

Localization of a 34-Amino-Acid Segment Implicated in Dimerization of the Herpes Simplex Virus Type 1 ICP4 Polypeptide by a Dimerization Trap

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The herpes simplex virus type 1 immediate-early protein ICP4 plays an essential role in the regulation of the expression of all viral genes. It is the major *trans* activator of early and late genes and also has a negative regulatory effect on immediate-early gene transcription. ICP4 is a sequence-specific DNA-binding protein and has always been purified in a dimeric form. The part of the protein that consists of the entire highly conserved region 2 and of the distal portion of region 1 retains the ability to specifically associate with DNA and to form homodimers in solution. In an attempt to map the dimerization domain of ICP4, we used a dimerization trap assay, in which we screened deletion fragments of this 217-amino-acid stretch for sequences that could confer dimerization properties on a heterologous cellular transcription factor (LFB1), which binds to its cognate DNA sequence only as a dimer. The analysis of these chimeric proteins expressed *in vitro* ultimately identified a stretch of 34 amino acids (343 to 376) that could still confer DNA-binding activity on the LFB1 reporter protein and thus apparently contained the ICP4 dimerization motif. Consistent with this result, a truncated ICP4 protein containing amino acids 343 to 490, in spite of the complete loss of DNA-binding activity, appeared to retain the capacity to form a heterodimer with a longer ICP4 peptide after coexpression in an *in vitro* translation system.

The $\alpha 4$ open reading frame of herpes simplex virus type 1 (HSV-1) encodes a protein of 1,298 amino acids known as ICP4 or Vmw175, with a predicted molecular mass of 132,835 Da. ICP4 is generally accepted to be the major *trans* activator of HSV-1 genes, and for this reason, it has been the subject of intensive study.

During the very early phase of infection, the $\alpha 4$ gene is transcribed and the ICP4 protein is synthesized in high amounts (reviewed in reference 33). This newly synthesized polypeptide is thought to mediate the transition from the immediate-early (IE) phase to the later phases of the viral life cycle by shutting off the transcription of the IE genes and by activating the expression of the early (E) and late (L) ones (4, 7, 30, 31). The protein has been shown to bind with nanomolar affinity to a bipartite consensus sequence, RTCGTCNNY NYSG (where R is A or G, N is any nucleotide, Y is C or T, and S is C or G) (6, 8), which is found in the promoter regions of the ICP4 and ICP0 genes. The binding of ICP4 to these sites is generally thought to be involved in the transcriptional downregulation of these genes (11, 22, 24, 32). Although the molecular mechanism of this process is subject to speculation at present, elegant *in vitro* studies carried out by De Luca and colleagues (2) demonstrated that the binding of ICP4 to the high-affinity consensus sequence at the start site of $\alpha 4$ transcription is strictly required for the repression of Sp1-induced transcription.

Numerous studies carried out in recent years led to extensive biochemical characterization of ICP4. The protein has been partially purified from infected-cell extracts and shown to have unimpaired binding properties with respect to the high-affinity consensus site (10, 11, 14–17, 23, 24, 29, 41). It is largely

believed to bind DNA as a dimer (21, 35), mainly because it has always been found to exist as such in solution (20). The protein was arbitrarily subdivided into five regions (Fig. 1), two of which, 2 and 4, are conserved among the functional homologs of ICP4 from other members of the *Alphaherpesviridae* subfamily (19). Recently, a combination of mutational and functional studies (3, 5, 27, 28, 37) showed that, like many other regulatory proteins involved in transcription, ICP4 is modular in composition, consisting of multiple discrete domains that collectively contribute to its function during viral infection. In particular, the integrity of region 2 is strictly necessary for the protein to bind to DNA and to regulate viral gene transcription. Everett et al. (9) showed that a domain encompassing the distal end of region 1 and a substantial part of region 2 could be liberated by proteinase K digestion as a functional unit that retains the ability to bind DNA. The same region could be expressed in *Escherichia coli* as a stable polypeptide, capable of sequence-specific DNA binding (8, 29, 44). This finding was further extended by the demonstration that a set of N- and C-terminal deletion mutants of this domain could bind to the consensus site so long as amino acids 275 to 490 were present (8, 44). Further deletions both at the N terminus (up to amino acid 306) and at the C terminus (up to amino acid 464) completely abolished binding to DNA, demonstrating that both the distal end of region 1 and the distal part of region 2 are required for binding and sequence recognition.

The minimal functional ICP4 DNA-binding domain (amino acids 276 to 523) expressed in bacteria was shown to exist as a dimer in solution (8), but no information is available about the multimerization state of the shorter inactive forms. It is thus unclear at present whether their inability to bind DNA is due to a lack of dimerization or to a loss of amino acid residues critical for sequence recognition. Studies aimed at resolving this dilemma have been frustrated by the fact that the DNA-binding and dimerization regions appear to either overlap or

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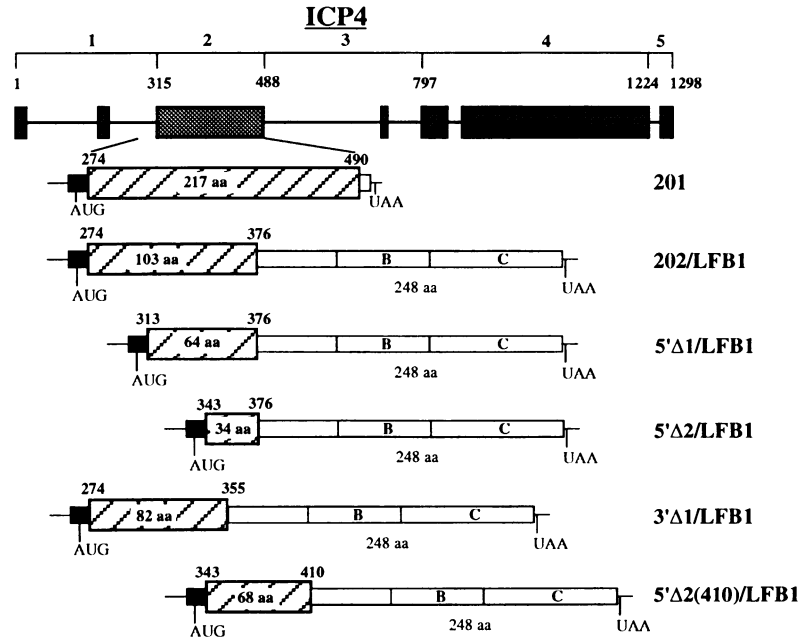


FIG. 1. Structures of ICP4-LFB1 chimeric constructs. (Top) Diagram of the five ICP4 polypeptide domains proposed by McGheoch et al. (19) is shown (modified from reference 5). Shaded boxes, regions of amino acid similarity with the ICP4 homologs from other alphaherpesviruses. (Bottom) Schematic representation of the 201 construct and of the ICP4-LFB1 chimeric constructs. Hatched boxes, ICP4 sequences. Boundaries are indicated by the original ICP4 amino acid (aa) numbers. Open boxes, LFB1 sequences; B, ψ POU domain; C, homeodomain; solid boxes, pT7.7 sequences.

be interspersed. It has thus not been possible to map the minimal dimerization domain of ICP4 within the context of the wild-type amino acid sequence and thus to demonstrate whether dimerization of ICP4 per se is required for binding to DNA or for one of its other associated activities.

In an attempt to map the dimerization domain of ICP4, we decided to adopt a strategy different from the deletion mutagenesis studies carried out to date in other laboratories. We substituted the dimerization domain of the cellular transcription factor LFB1 (for which high-affinity binding to DNA is strictly dependent on dimerization) (42) with progressively smaller portions of a region of ICP4 spanning amino acid residues 274 to 490 and examined these chimeric proteins for LFB1-specific DNA binding in the form of a dimer. In this way, we could assess the dimerization capacity of ICP4 independently of its DNA-binding properties. To determine whether dimerization is necessary for binding to DNA, we expressed in an *in vitro* translation system several polypeptides containing ICP4 DNA-binding domains of various lengths and analyzed their capacity to bind to the DNA high-affinity site as dimers. It was anticipated that the functional definition of a minimal ICP4 dimerization domain would allow us to search for compounds that could specifically impair dimerization and thus interfere with the function of ICP4 in the replicative cycle of HSV-1.

MATERIALS AND METHODS

Synthetic oligonucleotides and plasmid constructs. The following oligonucleotides, corresponding to the indicated binding sites and their respective complementary strands, were synthesised: LFB1 Pal, GCTTGGTAAATGATTAACC AAGC; LFB1 C, CACTGCCAGTCAAGTGTCTTGA (42); IE3 cap site ICP4, GTGAATTCCC GGGATCCGCGC

CGGTCGACTGCAGGTACGC; and BCU, GTGAATTCCC GGGATCCGCGCTTTCGAGTGTAAATCCCCAGATATAGC TATGGAGCCAGGTCGACTGCAGGTACGC. To obtain the ICP4-LFB1 chimeric constructs 201/LFB1 (not shown), 202/LFB1, 5'Δ1/LFB1, 5'Δ2/LFB1, 3'Δ1/LFB1, and 5'Δ2(410)/LFB1 (Fig. 1), the corresponding DNA fragments coding for amino acids 274 to 490, 274 to 376, 313 to 376, 343 to 376, 274 to 355, and 343 to 410, respectively, were amplified by PCR with suitable primers containing a *SacI* site (5' primer) and an *SphI* site (3' primer).

Plasmid pcICP4, which contains the *SalI-DdeI* restriction fragment encompassing bp -122 to +4363 from the translation start site of ICP4, was used as a template in PCR. Amplified DNA fragments obtained after digestion with *SacI* and *SphI* enzymes were inserted into the unique *SacI* and *SphI* sites of the LFB1 mutant vector, renamed LFB1/Δ8 in this article, generously donated by R. De Francesco and L. Tomei. This backbone vector is the modified version of LFB1 mutant Δ1 (25) described in reference 42. Briefly, it contains the N-terminal portion of LFB1, in which the dimerization domain of the protein (region A) has been replaced with a shorter sequence containing suitable restriction sites for cloning heterologous dimerization domains in frame with the distal part of the LFB1 DNA-binding domain (ψ POU/homeodomain, regions B and C, respectively). The chimeric ICP4/LFB1 fragments were cloned into the *EcoRI* (filled in with Klenow enzyme) and *BamHI* restriction sites of a pT7.7 expression vector containing an artificial stop codon. The LFB1 sequence was deleted from the 201/LFB1 chimeric construct, generating ICP4 mutant 201, in which the sequence coding for amino acids 274 to 490 was included between the *SacI* and *SphI* sites. The 5'Δ1BD200 and 5'Δ2BD200 mutants (see Fig. 4A) were generated by PCR amplification of the DNA fragments coding for amino acids 313 to 490 and 343 to 490 with the same

suitable primers and the same DNA template as for the chimeric constructs. After *SacI* and *SphI* digestion, the fragments were inserted into the unique *SacI-SphI* sites of the modified pT7.7 vector, isolated after *SacI-SphI* digestion of construct 201. All constructs contained an additional sequence encoding M A R I N S S S in front of the ICP4 N terminus.

Construct pT7110X was kindly provided by R. Everett and has been described in detail before (8, 29).

In vitro transcription and translation. In vitro transcriptions were performed with 2 μ g of linearized template DNA in a 40- μ l reaction volume containing 40 mM Tris-HCl (pH 8.0), 50 mM NaCl, 8 mM MgCl₂, 10 mM dithiothreitol, 2 mM spermidine, 0.5 mM each of the four nucleoside triphosphates (NTPs), 0.56 U of m7GpppG (Boehringer Mannheim), 80 U of RNasin, and 40 U of T7 RNA polymerase (Promega). The mixture was incubated for 1 h at 37°C, diluted to 100 μ l with H₂O, and extracted with phenol-chloroform. The in vitro-transcribed complementary RNA (cRNA) was purified through a 1-ml Sephadex G50 prespun column, ethanol precipitated, washed with 70% ethanol, dried, and resuspended in 40 μ l of H₂O. Translation of the cRNA (2.5 μ l) was carried out for 90 min at 30°C in a 30- μ l reaction mix containing 3 μ M amino acid mix lacking methionine, 30 μ Ci of [³⁵S]methionine (1,000 Ci/mmol, 10 mCi/ml; Amersham SJ115), 1 mM dithiothreitol, and 12 μ l of rabbit reticulocyte lysate (Promega). ³⁵S-labeled translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE), and the gel was treated with Amplify (Amersham), dried, and autoradiographed.

Expression and purification of protein 201 from bacteria. The 201 protein was expressed in *E. coli* BL21(DE3) as described before (40). After a 3-h induction with 0.4 mM IPTG (isopropyl- β -D-thiogalactopyranoside), cells were harvested and lysed with a French pressure cell in a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 8.0), 2 mM EDTA, 20% (vol/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (buffer A). The extract was treated with 20 μ g of DNase I and 100 μ g of RNase per ml for 10 min on ice. NaCl and polyethylenimine were then added to a final concentration of 0.5 M and 0.1% (vol/vol), respectively, and insoluble material was pelleted by centrifugation at 27,000 \times g for 30 min in a Sorvall SS34 rotor. The clarified supernatant was dialyzed against buffer A containing 0.1 M NaCl and subsequently purified by fast protein liquid chromatography (FPLC; Pharmacia) as follows. The 201 protein collected in the flowthrough of a DEAE column equilibrated in the same buffer as the final crude extract was loaded on a Mono S column and eluted with a 0.1 to 0.5 M NaCl gradient in buffer A. The 201 protein eluted as a sharp peak at 0.250 M NaCl. This process yielded about 0.5 mg of purified 201 from 1 liter of culture.

Gel retardation assays. Gel retardation experiments were carried out by mixing the appropriate amount of protein with the specific radiolabeled double-stranded oligonucleotide (40 fmol) in a 20- μ l reaction mix containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, and either 0.1% (vol/vol) Nonidet P-40, when binding of ICP4 mutants was tested, or 5 mM MgCl₂, when binding of LFB1 chimeras was tested. Either 0.1 or 2 μ g of poly(dI:dC)·poly(dI:dC) was used as nonspecific competitor DNA when proteins expressed either in vitro or in bacteria were analyzed. Binding reaction mixes were incubated for 30 min on ice. The samples were then loaded onto a 6% native polyacrylamide gel (30:1, acrylamide-bisacrylamide; 0.25 \times Tris-borate-EDTA electrophoresis buffer) and analyzed by autoradiography.

Gel filtration chromatography. The native molecular weight of the 201 protein purified from bacteria was determined on a Pharmacia Superdex 75 HR 10/30 prepacked column in a buffer containing 50 mM HEPES (pH 8), 0.2 M NaCl, 10% (vol/vol) glycerol, and 0.1 mM dithiothreitol. The flow rate was 0.5 ml/min, and 0.5-ml fractions were collected and analyzed by both SDS-PAGE and gel retardation assays. Bovine serum albumin (67 kDa) and chymotrypsinogen (25 kDa) were obtained from Sigma and used as standards for calibration of the column.

RESULTS

Construction of ICP4-LFB1 chimeric expression plasmids. We aimed to devise a dimerization trap which would allow us to separate the analysis of two distinct functions of ICP4, namely, binding to DNA and dimerization, a task that would otherwise be very difficult to accomplish because of the high stability of ICP4 dimers in solution in the absence of DNA (20) and the probable partial colocalization of the DNA-binding and dimerization functions within the same domain of the protein. Indeed, the minimal portion of ICP4 identified so far which retains DNA-binding activity was demonstrated to be a dimer in solution (8). As a reporter for dimerization, we used the ψ POU/homeodomain of LFB1, a liver-specific transcription factor that binds with high affinity to its cognate DNA palindromic sequence in the form of a homodimer. Previous studies showed that deletion of the dimerization domain of this protein resulted in the loss of DNA-binding activity but that this activity was restored in chimeric proteins containing the LFB1 DNA-binding domain linked to any one of a number of different heterologous dimerization motifs (42). On the basis of these findings, it was anticipated that the LFB1 ψ POU/homeodomain would also tolerate the ICP4 dimerization motif.

To make the chimeric constructs shown in Fig. 1, we used the backbone vector LFB1/ Δ 8, which contains the N-terminal portion of LFB1 in which the dimerization domain of the protein (32 N-terminal amino acids [region A]) was replaced with a shorter sequence containing restriction sites for convenient in-frame cloning with the LFB1 DNA-binding domain (region B, ψ POU; region C, homeodomain). We initially assembled the construct 201/LFB1 (not shown), in which the LFB1 dimerization domain was replaced with the sequence corresponding to amino acids 274 to 490 of ICP4. From previous mutational studies (8, 9, 29, 44), this region was predicted to represent the minimal functional DNA-binding domain of the protein and to maintain the capacity to dimerize. The polypeptide product of 201/LFB1, expressed in vitro, could bind neither the LFB1 nor the ICP4 cognate DNA consensus sequence (data not shown). We took this negative result to indicate a functional interference between the two adjacent DNA-binding domains contained in this polypeptide. In order to avoid a recurrence of this phenomenon, we decided to exclude from the other constructs amino acids 377 to 490, which correspond to the most highly conserved part of region 2 and have been demonstrated to be strictly necessary for binding to DNA (5, 27, 28, 37, 44). Insertion of an α 4 fragment encoding amino acid residues 274 to 376 into the LFB1/ Δ 8 polylinker gave construct 202/LFB1, into which were introduced two 5' deletions (5' Δ 1, amino acids 313 to 376, and 5' Δ 2, amino acids 334 to 376) and a 3' deletion (3' Δ 1, amino acids 274 to 355). In addition, we also cloned into a pT7.7 vector, modified as described in Materials and Methods, an ICP4 domain comprising amino acids 274 to 490 (region 2 and distal part of region 1), which encoded polypeptide 201 (Fig. 1).

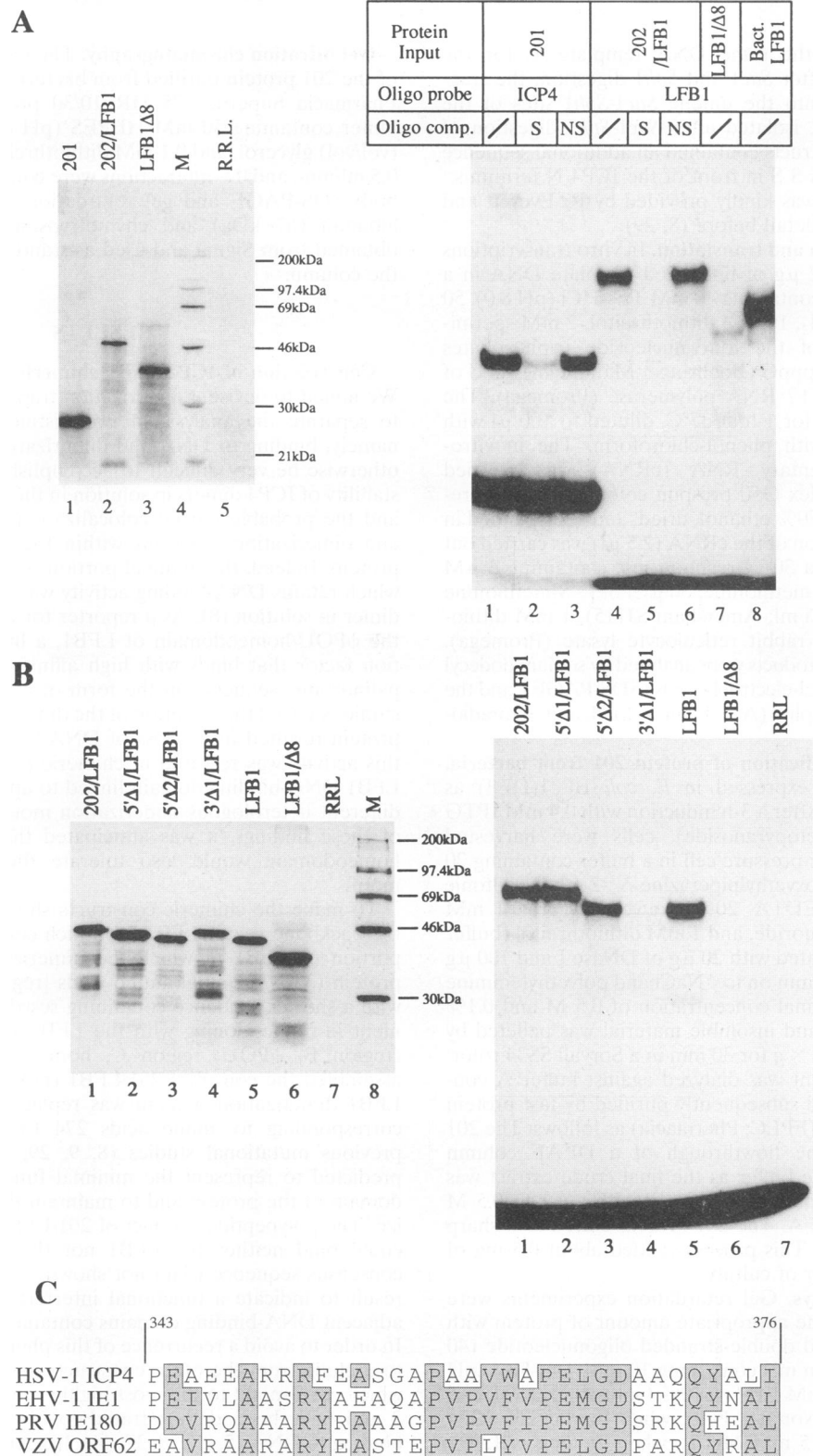


FIG. 2. Analysis of ICP4-LFB1 chimeric proteins synthesized in vitro. In vitro transcription-translation reactions were carried out as described in Materials and Methods. (A and B, left panels) A 1.5- μ l amount of the reaction mix containing the 35 S-labeled protein products was analyzed by SDS-PAGE (12% polyacrylamide) and autoradiography. R.R.L., in vitro translation reaction with no RNA included; M, 14 C-labeled size markers (Amersham). (A and B, right panels) Approximately equal amounts of protein were tested in a gel shift assay, as described in Materials and Methods. See the text for the oligonucleotide probes. Oligonucleotide competitors: S (specific), ICP4 oligonucleotide for 201 and Pal oligonucleotide for ICP4-LFB1; NS (nonspecific), BCU oligonucleotide for 201 and C oligonucleotide for ICP4-LFB1. (C) Sequence alignment of HSV-1 ICP4 amino acids 343 to 376 with the corresponding regions of ICP4 homologs from different alphaherpesviruses. EHV-1, equine herpesvirus 1; PRV, pseudorabies virus; VZV, varicella-zoster virus.

Analysis of ICP4-LFB1 chimeric proteins synthesized in vitro. The constructs depicted in Fig. 1 were used as linear templates in an in vitro transcription-translation system. We chose this system because it permits rapid analysis of the recombinant products because the proteins of interest need not be purified to homogeneity prior to use. In addition, no complications from nonspecific aggregation of the protein arise, as the concentration of the polypeptide in the translation mixture is relatively low.

The ³⁵S-labeled protein products were analyzed by SDS-PAGE (Fig. 2A and B, left panels) and were tested for DNA binding either to a 23-mer duplex oligonucleotide probe containing the LFB1 palindromic consensus sequence (chimeric) or to a 74-mer oligonucleotide probe bearing the ICP4 high-affinity binding site (201) (Fig. 2A and B, right panels). As shown in Fig. 2A, the 217-amino-acid region of ICP4 (polypeptide 201) synthesized in vitro (left panel, lane 1) behaved as a sequence-specific DNA-binding protein, giving a retarded band that could be specifically competed with by an excess of unlabeled ICP4 oligonucleotide (right panel, lanes 1 to 3). To our knowledge, this protein is the smallest version of an active nonfusion DNA-binding domain observed to date. However, the most significant result in Fig. 2A shows that the chimera 202/LFB1 (left panel, lane 2) interacted specifically with the LFB1 palindromic consensus sequence (right panel, lanes 4 to 6) with an affinity and specificity comparable to those of the intact LFB1 DNA-binding domain either purified from bacteria (Fig. 2A, right panel, lane 8) or synthesized in vitro (Fig. 2B, right panel, lane 5). In contrast, the in vitro-translated LFB1/Δ8 mutant (Fig. 2A, left panel, lane 3), lacking the N-terminal dimerization domain, produced only a very faint band (Fig. 2A, right panel, lane 7). Furthermore, the 201 polypeptide and the 202/LFB1 chimeric protein did not show any appreciable binding to the LFB1 probe or to the ICP4 probe, respectively (data not shown). It therefore appears that the ICP4 sequence between amino acids 274 and 376 can confer on LFB1 the capacity to bind to DNA specifically as a dimer, implying that the ICP4 dimerization domain is located in this region.

Figure 2B shows the results of a further deletion analysis of the putative dimerization domain from both the 5' and 3' directions. Approximately equal amounts of the in vitro-translated products shown in the left panel were tested in a gel shift assay for binding to the LFB1 oligonucleotide probe. As can be seen in lanes 2 and 3, mutants with 5' deletions up to amino acid 343 retained the capacity to bind DNA with high affinity. The specificity of binding was confirmed both by competition with an excess of LFB1-specific oligonucleotide and by the interaction with an LFB1-specific antiserum, which supershifted the retarded complexes (data not shown). On the other hand, the 3' deletion mutant truncated at amino acid 355 possessed very low DNA-binding activity (Fig. 2B, lane 4), comparable to the background activity of the negative control LFB1/Δ8 (lane 6). The stretch of 34 amino acids containing ICP4 residues 343 to 376 thus appeared to be sufficient to functionally replace the dimerization domain of the LFB1 reporter protein, and we therefore propose that it contains the ICP4 protein dimerization motif. Our data also demonstrate that no dimerization was apparent in the absence of the C-terminal 22 amino acid residues within this stretch (355 to 376).

Protein sequence analysis of this ICP4 peptide revealed a high degree of conservation with the corresponding regions of the ICP4 homologs from the other members of the *Alphaherpesviridae* subfamily, with a block of homology of 11 amino acids in the C-terminal part (Fig. 2C). On the other hand, it

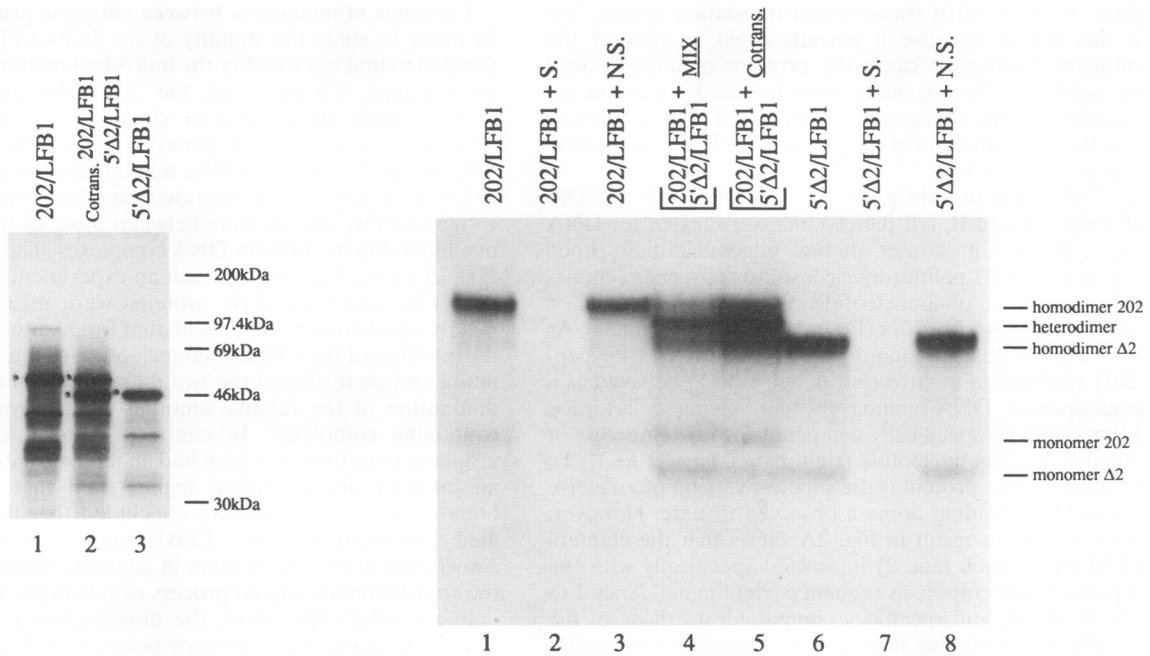
showed no homology with other known dimerization motifs, and no prediction of secondary structure could be made.

Exchange of monomers between chimeric proteins in vitro. In order to study the stability of the ICP4-LFB1 dimers, we decided to find out whether the individual monomers were free to exchange. To this end, the 202/LFB1 and 5'Δ2/LFB1 proteins were synthesized in vitro either separately or in cotranslation (Fig. 3A, left panel). Since the two polypeptides differ in size by about 7.6 kDa, a heterodimeric protein bound to DNA was expected to migrate through a polyacrylamide gel with a mobility intermediate between those of the two respective homodimeric protein-DNA complexes. The right panel of Fig. 3A shows the result of such an experiment. In lane 4, the 202/LFB1 and 5'Δ2/LFB1 proteins were mixed in approximately equal amounts and incubated for 30 min at 37°C before the addition of the LFB1 oligonucleotide probe. Both cotranslation and premixing of the two different chimeras resulted in diminution of the relative amounts of the two homodimer-containing complexes. In contrast, a novel complex (heterodimer) was formed, which had an intermediate mobility and an intensity about twofold higher than that of the single homodimeric complexes. We concluded that the ICP4-mediated dimerization of the LFB1 reporter occurred via the association of free monomers in solution, rather than during the translation-associated process of polypeptide folding.

In a similar experiment, the dimerization partner for the 5'Δ2/LFB1 protein was chimeric polypeptide 5'Δ2(410)/LFB1, which contains amino acids 343 to 410 of ICP4 fused to the LFB1 DNA-binding domain (Fig. 1). This protein differs from 5'Δ2/LFB1 only by the additional 35 amino acid residues at the C terminus of the ICP4 sequence. As shown in Fig. 3B, the two in vitro-synthesized proteins were able to exchange monomers both after mixing of the individually translated proteins in solution and during cotranslation (lanes 4 and 5), demonstrating that amino acids 274 to 343 are not required for dimerization. Furthermore, as free monomer exchange was still observed, the region of ICP4 containing amino acids 376 to 410 did not appear to contain residues that are involved in the stabilization of ICP4 dimerization.

Analysis of the ICP4 DNA-binding region expressed in bacteria. In addition to the truncated ICP4 protein 201 (amino acids 274 to 490; see Fig. 1), we inserted in the modified pT7.7 expression vector two further ICP4 segments in which additional amino acids were removed from the N terminus to yield polypeptides containing residues 313 to 490 (5'Δ1BD200) and 343 to 490 (5'Δ2BD200) (Fig. 4A). Our aim was to express and purify these proteins in large amounts to allow future biochemical and structural studies. Protein 201 was expressed in bacteria and purified by FPLC ion-exchange chromatography. It was shown to bind to the ICP4 consensus site with high affinity (Fig. 4B) and with the same specificity as the full-length protein (data not shown). In gel shift experiments, the protein gave rise to at least two protein-DNA complexes; the ratio between the slower- and the faster-migrating species increased with increasing protein concentration (Fig. 4B, right panel). This phenomenon was not observed with 201 expressed in vitro, which reproducibly formed only a single complex with the target DNA (Fig. 2A) migrating with a mobility similar to that of the faster-moving band seen in Fig. 4B. This result confirmed previous observations by Everrett et al. (8), who proposed that the slower-migrating complexes represent multiples of the basic dimeric form. Multimerization could derive either from nonspecific aggregation of two or more dimeric units at high protein concentrations or from the interaction of the additional dimers with the nonspecific DNA tails in the probe molecule.

A



B

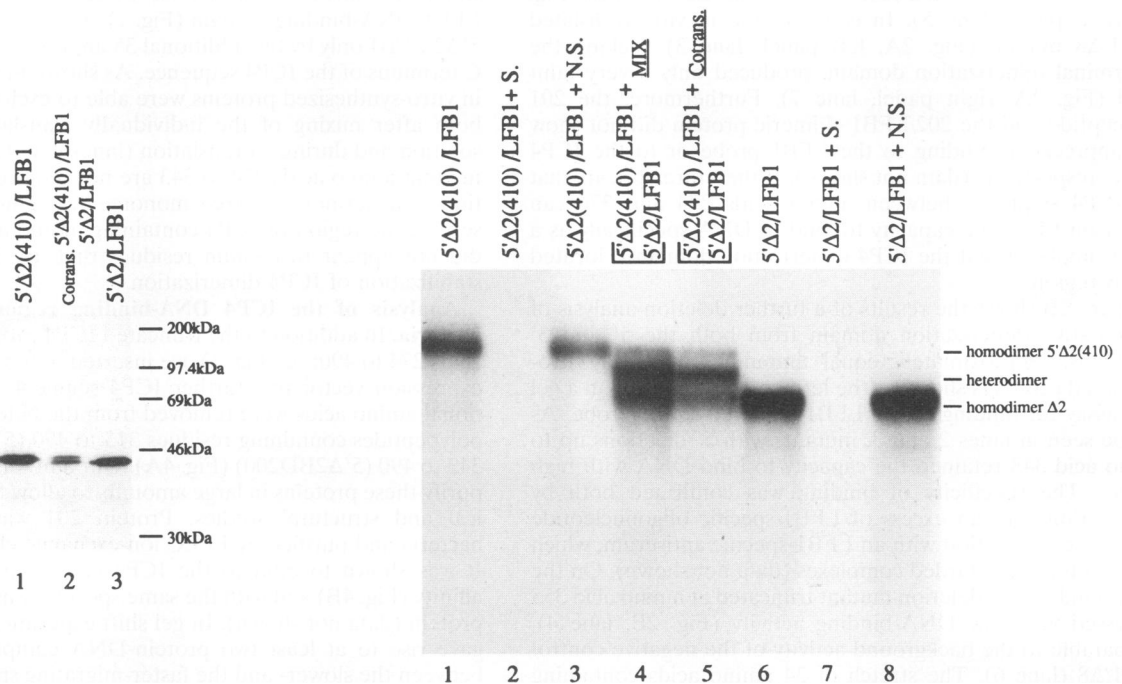


FIG. 3. Monomer exchange between different ICP4-LFB1 chimeric proteins synthesized *in vitro*. For protein synthesis, SDS-PAGE analysis (A and B, left panels) and gel retardation assays (A and B, right panels), the experimental conditions and the abbreviations were the same as in Fig. 2 and in Materials and Methods. In cotranslation reactions, 2.5 μ l of each cRNA species was included as for single translation reactions, and 3 μ l of the protein sample was analyzed by gel shift assay. In the premixing experiments, 1.5 μ l of each of the two proteins was mixed and incubated for 30 min at 37°C under DNA-binding conditions before the LFB1 Pal probe was added. The identity of the retarded complexes is indicated.

Physical analysis of 201 protein purified from bacteria. To investigate the multimerization state of 201, a purified preparation was applied to a Pharmacia Superdex 75 gel filtration column. The elution profile was monitored by UV absorbance, and the eluted fractions were analyzed by SDS-PAGE and gel

shift assays (Fig. 5A). The 201 protein eluted between the bovine serum albumin (67 kDa) and chymotrypsinogen A (25 kDa) markers, and its molecular mass was estimated to be about 45 kDa, in agreement with the predicted molecular mass of the dimeric form of 47.4 kDa (the faster-migrating band in

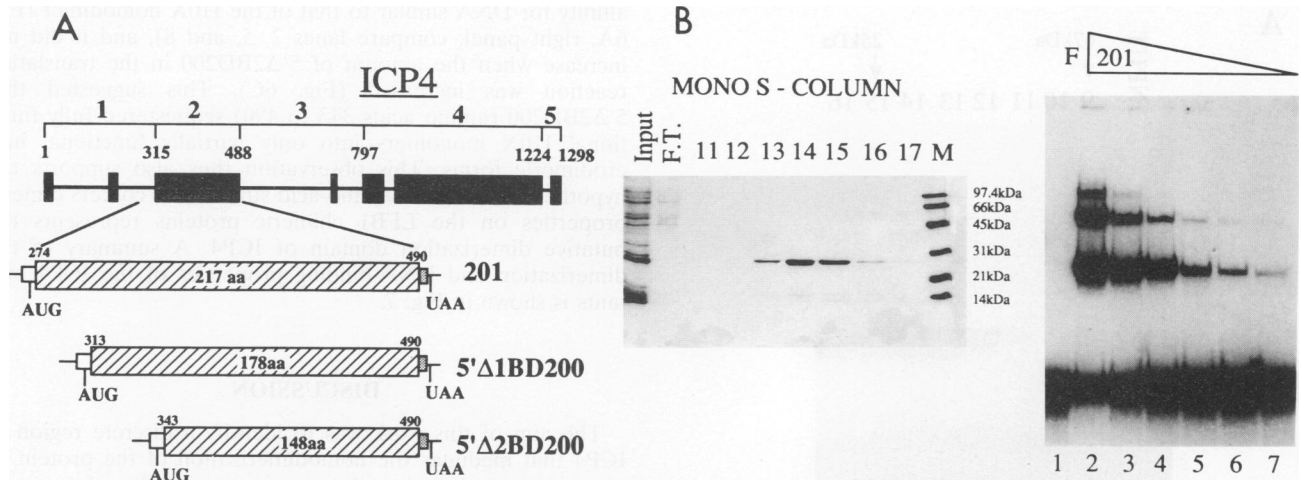


FIG. 4. Analysis of truncated ICP4 proteins expressed in *E. coli*. (A) Diagram of ICP4 mutant constructs. See the legend to Fig. 1 for details. (B) In the left panel, the 201 protein-containing fractions of a Mono S column eluted with a linear NaCl gradient as described in Materials and Methods were analyzed on a 12% polyacrylamide-SDS gel stained with Coomassie blue. Lane M, size markers (Bio-Rad). F.T., flowthrough. In the right panel, fractions containing the 201 protein were pooled, and serial 1:2 dilutions of the sample were tested for binding to the ICP4 probe in a gel shift assay (lanes 2 to 7) as described in Materials and Methods. F, free probe.

the gel shift was most likely the result of proteolysis). To confirm this result, an aliquot of the 201 preparation was treated with increasing concentrations of the cross-linking agent glutaraldehyde, and the products were analyzed by SDS-PAGE. The result (Fig. 5B) shows clear formation of 201 dimers, supporting the finding that this protein retains the ability of the full-length ICP4 protein to exist in a dimeric form in solution. The fact that the cross-linking was not quantitative is probably the consequence of poor efficiency of the reagent used rather than of an existing equilibrium between dimers and monomers. This interpretation is also supported by our results with the *in vitro*-synthesized ICP4 proteins (see below).

Analysis of ICP4 deletion mutants *in vitro*. The two truncated polypeptides 5'Δ1BD200 and 5'Δ2BD200 expressed in bacteria were more difficult to isolate in large amounts because of their low solubility. We therefore had to resort to studying the dimerization and DNA-binding properties of these polypeptides in the *in vitro* transcription-translation system described above. In addition to the polypeptides already described, we also expressed *in vitro* the polypeptide I10X (from the pT7I10X vector, kindly provided by R. Everett) that extends 34 amino acids further towards the C terminus of ICP4 than our constructs (amino acids 276 to 523) and has been shown to exist in a dimeric form in solution (8, 28).

In order to analyze the dimerization capacity of each of the truncated proteins, we carried out a monomer exchange experiment similar to that shown in Fig. 3, this time with I10X as a partner for heterodimerization with either the 201, 5'Δ1BD200, or 5'Δ2BD200 polypeptide (Fig. 6A). Approximately equal amounts of the proteins synthesized *in vitro* (either separately or in cotranslation) (Fig. 6A, left panel) were tested in a gel shift assay for binding to the ICP4 DNA oligonucleotide probe (Fig. 6A, right panel). Incubation of the ICP4 probe with the cotranslation mix of I10X and 201 (Fig. 6A, left panel, lane 5) resulted in the appearance of a novel complex with a mobility intermediate between and an intensity about twofold higher than those of the respective bound homodimer complexes (Fig. 6A, right panel, lane 2). Interestingly, this new complex did not form after mixing and incubation of the two individual polypeptides before DNA binding

(Fig. 6A, right panel, lane 10). This demonstrated that a heterodimer can form solely during the translation of the ICP4 fragments and that, once formed, the individual monomer subunits are no longer able to exchange in solution. This observation is consistent with previous results (38), which showed that heterodimers between the wild-type ICP4 and the mutant truncated protein X25 formed in HSV-1-infected X25 cells but not *in vitro* when cell extracts containing the two homodimers were mixed.

The second noticeable result is that both 5'Δ1BD200 and 5'Δ2BD200 (Fig. 6A, upper panel, lanes 3 and 4) were not able to bind the DNA probe with high affinity (Fig. 6A, right panel, lanes 6 and 9). This is consistent with earlier observations (8, 29, 37, 44) indicating that the distal part of region 1 is involved in stabilizing the binding to DNA. However, in spite of the complete loss of DNA-binding activity upon deletion of amino acids 274 to 313, cotranslation of 5'Δ1BD200 with I10X (Fig. 6A, left panel, lane 6) produced a novel complex with a mobility and an intensity consistent with that of the heterodimeric form (Fig. 6A, right panel, compare lanes 4 and 5). This novel complex was shown to be specific by competition with a 100-fold excess of either specific or nonspecific unlabeled oligonucleotide (Fig. 6B, lanes 5 to 7). Since only the I10X shift was present when the two proteins were mixed before the DNA-binding assay (Fig. 6A, right panel, lane 11), 5'Δ1BD200 seemed to behave similarly to 201 in terms of its dimerization properties. An interesting and somewhat unexpected finding was that only one functional monomer subunit (I10X) in the heterodimer was sufficient to confer on the protein the ability to bind DNA.

Finally, cotranslation of 5'Δ2BD200 with I10X (Fig. 6A, left panel, lane 7) reproducibly caused an inhibition of I10X DNA-binding activity (Fig. 6A, right panel, compare lanes 7 and 8), which was demonstrated to be dependent on the amount of 5'Δ2BD200 RNA added to the *in vitro* translation reaction (Fig. 6C). As with 5'Δ1BD200, a novel complex was formed with a mobility and a specificity of binding consistent with a heterodimeric form (Fig. 6B, lanes 8 to 10). However, the relative intensity of this band was lower than would be expected if all the heterodimeric molecules formed had an

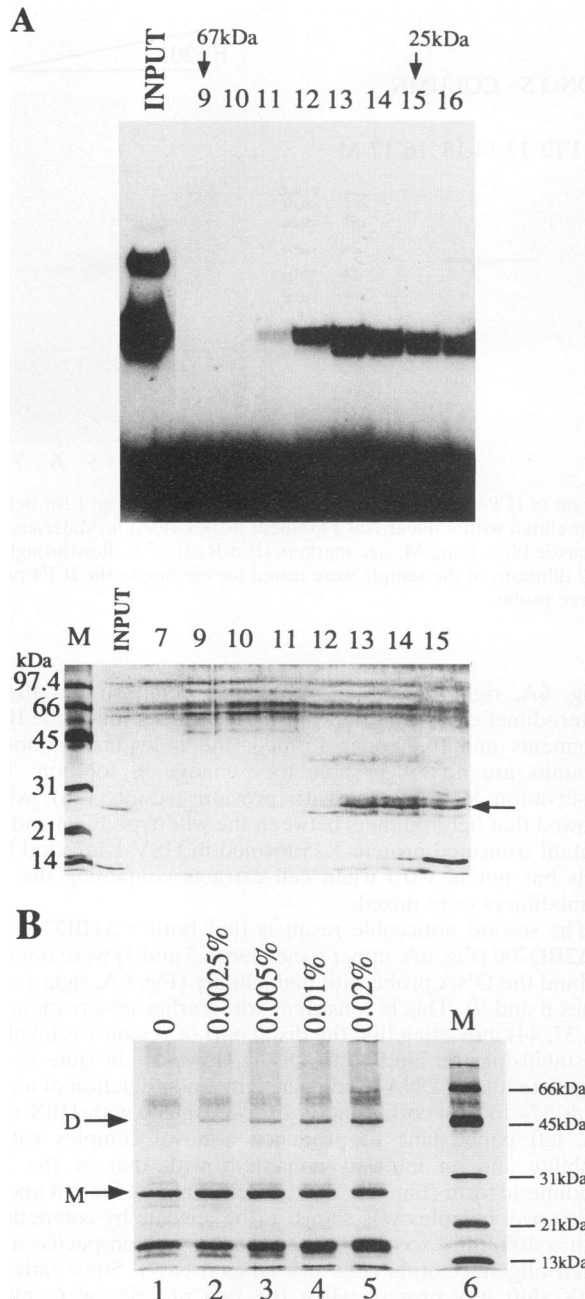


FIG. 5. The 201 protein is a dimer in solution. (A) Gel filtration analysis. A 100- μ g amount of the 201 preparation from bacteria was loaded on a Superdex 75 column, calibrated with 20 μ g of bovine serum albumin (67 kDa) and chymotrypsinogen (25 kDa). Fractions (0.5 ml) were collected, and appropriate samples (based on the UV elution profile) were analyzed for DNA-binding activity with the ICP4 probe (upper panel) and by SDS-PAGE and silver staining (lower panel). The positions of the two protein standards which eluted in fractions 9 (67 kDa) and 15 (25 kDa) are indicated by arrows in the upper panel. The peak of 201 protein (indicated by an arrow in the lower panel) and DNA-binding activity eluted in fraction 13. Lane M, size markers (Bio-Rad). The expected sizes for 201 protein were 23.7 kDa as a monomer and 47.4 kDa as a dimer. (B) Glutaraldehyde cross-linking of protein 201. The protein was treated with 0, 0.0025, 0.005, 0.01, and 0.02% (vol/vol) glutaraldehyde for 2 h at room temperature and analyzed by SDS-PAGE followed by silver staining. The positions of the monomeric (M) and cross-linked dimeric (D) species are marked. Lane M, size markers.

affinity for DNA similar to that of the I10X homodimer (Fig. 6A, right panel, compare lanes 2, 5, and 8), and it did not increase when the amount of 5' Δ 2BD200 in the translation reaction was increased (Fig. 6C). This suggested that 5' Δ 2BD200 (amino acids 343 to 490) sequestered fully functional I10X monomers into only partially functional heterodimeric forms. This observation thus also supports the hypothesis that the 34-amino-acid stretch that confers dimeric properties on the LFB1 chimeric proteins represents the putative dimerization domain of ICP4. A summary of the dimerization and DNA-binding properties of the ICP4 mutants is shown in Fig. 7.

DISCUSSION

The aim of this work was to identify a discrete region of ICP4 that mediates the homodimerization of the protein. It was anticipated that such a region would be of a novel, previously uncharacterized type, as dimerization interfaces such as the leucine zipper of the Fos-Jun heterocomplex or the helix-loop-helix motif of several other regulatory proteins (reviewed in reference 18) are not evident in its amino acid sequence. Mutational studies carried out by other groups (8, 44) indicated that the dimerization domain of ICP4 is located somewhere within the DNA-binding domain of the protein, consisting of all of region 2 and the distal part of region 1. Attempts to delineate this motif with more precision were frustrated by the fact that shorter versions of the ICP4 DNA-binding domain produced in bacteria (8, 44) no longer bound DNA, and their capacity to dimerize has not been studied by other means as yet.

In order to map the minimal functional dimerization domain of ICP4, we decided to undertake a molecular dissection of the sequence encompassing residues 274 to 490. We showed that protein 201 expressed both *in vitro* and in bacteria binds the cognate DNA sequence as a dimer and exists as a dimer in solution. Our results confirm those of Everett et al. (8), and extend them to demonstrate that the dimeric form of 201 is very stable and cannot freely exchange monomers in solution, in a way similar to native ICP4. Our first attempts to study the multimerization state of progressively shorter truncated polypeptides expressed in bacteria as nonfusion proteins failed because of their low solubility.

As the construction of hybrid DNA-binding proteins containing heterologous dimerization domains proved to be a useful tool in the molecular and functional dissection of many dimeric cellular transcription factors (reviewed in reference 18), we decided to adopt this approach and set up a dimerization trap assay, which would permit us to screen a series of 5' and 3' deletion fragments of this 217-amino-acid stretch for sequences that could confer dimerization properties on a heterologous sequence-specific DNA-binding protein. A similar strategy, using the DNA-binding domain of the bacteriophage λ repressor as a reporter for dimerization, allowed the identification of previously unknown heterologous dimerization domains (1) as well as the genetic analysis of already characterized motifs (13). We selected as a reporter the ψ POU/homeodomain of LFB1, a liver-specific transcription factor, which binds with high affinity to its cognate DNA sequence, provided that it is linked at the N terminus to a dimerization motif (42). Thus, among a series of ICP4 polypeptides linked to the LFB1 ψ POU/homeodomain (see Fig. 1), only those containing ICP4 residues 343 to 376 demonstrated a DNA-binding phenotype in gel shift assays and were therefore judged to contain a dimerization motif. Con-

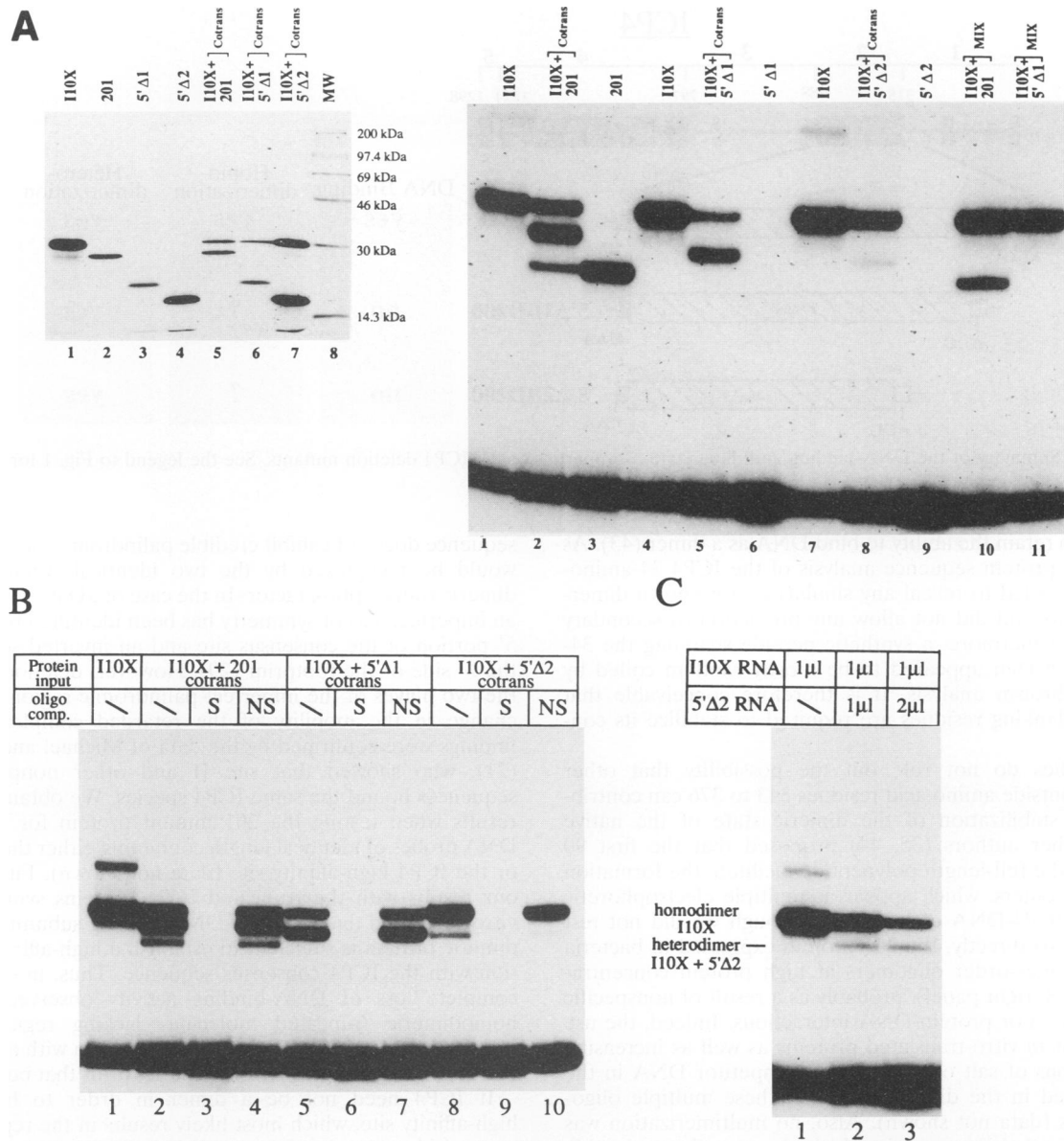


FIG. 6. Analysis of ICP4 deletion mutant proteins synthesized in vitro. In vitro transcription-translation reactions were carried out as described in the legends to Fig. 2 and 3. (A) Proteins 201, 5'Δ1BD200, and 5'Δ2BD200 were synthesized singly or together with protein I10X (8). Either 1.5 μl of the samples containing single ³⁵S-labeled proteins or 3 μl of the cotranslation reaction mixes was analyzed by SDS-PAGE (12% polyacrylamide) and autoradiography (left panel). Lane MW, ¹⁴C-labeled size markers (Amersham). Approximately equal amounts of protein were tested for binding to the ICP4 oligonucleotide probe in a gel shift assay (right panel). Suitable volumes of either 201 or 5'Δ1BD200 samples were also mixed with the I10X preparation at an approximately 1:1 protein ratio for 30 min at 37°C and then analyzed for DNA-binding activity (lanes 10 and 11). (B) The 201-I10X, 5'Δ1BD200-I10X, and 5'Δ2BD200-I10X heterodimeric forms all bind DNA in a specific manner. Suitable amounts of the cotranslation reaction mixes shown in panel A were tested for binding to the ICP4 probe in the absence or presence of a 100-fold excess of either specific (S) or nonspecific (NS) unlabeled oligonucleotide and analyzed by the gel shift assay. (C) Inhibition of I10X DNA-binding activity is dependent on the amount of 5'Δ2BD200 synthesized in the cotranslation reaction. I10X cRNA (1 μl) was cotranslated with 0, 1, or 2 μl of 5'Δ2BD200 cRNA, and equal protein samples were analyzed for DNA binding by the gel shift assay. The identity of the retarded complexes is indicated. Electrophoresis was carried out for a shorter time in panels B and C than in panel A.

sistent with this result, a truncated ICP4 protein as small as 5'Δ2BD200 (amino acids 343 to 490), in spite of the complete loss of DNA-binding activity, appeared to retain the capacity to form a heterodimer with a longer polypeptide after coexpression in the in vitro translation system.

The identified 34-amino-acid stretch exhibits significant sim-

ilarity to the corresponding region of the ICP4-related proteins from other alphaherpesviruses in that at its C-terminal end, which is strictly necessary for dimerization, there is a block of homology of 11 amino acids. Interestingly, a truncated form of the varicella-zoster virus protein ORF62, approximately corresponding to our ICP4 deletion mutant 5'Δ2BD200, was dem-

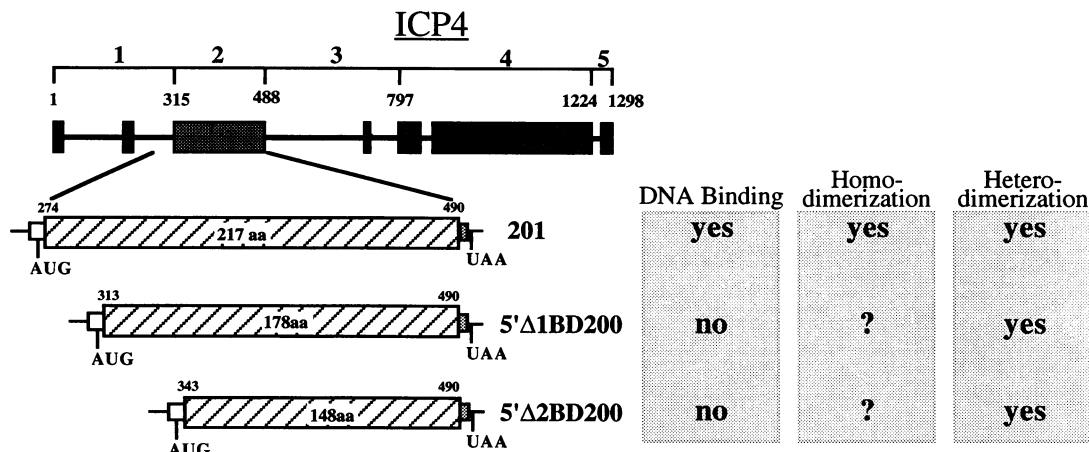


FIG. 7. Summary of the DNA-binding and dimerization properties of the ICP4 deletion mutants. See the legend to Fig. 1 for details.

onstrated to retain the ability to bind DNA as a dimer (43). As anticipated, protein sequence analysis of the ICP4 34-amino-acid stretch failed to reveal any similarity with known dimerization motifs and did not allow any prediction of secondary structure. Furthermore, a synthetic peptide spanning the 34-amino-acid stretch appeared to be mostly random coiled by circular dichroism analysis. It is therefore conceivable that additional flanking residues are required to stabilize its conformation.

Our studies do not rule out the possibility that other sequences outside amino acid residues 343 to 376 can contribute to the stabilization of the dimeric state of the native protein. Other authors (35, 44) proposed that the first 90 residues of the full-length polypeptide facilitate the formation of ICP4 oligomers, which appear as multiple electrophoretic variants of ICP4-DNA complexes. Although we did not test this hypothesis directly, the 201 protein expressed in bacteria did form higher-order oligomers at high protein concentrations (Fig. 4B, right panel), probably as a result of nonspecific protein-protein or protein-DNA interactions. Indeed, the use of diluted or *in vitro*-translated proteins as well as increasing concentrations of salt or nonspecific competitor DNA in the assay resulted in the disappearance of these multiple oligomeric forms (data not shown). Also, no multimerization was observed in gel shift assays in which shorter oligonucleotide probes containing the ICP4 DNA-binding site were used, and only the dimeric form was evident in solution by both gel filtration and protein-protein cross-linking.

The main difference between the ICP4-LFB1 hybrid polypeptides and the corresponding ICP4 truncations was that the chimeric proteins could freely exchange monomers in solution, whereas the ICP4 mutants, like the native HSV-1 protein (36, 38), could heterodimerize only upon cotranslation. We propose that amino acid residues within the C-terminal portion of region 2 (410 to 490) contribute to this increase in the stability of the truncated ICP4 dimers. This hypothesis is based on our observation that the chimeric protein 5'Δ2(410)/LFB1, containing amino acids 343 to 410 of the ICP4 sequence, retained the ability to exchange monomers in solution, which indicated that the region between residues 376 and 410 does not augment the stability of the dimer, whereas that delineated by residues 410 to 490 clearly does.

Whether dimerization is strictly necessary for binding of ICP4 to DNA is a question that has been debated by several authors. The first consideration is that the ICP4 consensus

sequence does not exhibit credible palindromes or repeats that would be recognized by the two identical subunits of the dimeric transcription factor. In the case of gD promoter site II, an imperfect axis of symmetry has been identified between the 5' portion of the consensus site and an inverted sequence at the 3' side of the footprint (29). However, deletion of one of the two halves of the imperfect palindromic region caused no change in the mobility of the retarded complexes. These findings were confirmed by the data of Michael and Roizman (21), who showed that site II and other nonpalindromic sequences bound the same ICP4 species. We obtained similar results when testing the 201 mutant protein for binding to DNA probes of identical length containing either the gD II site or the ICP4 high-affinity site (data not shown). Furthermore, our results with the truncated ICP4 proteins synthesized *in vitro* suggested that only one DNA-binding subunit within the dimeric protein is sufficient to establish a high-affinity interaction with the ICP4 consensus sequence. Thus, in spite of the complete loss of DNA-binding activity observed with the homodimeric truncated molecules lacking region 1 (5'Δ1BD200 and 5'Δ2BD200), their cotranslation with a functional ICP4 variant generated heterodimeric forms that bound DNA.

If ICP4 need not be a dimer in order to bind to its high-affinity site, which most likely results in the repression of transcription, the significance of ICP4 dimerization most likely relates to some other function of the protein. As a good correlation exists between mutations in region 2 that impair DNA binding and the loss of some of the ICP4 *trans*-activation functions (5, 27, 28, 37) and since region 2 appears to be responsible for both high- and low-affinity binding to DNA, it is tempting to speculate that dimerization may be a prerequisite for *trans* activation by the protein. Several low-affinity DNA-binding sites were found in the vicinity of early and late ICP4-activated promoters (12, 14, 23, 26, 34, 41). As these sites do not contain an apparent consensus sequence, it is likely that an interaction of an ICP4 monomer with these sites would be too labile to be functional. It would thus need to be further stabilized, most likely by interaction with other proteins bound in the vicinity with high affinity. Alternatively, were ICP4 to bind as a dimer, it could effectively double the number of contacts with the DNA and thus stabilize the interaction. However, no evidence in favor of this hypothesis has as yet been provided.

Alternatively, as indicated by previous results (38, 39), the dimeric state could represent a necessary constraint on the

conformation of a domain of ICP4 which participates in protein-protein interactions required for activating transcription. These studies indicated that the stable expression of a truncated ICP4 peptide lacking region 4 and therefore unable to transactivate viral genes (5, 35) led to increased resistance to infection with HSV-1 both in tissue culture and in transgenic mice (38, 39). This *trans*-dominant inhibition was attributed to the formation of nonfunctional heterodimers between the truncated and wild-type ICP4 proteins. As these heterodimers could be isolated from cells and demonstrated in gel shift experiments to be capable of DNA binding, their failure to *trans* activate was ascribed to the conformational alteration in the C-terminal region, genetically implicated as important for *trans* activation.

Construction of a recombinant ICP4 gene lacking sequences encoding amino acids 343 to 376 should yield a protein that is unable to dimerize. Such a protein ought to provide us with invaluable information about the real role of dimerization in the function of this important HSV-1 transcriptional regulator. These experiments are in progress.

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