980; Watson & Clements, 1980; DeLuca & Schaffer, 1985; O'Hare & Hayward, 1985a, b). Mutational analysis of the IE175K protein has revealed that it is modular in
nature, and contains a DNA-binding domain in addition to regions specifically required for trans-activation (Muller, 1987; DeLuca & Schaffer, 1988; Faber & Wilcox, 1988; Paterson & Everett, 1988a, b; Shepard et al., 1989; Wu & Wilcox, 1990; Everett et al., 1991; Pizer et al., 1991). IE175K binds to DNA sequences that contain the consensus core motif ATCGTC (Faber & Wilcox, 1988; DiDonato et al., 1991; Everett et al., 1991), but also binds to a variety of sequences that have diverged from the consensus (Michael et al., 1988; Tedder & Pizer, 1988; Michael & Roizman, 1989; Imbalzano et al., 1990; DiDonato et al., 1991; Papa- vassiliou et al., 1991). Although the role of IE175K–DNA binding in the trans-activation of early/late genes has not been conclusively resolved (Paterson & Everett, 1988a, b; Tedder & Pizer, 1988; Shepard et al., 1989; Imbalzano et al., 1990; Shepard & DeLuca, 1991a, b; Smiley et al., 1991, 1992), IE175K–DNA binding is directly involved in autoregulation. In cotransfection experiments, under conditions in which a variety of promoters were unaffected or activated by IE175K, the IE175K promoter was repressed by its own gene product (O’Hare & Hayward, 1985b; Roberts et al., 1988). Autoregulation of the IE175K promoter was dependent on IE175K binding to an ATCGTC motif which overlaps the transcription initiation site (Muller, 1987; Paterson & Everett, 1988a, b; Roberts et al., 1988; DiDonato & Muller, 1989; Kattar-Cooley & Wilcox, 1989).

We have previously demonstrated that LAP was downregulated by IE175K. In cotransfection experi-
ments LAP was efficiently repressed by IE175K and deletion of LAP cap site sequences effectively abolished IE175K repression (Batchelor & O'Hare, 1990). To extend these results, in this study we have used probes spanning the LAP cap site sequences to demonstrate IE175K binding to the region. Surprisingly, in comparative analysis, IE175K formed a significantly larger complex on the LAP cap site (termed C2) compared with the complex formed on the IE175K cap site (C1). Mutational analysis of the nucleotide sequence requirements for C2 formation suggested that the LAP cap site could not be considered a ‘conventional’ IE175K binding site, broadening the range of previously defined nucleotide sequences which are recognized by IE175K. Although purified intact IE175K was capable of forming a stable C2 complex, the IE175K ‘DNA-binding domain’ was unable to interact efficiently with the LAP cap site probe. We propose a model to explain the novel manner in which IE175K forms a complex at the LAP transcription initiation site where, unlike at the IE175K cap site, a higher order oligomer, probably tetrameric, is obligatory but the result is a complex with significantly more stable binding.

**Methods**

*Cell extracts and gel retardation assays.* HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 10% newborn calf serum. Subconfluent cell monolayers were infected with HSV-1 strain MP at an m.o.i. of 10, and harvested 8 h after infection. Whole-cell extracts were made as described previously (Batchelor & O’Hare, 1992) except that only 0.2 mg of lysine chloroform/methyl ketone was used as an additional protease inhibitor.

Gel retardation analyses were performed as described previously (Batchelor & O’Hare, 1992) except that binding reaction mixtures contained 150 mM-NaCl unless otherwise indicated in the figure. Extracts were preincubated with poly(dI–dC) for 5 min and then for 30 min with probe at room temperature. DNA-binding species were separated from free probes by electrophoresis in 4% polyacrylamide gels. Autoradiographic analysis of equal amounts of the probes after electrophoresis in 4% polyacrylamide gels was used to ensure that the specific activities of the DNA probes used in this work are as follows.

**LAP cap site oligonucleotides:**

A. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

B. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

C. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

D. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

E. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

**HSV-2 LAP cap site oligonucleotides:**

M. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

**IE175K cap site oligonucleotides:**

R. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

L. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

**HSV-2 LAP cap site oligonucleotides:**

R. 5' -CTAGGGGGGGCGCGGCCGTGCCGATCGC GG GTGGTGCGAAAGACT TCCCCCGCGCGCGCAGGCCAATTTGCTCGACCACTCTTTGAGATC-5'

Specific alterations from the wild-type sequence are indicated by underlining. Annealed probes were radio labelled with [α-32P]dCTP using the Klenow fragment of Escherichia coli DNA polymerase I and purified by electrophoresis in 12% polyacrylamide gels. Autoradiographic analysis of equal amounts of the probes after electrophoresis in polyacrylamide gels was used to ensure that the specific activities of the labelled probes did not vary significantly.

**Plasmid constructions.** pBB-13 contains the LAP PstI promoter fragment fused to the chloramphenicol acetyltransferase (CAT) open reading frame (ORF) (Batchelor & O’Hare, 1990). Mutations were introduced into pBB-13 promoter sequences by subcloning the PstI fragment into pTZ vectors (Mead et al., 1986) and performing oligonucleotide-directed mutagenesis on single-stranded derivatives with a Bio-Rad Muta-Gen kit. Mutagenesis of the resulting promoter–CAT plasmids, pBB-54, pBB-55 and pBB-56, was confirmed by dideoxynucleotide sequencing of alkali-denatured plasmids as described previously (Batchelor & O’Hare, 1990).

The IE175K expression vector, pBB-37, contains the IE175K ORF from HSV-1 strain KOS under the control of the human cytomegalovirus immediate early (HCMV IE) promoter. pBB-37 was constructed by inserting a 5 kbp XhoI–SalI fragment from pXhoI-C (O’Hare & Hayward, 1985) into pBB-13 and performing BspI–SalI fragment cloning into pMB-37. The BspI site in pMB-37 is in the IE175K leader (position 146987). The Drai site is close to the long repeat–short repeat boundary (position 361). Downstream of the IE175K polyadenylation sequence, pCMV19A contains the HCMV IE promoter fused to downstream polylinker sequences and is a derivative of pCMV19 in which the ATG initiation codons within the polylinker SpeI site was removed by mung bean nuclease treatment and religation. pCMV19 was constructed by inserting the HindIII–EcoRI polylinker
**Fig. 2.** (a) Probes used to analyse DNA sequence requirements for formation of the LAP–IE175K complex. Probe A contains two potential IE175K binding sites (dashed arrows) in addition to a potential Spl binding site. Probe H is the HSV-1 strain 17+ or F equivalent of probe A, with a G residue in place of a C in the downstream IE175K binding site homology. Probes I, O and P are variants of probe A substituted at two consecutive bases. Mutated sequences are underlined. The probe J sequence largely overlaps A but lacks sequences in the region of the putative Spl binding site. Probe M spans a region of HSV-2 LAP which is homologous to the transcription initiation region of HSV-1 LAP. Conserved residues are indicated by an asterisk. In probe L the right-hand portion of probe K is repeated to create a palindromic sequence where the centre of symmetry of the palindrome is in between the central A and T residues, resulting in two IE175K binding sites positioned in the same manner as in the LAP cap site probe A. (b) Binding of the probes described above to infected HeLa extracts. Gel retardation assay in which binding reaction mixtures contained 100 mM-NaCl and were pretreated with 8 pmol of Spl competitor (approximately 400-fold molar excess over the amount of radiolabelled probe). Spl competitor was added so that C1 complex formation on probe A derivatives was not obscured. Duplicate assay mixtures were incubated in the absence (odd-numbered lanes) or presence (even-numbered lanes) of the anti-IE175K monoclonal antibody 58S. Complexes were
fragment from pUC19 into pCMV-IL2 (Cullen, 1986) digested with HindIII and EcoRI (R. Greaves, unpublished data).

Transfection procedures and CAT assays. HeLa cells were grown and transfected as described previously (Batchelor & O'Hare, 1990, 1992). In the figures, the amounts of radioactivity appearing in the acetylated products are given below each lane (10^3 c.p.m.).

Results

Binding of IE175K to LAP cap site sequences

We have shown previously that expression from the LAP of HSV-1 was efficiently repressed by the IE175K protein and predicted that this was likely to be due to direct IE175K binding around the initiation site (Batchelor & O'Hare, 1990). Fig. 1 illustrates the nucleotide sequences around the transcription initiation sites of the LAP and the IE175K promoter. The location of the IE175K recognition sequence at the IE175K cap site has been resolved by a variety of techniques including footprinting, missing contact analysis and systematic substitution (Faber & Wilcox, 1988; DiDonato & Muller, 1989; DiDonato et al., 1991; Everett et al., 1991) and has been demonstrated to span the bases ATCGTCCACA-CGG (indicated by the solid arrow in Fig. 1a). A consensus IE175K recognition site of the form ATCGTCCACAN(Y/C)G (n is any base) has been proposed in several studies (Faber & Wilcox, 1988; DiDonato et al., 1991; Pizer et al., 1991). Two potential IE175K binding sites in LAP, which are similar to the consensus and form an imperfect inverted palindrome, are indicated by dashed arrows (Fig. 1a). After examining the sequences of 12 known IE175K binding sites DiDonato et al. (1991) proposed a matrix mean model for predicting IE175K recognition sequences. By their criteria, both of the sequences in LAP could be weak IE175K binding sites, and the site oriented in the opposite direction to the promoter (upstream of the cap site) would be the stronger of the two sites.

To determine whether IE175K did in fact bind to the LAP cap site region, we synthesized probe A, the sequence of which spanned both of the potential IE175K binding motifs with the shared AT residues at the centre (Fig. 1a) and tested its binding using a nuclear extract of HSV-1-infected cells. For comparative purposes, we examined binding to probe K which contained sequences from the IE175K promoter cap site (Fig. 1a). Probes A and K were designed to be of equal length and have the AT of the core motif positioned centrally. Certain results have previously indicated salt lability of IE175K DNA-binding complexes (Muller, 1987; DeLuca & Schaffer, 1988) although in other studies IE175K complexes were stable at least up to 300 mM salt (Paterson & Everett, 1988a, b). We therefore initially examined binding of probes A and K at a range of salt concentrations from 50 to 250 mM. The results show that at each of the salt concentrations between 100 and 250 mM the IE175K cap site probe K bound a major complex, C1, with no significant decrease in binding at the higher concentrations (Fig. 1b). A non-specific band (n.s.) was also detected but was not consistently observed and was not out-competed by unlabelled probe K (see below). In contrast, the major complex formed with probe A, C2, had a much slower mobility and was less stable at the highest salt concentrations (200 and 250 mM-NaCl). Relatively minor amounts of a complex that comigrated with C1 were also observed with probe A. At the lowest salt concentration tested (50 mM-NaCl) neither probe formed C1 or C2 and probe A formed a complex with a higher mobility component. It is likely that this component may be Sp1, since probe A contains a G/C-rich region at its 5' end, it comigrated with Sp1 bound to previously characterized sites and it was out-competed by oligonucleotides containing Sp1 binding sites (data not shown). However this factor is not involved in C2 formation since LAP probes lacking the G/C-rich 5' region were still able to form C2 (see Fig. 2). In all subsequent experiments binding reactions were performed in 100 or 150 mM-NaCl.

To determine whether the complexes observed with probes A and K contained IE175K protein, antibody super-shift experiments were carried out as indicated in Fig. 2(b). After the addition of anti-IE175K monoclonal antibody 58S, as expected the probe K complex, C1, was super-shifted to give complex C1s (Fig. 2b, lanes 15 and 16). (Note that the low level non-specific complex was unaffected by the antibody.) With probe A the abundant C2 complex was also shifted to give the complex C2s which barely entered the gel (Fig. 2b, lanes 1 and 2). A small amount of C1 could be detected with probe A and this was also shifted to give C1s. C1s was more abundant than C1, possibly due to 58S stabilizing IE175K binding to probe A in the C1 configuration. Complexes C1 and C2 were not detected in mock-infected HeLa cell extracts, but were detected in extracts of cells transfected with an IE175K expression vector (data not shown) and with purified IE175K protein (see below). These results

separated by electrophoresis in polyacrylamide gels for 3 h by which time free probes had run off the end of the gel. In this experiment probes A, H and I had approximately twofold lower specific activities compared to the other probes. Lanes 1 and 2, probe A; lanes 3 and 4, probe H; lanes 5 and 6, probe I; lanes 7 and 8; probe O; lanes 9 and 10, probe P; lanes 11 and 12, probe J; lanes 13 and 14, probe M, lanes 15 and 16, probe K; lanes 17 and 18, probe L.
indicate that IE175K formed two different complexes on probes A and K. At the IE175K cap site, IE175K formed a previously detected complex, C1, and little if any C2, whereas IE175K was associated with LAP cap site sequences in a considerably larger complex, C2, and only minor amounts of C1 were observed.

**Mutational analysis of LAP–IE175K complex formation**

To examine the DNA sequence requirements for C2 formation and the basis for the difference between the major species observed on the LAP versus the IE175K cap site regions, we analysed binding to a series of oligonucleotides containing mutations in probe A (Fig. 2a). The sequence homologies to the IE175K binding consensus are indicated by dashed arrows. Specifically we wished to test the possibility that the formation of the larger C2 complex was due to the pseudopalindromic nature of the LAP site alluded to above. Therefore double substitutions at positions within the core sequence shown to be critical for IE175K binding (Everett et al., 1991) were used to disrupt either the upstream homology, probe O, or the downstream homology, probe P, around the LAP cap site. In probe I the central AT was mutated, disrupting both of the IE175K consensus homologies. Furthermore it should be noted that probe A contains the LAP sequence of HSV-1 strain KOS (Batchelor & O’Hare, 1990) which differs by one nucleotide from the LAP sequences of HSV-1 strains 17+ and F, in that the sequence ATCGCC is changed to ATCGCG in the latter two strains (Perry & McGeoch, 1988; Wechsler et al., 1989). This C to G transversion would disrupt the downstream IE175K binding site homology according to the matrix mean model of DiDonato et al. (1991). Probe H, incorporating this transversion, was therefore used to test whether C2 formation was unique to the HSV-1 strain KOS sequence. Finally the probe A variant, probe J, which lacked the potential Sp1 site indicated in Fig. 2(a) was tested to ensure that the 5’ G/C-rich region was not required for C2 formation.

The probes described in Fig. 2(a) were examined for their ability to bind to IE175K in infected HeLa cell extracts (Fig. 2b). Probe J, which lacked the putative Sp1 binding site (Fig. 2a) and no longer bound to Sp1, was still capable of forming complex C2 (Fig. 2b, lanes 13 and 14). In the experiments with the remainder of the probes which contained the 5’ G/C-rich segment, extracts were pretreated with excess Sp1 competitor so as to minimize obscuring of the C1 and C2 complexes by Sp1. As anticipated, substitution in the central AT positions completely abolished C2 formation (Fig. 2b, probe I, lanes 5 and 6). With respect to the pseudopalindromic nature of the LAP site, the variant probe H promoted C2 formation with equal efficiency compared with probe A (Fig. 2b, lanes 3 and 4) indicating IE175K binding to the LAP cap sites of HSV-1 strains 17 and F despite the substitution within the downstream homology. Furthermore, the double substitutions in probe P which were designed to disrupt this downstream homology had no effect on C2 formation (Fig. 2b, lanes 9 and 10). In contrast, probe O, in which the upstream homology was disrupted, no longer formed complex C2, although a weak IE175K interaction with probe O stabilized by 58S could be observed comigrating with C1s complexes (Fig. 2b, lanes 7 and 8). Therefore an intact upstream IE175K binding consensus homology is necessary for C2 formation whereas a downstream homology is dispensable. These results were supported by a series of competition experiments wherein oligonucleotides A, H and P competed with equal efficiency for IE175K, whereas oligonucleotides I and O were ineffective competitors (data not shown).

To provide further evidence that the formation of the larger C2 complex on the LAP probe did not involve the pseudopalindromic arrangement of consensus sites we synthesized probe L in which the core site within the IE175K cap site probe was repeated to form an inverted palindrome (Fig. 2a). The centre of symmetry of probe L, between the central AT, was positioned so as to mimic the inverted palindrome at the LAP cap site. As expected, the IE175K promoter probe, K, bound to infected HeLa extracts to form complex C1. However, repeating the IE175K binding motif in probe L had no effect on the binding profile, and did not result in the appearance of the C2 complex (Fig. 2b, lanes 15 to 18). These results, together with those showing efficient formation of the C2 complex on the mutant probes P and H, indicate that C2 formation does not simply result from the pseudopalindromic arrangement of core motifs around the LAP cap site region.

The TATA box and LAP cap site region are within one of the blocks of more highly conserved sequence between HSV-1 and HSV-2 at the LAT locus. At the consensus core IE175K binding site there are eight consecutive bases which are identical between HSV-1 and HSV-2 (Krause et al., 1991; McGeoch et al., 1991) and this conserved region coincides with the potential upstream IE175K binding sequence in probe A. We therefore wished to determine whether binding would be observed in the analogous region of the HSV-2 LAP and the corresponding probe, M, was synthesized and tested in parallel with probe A. The asterisks below probe M (Fig. 2a) indicate the bases conserved between the HSV-1 and HSV-2 sequences. Surprisingly, probe M was completely deficient in C2 and C1 complex formation (Fig. 2b, lanes 13 and 14), and was unable to compete for IE175K even at high concentrations (data not shown). However the precise position of the HSV-2 LAP
promoter activity from pBB-13 was efficiently repressed we have shown previously (Batchelor & O'Hare, 1990), were tested for repression by cotransfection with the IE175K expression vector, pBB-37. The total amount of transfected DNA was equalized in all cases by addition of pUC19 DNA. Cells were harvested 40 h after transfection and assayed for CAT activity. The incorporated radioactivity is listed under each lane.

transcription initiation site has not yet been mapped and this result does not necessarily indicate the absence of binding of IE175K, or its HSV-2 homologue, to the HSV-2 LAP cap site (see also Discussion). The result does indicate though that the sequences conserved in probes A and M were not sufficient for IE175K binding.

Correlation of IE175K binding in gel retardation assays with LAP repression in cotransfection studies

C2 complex formation was normal on the mutant probe P, which lacked the downstream homology to the IE175K consensus binding site, but was abolished on mutant probe O which lacked the upstream homology and probe I in which both consensus sites were disrupted. To determine whether these in vitro binding results correlated with IE175K repression in cotransfection experiments, the corresponding mutations were made in the LAP–CAT construct pBB-13, pBB-13 contains the LAP sequences spanning the transcription initiation site (corresponding to the sequence in probe A), as well as upstream elements sufficient for strong promoter activity in HeLa cells (Batchelor & O'Hare, 1990). Derivatives of pBB-13 were constructed by site-directed mutagenesis to give the variants pBB-54, pBB-55 and pBB-56 which have LAP cap site sequences corresponding to probes I, O and P respectively. The wild-type and mutant versions were tested for repression by cotransfection with the IE175K expression vector, pBB-37.

The results of a typical assay are shown in Fig. 3. As we have shown previously (Batchelor & O'Hare, 1990), promoter activity from pBB-13 was efficiently repressed by IE175K resulting in a 20-fold decrease in expression (Fig. 3, lanes 1 and 2). In contrast, pBB-54 which contained transversions at the central AT bases, eliminating both consensus sites, was virtually unaffected by IE175K, although a slight (1.7-fold) decrease was observed (Fig. 3, lanes 3 and 4). We have observed decreases of this level on independent promoters completely lacking any homology to the IE175K binding site and it may result from a non-specific effect. pBB-55, which contained transversions corresponding to probe O selectively disrupting the upstream site while retaining the downstream site, exhibited inefficient repression, similar to that obtained with pBB-54 (Fig. 3, lanes 5 and 6). By comparison, pBB-56, in which the downstream homology was now disrupted but the upstream homology retained, was clearly repressed by IE175K, although the degree of repression (sevenfold) was less than that observed with the wild-type construct pBB-13 (Fig. 3, lanes 7 and 8). Over the course of this work repression of pBB-56 was on average twofold less efficient than repression of pBB-13 but was still at least as efficient as that obtained on the IE175K promoter itself (Batchelor & O'Hare, 1990; data not shown). In contrast, pBB-54 and pBB-55 were no longer significantly repressed by IE175K. These results are consistent with those from the gel retardation studies and indicate that the IE175K binding results correlated very closely with the repression. The constructs pBB-13 and pBB-56 were repressed by IE175K, and the corresponding probes A and P could form complex C2 whereas pBB-54 and pBB-55 were not efficiently repressed by pBB-13, and probes I and O no longer formed C2 and could not compete for IE175K binding.

Examination of the sufficiency of the upstream IE175K binding motif in probe A to form complex C2

The results shown in Fig. 2 revealed that mutations in the upstream IE175K binding site homology in probe A could abolish formation of the IE175K complex, C2. To determine whether this consensus site was sufficient for IE175K binding, we tested binding of IE175K to two variant probes. Firstly, in probe Q all of the sequence of the left-hand side of the parent LAP probe A, including the upstream consensus site, was retained but multiple substitutions were incorporated on the right-hand side of the central AT (Fig. 4a, probe Q). In parallel comparisons, while the major complex C2 and minor complex C1 were observed with probe A, the mutations on the right-hand side (probe Q) virtually abolished formation of both complexes (Fig. 4b). An extremely faint C1 complex could just be detected with probe Q and was more clearly observed in the presence of 58S antibody (Fig. 4b, compare probe Q, −/+ 58S, lanes 3 and 4, respectively). This result indicates that specific bases on
(a) HSV-1 LAP

\[
\text{ctAGaGGGGGCGGCCGGCGTGC} \quad \text{ctAGaGGGGGCGC~GGCCGT} \quad \text{GCGAAGACTcTag}
\]

Probe A

\[
\text{ctAGaGGGGGCGGCCGGCGTGC} \quad \text{ctAGaGGGGGCGC~GGCCGT} \quad \text{GCCGATCGCCGGTGCGAAAGACTcTag}
\]

Probe Q

IE175K promoter

\[
\text{ctagaGAGCCCGAGGACGCCCCGATCGTCCACACGGAGCGCGGCTctaG}
\]

Probe K

\[
\text{ctagaGAGCCCGAGGACGCCCCGATCG} \quad \text{ctagaGTcgcaggtcTag}
\]

Probe R

(b) 

![Gel retardation assay](image)

Fig. 4. (a). Diagram of probes used to determine whether the similarity to the IE175K consensus sequence on the upstream side of probe A was sufficient for the formation of complex C2. The upstream IE175K binding site similarity is indicated by the dotted arrow. Probe Q contains a series of substitutions in the region downstream of the LAP cap site. The extent of the substitutions is underlined and individual alterations are indicated in lower case. In probe R, the upstream IE175K binding homology from LAP has been placed in the context of the IE175K cap site, i.e. transferred into probe K. (b) Binding of probes Q and R to infected HeLa extracts. Gel retardation assays were performed as in Fig. 2. Anti-IE175K antibody, 58S, was added to the samples in even-numbered lanes. Complexes were separated in polyacrylamide for 3 h, by which time free probe had run off the end of the gel. Lanes 1 and 2, probe A; lanes 3 and 4, probe Q; lanes 5 and 6, probe R; lanes 7 and 8, probe K.

the downstream side of the LAP cap site are necessary for IE175K binding and C2 formation but, on the basis of normal complex formation with mutant probes H or P, this does not reflect the requirement for an additional IE175K consensus site.

Secondly we transferred the upstream consensus site, which from the result with mutant probe O is clearly a required site for C2 formation, from the LAP probe to the context of the IE175K cap site probe (Fig. 4a, dashed arrow in probe A transferred to probe K to give probe R). This effectively results in the substitution of six bases of the IE175K cap site region but transfers the important LAP cap site motif to the context of a previously characterized IE175K binding site. Strong C1 (but no C2) formation was observed with probe K (Fig. 4b, lanes 7 and 8), but complex formation was virtually undetectable on probe R (Fig. 4b, lanes 5 and 6). (Again an extremely low level of C1 formation could be observed when it was stabilized by the 58S antibody.) No bases outside of the core consensus site have been shown to be required specifically for IE175K binding to its own cap site (Everett et al., 1991) and the results therefore indicate that although the upstream IE175K binding homology is critical for C2 formation on the LAP probe, it is intrinsically a very weak binding site. This is consistent with the result obtained with probe Q which, despite the retention of the upstream region including this site, binds IE175K extremely poorly, if at all. The specific activities of all the probes in this experiment were estimated to be roughly equal, and previous competition experiments confirmed that oligonucleotides Q and R did not bind efficiently to IE175K (Batchelor, 1992).
HSV-1 IE175K protein binds the LAT promoter

Comparison of the kinetic stability of complexes C1 and C2

Despite the observation that the upstream consensus site was intrinsically a weak IE175K binding site, we routinely observed as much C2 formation on the wild-type LAP cap site probe, or even mutants with the downstream homology disrupted, as there was C1 formation on the wild-type IE175K cap site probe. To compare the relative stability of IE175K on the LAP and IE175K probes the dissociation kinetics of complexes C1 and C2 were examined. Bulk reaction mixtures were made containing infected HeLa cell extract, poly(dI–dC), and radiolabelled probes A or K. Binding reactions were incubated for 20 min, at which time equilibrium had been established (data not shown). The mixtures were then divided into aliquots, and a large excess of competitor A or K was added at various time points. An example of one such experiment is shown in Fig. 5(a).

Strikingly, whereas the C1 complex was unstable and had almost completely dissociated from probe K within 5 min (lanes 7 to 12), C2 complex on probe A was highly stable in comparison and could still be detected after 40 min (lanes 1 to 6).

C1 and C2 dissociation curves are shown in Fig. 5(b). Results from a separate experiment in which K was used as a competitor are also included in the graph (dotted lines) and show no significant differences from those obtained using probe A as the competitor. The linear plot revealed that C2 dissociated in a standard exponential manner with a half-life of approximately 15 min. For C1 there was an initial rapid dissociation of the majority of the complex, followed by a slower rate of dissociation for the remainder. An upper limit for the half-life of the initial complex of less than 1–2 minutes could be estimated from the slope between the first two time points. The half-life of the remaining complex was around 13 or 14 min. This biphasic dissociation of C1 was reproduced in five separate experiments under a
variety of conditions (Batchelor, 1992) although the precise basis for it is at present uncertain. One possibility was that the probe K-binding IE175K species were heterogeneous, and that a particular IE175K subspecies formed a more stable complex on probe K. Different species of IE175K have in fact been reported to bind certain probes with different affinities (Michael et al., 1988; Papavassiliou et al., 1991). The probe K-stable species would, however, represent only a minor percentage of the total IE175K binding activity. Overall, for most of the IE175K binding activity, there was a significant difference in the rate of dissociation of the C1 and C2 complexes of at least 12-fold.

In contrast to the differences in the kinetic stability of complexes C1 and C2, in a series of dose–response competitor experiments carried out in conditions close to equilibrium there was found to be no significant difference in the total abundance of C1 and C2 complexes and no difference in the ability of oligonucleotides A and K to compete for IE175K (see e.g. Fig. 7 and Batchelor, 1992). Therefore, the overall binding affinity, determined by the association and dissociation rates, of IE175K for probe A or K was very similar despite the difference in dissociation rates. One possible explanation for this would be if the rate of association of C2 was considerably slower than that of C1, and this could readily be explained if C2 contained two or more separable protein components. The multimeric C2 complex would be more stably bound to DNA, but formation of C2 would be slower because it required that at least two protein components interacted at the same DNA site, and individually the protein components did not bind efficiently to the LAP cap site sequence (see Discussion). Whatever the precise explanation, our results indicate that despite the lower fit to the IE175K consensus site and the observation that the upstream homology is intrinsically a weak site, the LAP cap site region binds IE175K in an extremely stable fashion and, in terms of dissociation rate at least, is in fact a much stronger site than the IE175K cap site itself.

Protein requirements for C2 formation

The LAP cap site IE175K complex, C2, had a considerably lower mobility in gel retardation experiments than the complex observed at the IE175K cap site, C1. It was possible that complex C2 contained an additional protein component, which may have been a cellular factor (although as described above Sp1 was ruled out as a potential candidate). Purified IE175K has been shown previously to bind the IE175K cap site. To investigate the nature of the interaction at the LAP cap site, we examined whether purified IE175K could also bind to the LAP probe A and in particular whether, at the same input level of pure protein, the main form of binding to the LAP site was as a C2-type complex, different from the main form on the IE175K probe. (As before, each of the extracts was examined in both the presence or absence of 58S antibody to confirm the involvement IE175K in any complexes detected.) As expected, purified IE175K was still capable of forming C1 with probe K but, strikingly, in parallel the purified protein was able to form complex C2 efficiently and these complexes had mobilities identical to the C1 and C2 complexes observed with IE175K in crude infected HeLa cell extracts [Fig. 6, compare probe K and A for the infected cell extract (lanes 1 to 4) and purified IE175K (lanes 5 to 8)]. The purified IE175K (fraction IX) had been isolated from nuclear extracts of HSV-1-infected Vero cells by precipitation with ammonium sulphate followed by gel filtration, ion-exchange chromatography, and site-specific DNA affinity chromatography (Kattar-Cooley & Wilcox, 1989). Although the possibility remains that additional factors were present in fraction IX and contribute to binding, the most likely interpretation of the results is that IE175K is sufficient for C2 formation. C2 thus consists of multiple IE175K molecules bound to the LAP cap site in a higher order assembly.

The results of DeLuca & Schaffer (1988) indicated that large regions of IE175K could be deleted without affecting DNA binding. Moreover, small recombinant IE175K variants expressed in E. coli were sufficient for
HSV-1 IE175K protein binds the LAT promoter

Fig. 7. Competition analysis of A and K binding to FP449 and FP525. Binding mixtures contained purified IE175K (lanes 1 to 5), or bacterial fusion proteins FP449 (lanes 6 to 10) or FP525 (lanes 11 to 15). Samples were treated with 0.6 (lanes 2, 4, 7, 9, 12 and 14) or 3 (lanes 3, 5, 8, 10, 13 and 15) pmol of competitors A (lanes 4, 5, 9, 10, 14 and 15) or K (lanes 2, 3, 7, 8, 12 and 13) (approximately 30- and 150-fold excesses over probe) prior to the addition of radiolabelled probe K. Complexes were separated in 4% (19:1) polyacrylamide gels for 85 min.

DNA binding and for dimerization (Wu & Wilcox, 1990; Everett et al., 1991; Pizer et al., 1991). Bacterial fusion proteins FP449 and FP525, containing IE175K residues 262 to 490 that span the DNA-binding domain (Wu & Wilcox, 1990), were therefore tested to determine whether they retained the capacity to form complex C2. As expected from the results of Wu & Wilcox (1990), both of these fusion proteins were capable of binding to the IE175K cap site probe K (Fig. 6, lanes 9 and 13). In this case, the fusion proteins do not contain the 58S epitope which has been mapped to the carboxy-terminal 214 amino acids of IE175K (Shepard et al., 1989) and unlike the intact protein are not shifted in the presence of the antibody (Fig. 6, compare -/+ 58S for probe K with purified IE175K (lanes 5 and 7) versus the bacterial fusion proteins in lanes 9, 11, 13 and 15). Surprisingly, in view of the results with the intact protein and the stability of the C2 complex, neither of the truncated fusion proteins bound efficiently to the LAP cap site probe A (Fig. 6, lanes 10 and 14). Inefficient binding to the fusion proteins FP449 and FP525 was confirmed by competition analysis (Fig. 7). Using K as the radiolabelled probe, binding of purified intact IE175K was efficiently competed with by both unlabelled A and K (Fig. 7, lanes 1 to 5). There was little difference in the efficiency of competition and if anything probe A competed marginally better. In contrast, binding of FP449 and FP525 was competed with by K but was virtually unaffected by unlabelled probe A (Fig. 7, lanes 6 to 15). Together with the results of Fig. 6 the data indicate that although the LAP cap site is a strong IE175K binding site, the ‘DNA-binding domain’, which readily binds the IE175K cap site, is not sufficient for binding.

Discussion

We have previously shown that the LAP of HSV-1 was efficiently repressed by the major regulatory protein, IE175K, in cotransfection studies (Batchelor & O'Hare, 1990). Repression was shown to be abolished if sequences spanning the LAP cap site were deleted. IE175K repression of LAP was therefore analogous to IE175K repression of its own promoter, which has been shown to be achieved by IE175K binding to sequences spanning the IE175K promoter transcription initiation site (Roberts et al., 1988). In this study we show by gel retardation analysis that IE175K binds efficiently to probes spanning the LAP cap site. However, in parallel analysis, the IE175K complex formed on the LAP cap site, C2, had a significantly lower mobility than the IE175K cap site binding complex C1. C2 also exhibited an increased sensitivity to NaCl concentration. Purified IE175K retained the ability to assemble as the C2 complex suggesting that the major IE175K species which binds to the LAP cap site is a higher order assembly from the major species normally found binding to the IE175K cap site. Size estimation of IE175K by sedimentation analysis or gel filtration indicates that IE175K actually exists as a homodimer in solution (Metzler & Wilcox, 1985). Moreover, the C1-type DNA-binding complex has been shown to consist of two IE175K molecules bound to DNA (Michael & Roizman, 1989; Shepard & DeLuca, 1991a). C1 therefore consists of a single IE175K homodimer bound to DNA. In addition to IE175K polypeptide dimerizing, the IE175K homodimers are also able to multimerize. A tetrameric form of IE175K has been observed in solution (Kattar-Cooley & Wilcox, 1989), and the addition of excess IE175K to labelled probe results in higher order complexes in gel retardation assays. (It is noteworthy that the first of these higher order complexes, i.e. probably a tetramer, comigrates with the C2 complex formed on the LAP probe.) We suggest that a single homodimer is sufficient for stable binding to the IE175K cap site but is not sufficient for stable binding to the LAP cap site which requires the interaction of two IE175K homodimers to form a stable complex.

Considering this, and the relatively poor fit of the LAP cap site sequence to the IE175K consensus site, there was a striking difference in the kinetic stability of the two
HSV-1 LAP

Complex C2
Forms on LAP:
stable

Probe A

ctAGaGGGGGCGCGGCCGTGCCGATCGCCGGTGGTGCGAAAGACTcTag

LAP composite site

Core domain
not sufficient

ctAGaGGGGGCGCGGCCGTGCCGATCGCCGGTGGTGCGAAAGACTcTag

IE175K promoter

Core domain
binds efficiently

ctagaGAGCCCGAGGACGCCCCGATCGTCCACACGGAGCGCGGCTctag

Probe K

Complex C1

tagaGAGCCCGAGGACGCCCCGATCGTCCACACGGAGCGCGGCTctag

Fig. 8. Summary of mutagenesis results together with a possible model for complex C2. Probes A and K are indicated together with the region corresponding to the IE175K consensus binding sequence in probe K (solid arrow) or the potential binding sequence in probe A (dashed arrow). The region covered by lines and boxes below probe A is proposed to be sufficient for C2 binding. (The region indicated is the overlap between A and J which bind C2 equally well.) Black boxes indicate nucleotide pair mutations in I and O (pBB-54 and pBB-55) which abolished IE175K binding and repression. Grey boxes indicate sequences mutated in probe Q which no longer formed C2 but were still partially repressed by IE175K in the LAP construct pBB-8 (Batchelor & O'Hare, 1990). White boxes indicate sequences mutated in H and P (pBB-56) which did not significantly alter C2 formation or repression. In the proposed model two IE175K homodimers are binding to probe A in complex C2, whereas only one homodimer binds to probe K (complex C1). Grey shaded proteins are binding to 'consensus' IE175K DNA sequences. As discussed in the text, IE175K is proposed to contain a region involved in tetramerization or contacting DNA, which is necessary for C2 formation, but is outside the DNA-binding domain of the protein. Here we show one of these possibilities. The IE175K homodimer indicated by the vertical lines is contacting nucleotides in IE175K complexes C1 and C2 in favour of C2. From comparison of the dissociation kinetics, the half-life of LAP C2 complex was at least 12-fold greater than the half-life of C1 (at least for over 95% of the IE175K binding species). In contrast, in competition experiments there was little difference between the affinity of IE175K for the two DNA sequences. This implies that the rate of association of C2 was considerably slower compared to that of C1. Again these results would be consistent with the proposal that C2 contained two IE175K homodimers. The C2 complex could potentially contain a greater number of protein–DNA contacts resulting in the complex being more kinetically stable. However, formation of the C2 complex would be less likely due to the instability of intermediate complexes which could be either a complex of two IE175K dimers in the absence of DNA, or a single IE175K homodimer bound to DNA (a low abundance C1 complex was reproducibly detected with probe A).

Analysis of the LAP cap site sequence indicated that there were two potential motifs that were related to the IE175K consensus binding sequence arranged in a pseudopalindromic manner (Fig. 1). Mutations, which from previous analyses would be predicted to strongly disrupt the downstream motif had no effect on C2 formation, whereas disruption of the upstream motif virtually abolished binding. Moreover attempts to recapitulate this arrangement within the IE175K cap site did not result in C2 formation. Other mutations in the region downstream of the LAP cap site did prevent complex formation and from the combined results indicate that the sequence requirements in the downstream half of the LAP cap binding site did not involve a second IE175K binding motif. Therefore, in the model proposed for C2 (see Fig. 8), a single IE175K homodimer is shown binding to the upstream motif with a second IE175K homodimer in C2 contacting sequences in the downstream half of the LAP site in a different manner. We are currently refining the requirements within this downstream element by systematic mutagenesis of the region. Although IE175K did not bind to the sequence within the HSV-2 LAP region, the precise position of the HSV-2 LAP transcription initiation site has not yet been mapped. The HSV-2 motif used here to test for IE175K binding (i.e. the closest to the consensus), was 20 bp further from the presumptive TATA box than its equivalent in HSV-1. Thus this result does not necessarily indicate the absence of binding of IE175K, or its HSV-2 homologue (IE183K), to the HSV-2 LAP cap site.

the downstream half of probe A with a region of the protein outside the DNA-binding domain. In this case the tetramerization region of the protein is shown as being located in the DNA-binding domain.
However, although it is further possible that HSV-2 IE183K has different sequence requirements for binding, it may also be that the HSV-2 LAP is not subject to repression. We are currently testing this proposal.

The total abundance of intact IE175K binding was very similar for the LAP and IE175K cap sites. In contrast, recombinant IE175K variants FP449 and FP525, which contain the IE175K DNA-binding domain, bound efficiently to the IE175K cap site but extremely poorly to the LAP cap site. In addition, the low abundance FP449 or FP525 complexes observed with LAP site were of the same mobility as those on the IE175K cap site in contrast to the situation with the intact protein (see above). Thus although these, and related truncated proteins containing the DNA-binding domain, are able to dimerize (Wu & Wilcox, 1990; Everett et al., 1991; Shepard and DeLuca, 1991a), the simplest explanation of our results is that they lack a region of IE175K required for C2 formation, i.e. for the tetramerization necessary for a stable complex on the LAP site. (Note that although the truncated proteins form bands of lower mobility than the major dimeric species, our results would indicate that this may be unrelated to the oligomerization of the intact native protein and may perhaps represent aggregates of the fusion proteins due to interactions between regions not normally exposed in intact IE175K.) An alternative explanation for the lack of complex formation between FP449 or FP525 at the LAP cap site could be that they still retain the region involved in specific tetramerization, but lack an additional function necessary for C2 formation, possibly an additional region of IE175K outside the core DNA-binding domain but required for contacting downstream sequences at the LAP cap site. Analysis of additional truncation mutants should help identify additional requirements within IE175K for the C2 arrangement. It is noteworthy that previous studies have demonstrated that certain sites may selectively promote higher order IE175K binding complexes. For example, of three binding sites in the HSV-1 gD gene, two promoted the formation of two complexes with different mobilities while a third formed only the faster mobility complex (Tedder et al., 1989). Curiously the site forming only the single complex was the one whose sequence was closest to the consensus core motif. In addition, one of the gD binding sites has a considerably extended footprint compared to the other sites. It is possible that certain sites preferentially form the tetrameric complex, although unlike the case for the gD sites, tetramerization was obligatory for binding to the LAP cap site. A further possibility is that alterations in the arrangement of IE175K binding to different sites reflects their involvement in qualitatively distinct aspects of IE175K function. Analysis of the relative efficiencies of binding of the intact protein compared to the core binding domain to a number of sites may identify those sites where tetramerization was necessary.

The main purpose of the experiments described in this study was to give some insight into the mechanism by which IE175K represses the LAP. Overall, DNA sequence mutations that abolished C2 formation correlated well with IE175K repression results in cotransfection studies. Probe I and O mutations that disrupted C2 formation prevented IE175K repression of LAP expression, but the probe P mutation which did not affect C2 formation did not significantly interfere with IE175K repression of LAP. There was one discrepancy in that probe Q, which was unable to form C2 (Fig. 4), actually contains the nucleotide sequence of the LAP–CAT construct pBB-8 which was still repressed by IE175K, albeit less efficiently than the wild-type construct (Batchelor & O’Hare, 1990). This inconsistency may result from differences in in vivo and in vitro binding conditions, but overall the results were broadly in agreement with the conclusion that C2 was responsible for the repression of LAP. An interesting feature of our previous results was that repression of expression from LAP was considerably more efficient than the repression observed from the IE175K promoter itself (Batchelor & O’Hare, 1990; Batchelor, 1992). It is reasonable to propose that IE175K represses expression by preventing the RNA polymerase transcription initiation complex from forming and C2, being a larger complex, may be able to sterically hinder the formation of an initiation complex more efficiently. In addition the significantly longer half-life of complex C2 may contribute to more effective repression.

One important feature of the LAP–IE175K binding site is that it differs from previously identified IE175K binding sites in that IE175K binds most stably to LAP sequences in a multimeric configuration, probably in the form of two homodimers. The upstream IE175K binding site homology in probe A was identified in the HSV-1 genome by DiDonato et al. (1991) but their model would predict this to be a weak IE175K binding site. Our results therefore indicate that sequences which would previously have been predicted to be weak IE175K binding sites could potentially form a strong interaction with two IE175K homodimers, depending on the neighbouring nucleotide sequence. For the LAP the result is that in contrast to the prediction, the cap site is actually a very strong binding site with a much slower dissociation rate than the IE175K cap site and much more efficient repression. The results presented here indicate that IE175K is capable of interacting strongly with sites on the HSV-1 genome which would previously have been predicted to be poor matches to the consensus IE175K binding sequence.
Whether LAP activity is repressed by IE175K during infection remains to be established but transcription from this locus is at an extremely low level and does not proceed with the normal kinetics of a typical delayed early or late promoter during productive infection. Moreover a decrease in LAT expression has been observed as an early event after virus reactivation from a latent infection (Spivack & Fraser, 1988). We are currently attempting to construct mutant viruses with refined substitutions at the LAP cap site in order to test this proposal of efficient LAP repression by IE175K during infection.

References


O'Hare, P. & Hayward, G. S. (1985). Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early


